Fish-microarray: miniaturized platform for single-embryo high-throughput screenings

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Abstract

Small molecule high-throughput screenings using cell systems are essential for the fields of drug discovery and toxicology. Hundreds of millions of compounds are screened every year. Nevertheless, 2D cell culture models do not always reflect the whole complexity of in vivo cellular microenvironments leading to a large number of false results. Scientists are trying to move towards more physiologically relevant 3D cell culture models. However, all existing 3D cell culture models are still far from in vivo situations and animal models are needed for better screening applications. Danio rerio (zebrafish) represents a promising animal model that is able to bridge the required in vivo physiological environment with the possibility of high-throughput screening applications. Drug and toxicity screenings using whole organisms are more physiologically relevant because they can address complex processes such as for example, behavior, pain or vascularization. Therefore, such screenings lead to more predictive results. However, due to challenges in automation of animal-based experiments, as well as high costs and absence of miniaturized solutions for whole-organism screenings, high-throughput screenings based on animal models are still in its infancy. Here we present a platform and methodology for miniaturized high throughput whole-organism screenings. The new platform is based on patterns of hydrophilic spots separated from each other by superhydrophobic, water repellant, regions. The difference in wettability of spots and borders generates the effect of discontinuous dewetting and allows for formation of arrays of microdroplets that can incorporate single fish embryos. Due to the flat border-less nature of the platform, the system is compatible with single-step collection of embryos and pipetting-free parallel addition of chemical libraries using the “sandwiching method”. The system is miniaturized and allows for incubation of embryos in volumes as low as 5 µl. Finally, the platform realizes surface-tension-based immobilization of single embryos inside of each microcompartment and permits high-throughput microscopic analysis directly on the platform. Thus, this method combines the advantages of microarrays, such as high-throughput capabilities and simplicity, with the power of whole animal in vivo experiments.
Introduction

High throughput screening (HTS) is used to perform numerous bio-chemical, phenotypic and genetic experiments in parallel. The classical goal of HTS is to rapidly identify the most promising compounds from a large chemical library space. In these screenings, compounds are tested against molecules (biochemical screens), cells, 3D cellular systems and even whole organisms.\textsuperscript{1-4} With raising demand for new drugs and bioactive compounds, the amount of screens performed per day is continuously growing reaching almost 100,000 compounds a day for big pharmaceutical companies.\textsuperscript{5, 6} Typically, HTS are conducted using microtiter plates (MTPs) and the last several decades have seen a systematic evolution of MTP formats from 6 to 3072 wells per plate.\textsuperscript{7} The main drivers for such an evolution are (1) the increasing demand for higher throughput; and (2) the need to decrease the costs of screenings by reducing the consumption of compounds, reagents and cells. Further miniaturization of MTPs is barely possible because of physical limits in pipetting into small wells due to capillary effects. Another important parameter to consider in HTS is using physiologically relevant models such as primary and stem cells, 3D cell cultures (organoids, spheroids, embryoid bodies) and whole organism for obtaining relevant screening data. The use of such models for large screens is very often restricted due to high costs (stem cells, animal models), limited availability of materials (stem cells, primary cells) and incompatibility with HTS workflows (mouse models). Due to these reasons, the majority of primary screens is performed using robust 2D cell cultures leading to a lot of false positive “hits” in primary screens that do not make it to clinical trials. As a result, it takes about 1 billion US dollars and about 12 years after the identification of a primary “hit” for a drug to be approved for clinical use.\textsuperscript{6, 8} In order to change this situation there is a need for novel alternative technologies that would allow miniaturized HTS using physiologically relevant models.\textsuperscript{6, 8, 9}

Whole organism experiments are more physiologically relevant compared to cell culture models and can address complex processes such as behavior, pain, immunity, tumor metastasis and vascularization that are out of reach for cell-based assays.\textsuperscript{10} HTS based on whole organisms is an indispensable method, which can allow for compound screenings in a context of a whole body, practically combining primary and secondary screenings, toxicity validation and animal experiments in one screen. All these screens are classically performed consequentially one after another using different models with increasing complexity, usually moving from cell-based assays to tissues and animal experiments. This takes time and resources and a big part of firstly identified “hits” do not confirm in following experiments based on different models. Animal-based screenings are, however, expensive, methodologically challenging and ethically problematic. Typically used mouse models are not compatible with HTS workflow, therefore, small animal models such as the nematode Caenorhabditis elegans, the fruit fly Drosophila melanogaster or the zebrafish Danio rerio are the main alternatives for high-throughput applications. Zebrafish Danio rerio has become a widely used model for studying genetics, development, diseases, as well as for drug discovery and toxicology, due to its small size, short reproductive cycle, high fecundity, transparent embryos, 70 \% genetic similarity with humans, fully sequenced genome and availability of a large number of established and well characterized transgenic lines.\textsuperscript{11} Due to the similar genetic structure with humans, on the one hand, and compatibility with screenings, on the other, zebrafish have become an important tool for high throughput phenotypical screenings in order to characterize novel drug candidates and assess
their toxicity. Zebrafish are also a powerful model for chemical toxicity studies and for eco-environmental monitoring and evaluation of potential toxicity of pollutants. The majority of large pharmaceutical companies report the use of zebrafish for toxicology and the Organisation for Economic Co-operation and Development (OECD) has developed and established rigorous guidelines for Fish Embryo Testing based on the *Danio rerio* model. However, only about 65 chemical screens using zebrafish were published to date which is extremely few compared to the numerous cell-based screens being reported every year. This is because such screens are costly and technologically challenging. The reasons for that are the (1) large volumes required for incubation of fish, leading to high consumption of compounds and reagent; (2) difficulties to automate screenings due to incompatibility of standard pipetting robotics with animal sizes and shapes and absence of affordable commercially available solutions for that; and (3) challenges in microscopy and image analysis of a complex 3D animal structures. Due to the unavailability of commercially available robotics, there is a need for large amount of manual work. Which dramatically increases the time of experiments limiting the number of compounds possible to analyze. Despite a few available commercial solutions such as the COPAS Biosort system (Union Biometrica, MA, USA), most of the performed whole organism screenings are based on semi-automated in-house developed workflows.

Reported alternative platforms for screening of whole animals, such as *Caenorhabditis elegans* and *Danio rerio*, are based on microfluidic principles allow for immobilization of the animals in two-dimensional arrays with defined physical locations and microscopic analysis directly on chips. Such screening platforms are good for perfusion and dynamic culturing in miniaturized formats, however, they are barely compatible with screenings of large chemical libraries. Therefore, there is a clear need for novel technologies capable of solving the problems of high experimental costs and embryo handling allowing for affordable in vivo screening of large compound libraries. Using novel synthetic materials, in particular patterned surfaces with different wettability, as platforms for biological applications was reported previously. In this study we developed a miniaturized system, Droplet-Microarray (DMA) chip, for screening of zebrafish embryos based on the use of arrays of highly hydrophilic spots separated by superhydrophobic barriers. Highly hydrophilic spots possess perfect wettability with a static water contact angle (WCA) of less than 10°. Superhydrophobicity on the other hand has the opposite property – it shows extreme water repellency with both advancing and receding WCA above 150° and very low sliding angles being less than 5°. Such combination of two opposite properties on the same surface in precise micropatterns leads to the effect of discontinuous dewetting, which allows for a pipetting-free formation of nano-to-microliter sized droplets on the hydrophilic spots by rolling a source droplet across such a pattern. We demonstrated that the effect of discontinuous dewetting can be applied to suspensions of zebrafish embryos to enable manual spreading of zebrafish embryos in an one-embryo-per-spot manner to form a microarray of fish embryos trapped in aqueous microcompartments. Simple collection of the embryos from the DMA chip, parallel single-step pipetting-free addition of compounds, direct on-chip microscopy, defined positioning of embryos in 2D array were demonstrated. We evaluated the DMA platform by performing two small proof-of-principle screenings on zebrafish embryos. We investigated organ specificity of several fluorescently labelled peptoids and evaluated toxicity of ZnCl₂ and AgNO₃ within a range of concentrations. The results obtained in these screenings were in good agreement with
the results obtained in standard microtiter plates. Such system carries several advantages compared to conventional microtiter plates (MTPs) including (1) screening on single embryos, excluding influence of neighboring embryos, (2) small reaction volumes down to 5 μL per embryo, (3) pipetting-free handling (spreading of embryos, collection of embryos, introduction of compounds), and (4) easy microscopy due to fixed position of fish embryos.

Figure 1. Spreading of zebrafish embryos on Droplet-Microarray (DMA) platform. (a) Schematic representation (left) and images (right) of the process of spreading zebrafish embryos using the effect of discontinuous dewetting. (b) An image of DMA containing zebrafish embryos 24 hours post fertilization spread using the effect of discontinuous dewetting.
Results

Spreading, maintenance and collection of zebrafish embryos on Droplet-Microarray platform

In the current study we used the Droplet-Microarray (DMA) chip with dimensions of a microscope glass slide (7.5 cm x 2.5 cm) consisting of 108 square, highly hydrophilic (HH) spots with 3 mm side length and 1mm wide superhydrophobic (SH) border (Fig. 1a, S1a). To prepare DMAs, microscope glass slides were coated with a thin (2-4 μm) layer of nanoporous poly(2-hydroxyethyl methacrylate)-co-(ethylene dimethacrylate) (HEMA-EDMA) polymer followed by esterification of the polymer using 4-propynoic acid and consequent modification of the surface using the photoclick thiol-yne reaction by covalent attachment of hydrophilic and hydrophobic groups to create HH and SH areas, respectively.\(^{28-30}\) Due to extreme hydrophilicity (static water contact angle 4° and receding contact angle 0°) and superhydrophobicity (static water contact angle 170° and receding contact angle 164°), aqueous solutions applied onto patterned surface of DMA slide will spontaneously split to form an array of separated droplets (Fig. 1a, S1b, Video S1, S2). This process, known as discontinuous dewetting,\(^{27}\) can be used for pipetting-free spreading of solutions, substances, particles and cells.\(^{28, 29, 31-34}\) We utilized the effect of discontinuous dewetting to create a single-embryo array, an array of droplets each containing a single embryo (Fig. 1a, Video S2). This process is based on spreading zebrafish embryos using a simple pipette without the need for multiple pipetting steps or robotics (Fig. 1a and b, Video S2). Embryos are taken up with a Pasteur pipette with an opening of 3 mm and aligned inside the pipette in one row. The pipette is moved with constant speed along the rows of hydrophilic spots to gradually push single embryos one after another from the pipette. Each embryo comes to the tip of the pipette by gravity and is pushed out onto a hydrophilic spot with liquid around it. The droplet breaks at the edge of the hydrophilic spot while the pipette is moved over the superhydrophobic border and the embryo is trapped and confined to the droplet on the hydrophilic spot (Fig. 1a, Video S2). By using this method we managed to achieve on average up to 95% of droplets containing single embryo. Obtaining such high percentage of droplets with single embryos is unique and very important for high-throughput screenings. On average 7.4 % of droplets occurred to trap two embryos. In this case, the second egg was removed manually. Volume of produced droplets was on average 4.5 μL with standard deviation being approximately 10%, and 80% of droplets being 4±5.8% μL (Fig. 1b, right; Fig. S1d). Variability in droplet volumes and probability of occurrence of two embryos per droplet both depend on such parameters as width of pipette opening, angle of the pipette towards the surface, speed of pipette movement, and can be minimized by controlling these parameters.

Collection of embryos from MTPs is another technical challenge that usually requires manual pipetting. The advantage of the DMA method is that the formed aqueous compartments are not engulfed by solid barriers as in MTPs. The produced droplets are located on top of a flat polymer surface, which allows for the facile collection of all fish embryos from the DMA by washing off the droplets into a Petri dish. It is also possible to easily collect fish embryos of interest by dragging them with a pipette across the droplets of interest pooling embryos from particular locations, for example a row, without disturbing neighboring droplets (Video S3). The possibility of fast and easy collection of embryos, without the need for pipetting of each embryo, can be useful for multiple applications where zebrafish eggs are needed to be collected.
for further analysis after incubation with a compound of interest. Screenings of fish embryos are usually performed in MTPs in relatively large volumes, starting from 200 µL. In the case of the DMA, the volume used per embryo is reduced to 5 µL. In order to evaluate whether such minute volume can influence viability and development of embryos, we spread zebrafish embryos 24 hours post fertilization on a DMA slide and incubated them in the droplets for 24 hours (Fig. 2) in a double Petri dish containing wetted tissues (see Experimental section). The evaporation rate of droplets was on average 7% after 24 hours of incubation. Zebrafish embryos incubated in droplets did not show any obvious morphological abnormalities compared to control zebrafish embryos that were maintained in Petri dish (Fig. 2a). Eye is one of the earliest organ in zebrafish developmental process and several developmental defects are reflected in eye size. Zebrafish eye has elliptical shape and one way to quantify its size is to measure its two axes. We compared the eye sizes of fish incubated on Droplet-Microarray and in Petri dish, in order to ensure that there are no developmental delays or defects in zebrafish embryos incubated on our platform. Therefore, we compared the measurements of major and minor axis of the eyes of 216 embryos incubated in droplets versus in Petri dish. No significant difference in eye measurements of both embryo populations could be detected (Figure 2b) confirming that maintenance of embryos in small volume droplets for 24 hours does not influence their development. To check whether incubation in droplets can cause changes in development of zebrafish embryos at later stages, we washed down the embryos from a DMA into a Petri dish and let them develop and hatch normally (Fig. 2c). The hatching time of those embryos was the same compared to that of control embryos (Fig. S2a) and by 72 hours post fertilization nearly all embryos were hatched in both embryo populations (Fig. S2a). We also compared the measurements of minor and major axis of eyes of zebrafish larvae 72 hours post fertilization and did not observe any significant difference in eye development of larvae that were previously incubated in 5 µL droplets and control larvae cultured in Petri dishes containing 10 mL of water (Fig. 2d). To check if zebrafish embryos can be incubated in droplets for more than 24h, we spread zebrafish embryos 2 hours post fertilization on a DMA slide and observed them for 48 hours (Fig. S4). The embryos did not show any morphological abnormalities compared to control embryos incubated in a Petri dish. Viability of the embryos across the examined population at 48 hours post fertilization was 88.1 and 91.8% for DMA and Petri dish, respectively (Fig. S4b). 48 hours post fertilization embryos were transferred to a Petri dish and allowed to hatch (Fig. S4). There were no obvious differences in morphology of zebrafish larvae previously incubated on the DMA and in Petri dish. Similar results were obtained after incubating two eggs per single droplet (Fig. S4). These results demonstrate that zebrafish embryos can be maintained in individual droplets of about 5 µL volume on DMA for 48 hours without affecting their viability and normal development.
Figure 2. Maintenance of zebrafish embryos in individual droplets. (a) Microscope images of control zebrafish embryos maintained in Petri dish and embryos on DMA initially after spreading and after 24 hours of maintenance in individual droplet. (b) A graph showing the comparison of major and minor eye axis of 48 hours zebrafish embryos maintained in Petri dish versus in individual droplets for a period of 24 hours between the age of 24 and 48 hours. Total 216 zebrafish embryos maintained on 6 different Droplet-Microarrays were analyzed. (c) Microscope images of 72 hours old zebrafish larvae maintained in Petri dish and in individual droplet for a period of 24 hours between the age of 24 and 48 hours. (d) A graph showing the comparison of major and minor eye axis of 72 hours zebrafish larvae maintained in Petri dish versus in individual droplets for a period of 24 hours between the age of 24 and 48 hours. Total 216 zebrafish embryos maintained on 6 different Droplet-Microarrays were analyzed.

Parallel addition of chemical libraries using the sandwiching method

In order to be able to use the DMA platform for screening applications and to treat zebrafish embryos confined in individual droplets with different substances, we utilized the sandwiching method depicted in Figure 3a,b.29 Thus, a fluorescently labeled dye (fluorescently labeled peptoid MlysMlysNpheNlysRhodB, ID number 175) was printed onto a microscope glass slide using a non-contact liquid dispenser in a geometry corresponding to that of a DMA
slide either in checker board pattern or in different amounts (1, 2, 4, 6, 8, 10 nmol), followed by drying the slide (Fig. 3d). Afterwards parallel addition of compounds was performed by sandwiching the DMA slide containing embryos with a glass slide containing preprinted compounds using an aligner shown in Figure 3b (for detailed description of procedure see Experimental section). The sandwiched slides were kept in an incubator for 15 minutes to ensure dissolution and diffusion of the drugs into the droplets (Figs. 3, S2b,c). Microscope images of DMA containing embryos after transfer of the dye presented in Figure 3d demonstrate that (1) no cross-contamination between neighboring droplets during the transfer (Fig. 3c) and (2) the ability to control the final concentration of the dye in droplets by preprinting different amounts of the dye. The sandwiching procedure also did not affect the zebrafish embryos’ viability. Thus, we can conclude that it is possible to use the sandwiching method for parallel addition of different compounds to droplet micro reservoirs containing zebrafish embryos.

**Screening of fluorescently labeled peptoids in zebrafish embryos using the DMA platform**

In order to further demonstrate applicability of our platform for miniaturized whole-organism screening applications, we performed a small screening of zebrafish embryos with 8 different fluorescently labeled cell penetrating peptoids (CPPos). A combinatorial library of 256 Rhodamine B-labeled tetrameric cell penetrating peptoids (CPPos) was synthesized by IRORI-radiofrequency tag-based solid phase synthesis \(^3\) for the generation of organ specific drug delivery agents. The tetrameric library was built up by a submonomer method using a subsequent coupling of bromoacetic acid and a permutation of four different amines (N-2-prop-2-yn-1-ylglycine (Nprg), N-(p-chlorobenzyl)glycine (Npcb), N-4-aminobutylglycine (Nlys) und N-benzylglycine (Nphe)). \(^3\) We then introduced zebrafish embryos to 8 chosen representative tetrameric CPPos from the library (Fig. 4a) using the DMA platform and compared their tissue localization with the results obtained in 96-well plates. Zebrafish embryos 24 hours post fertilization were spread onto the DMA slide and the CPPos were added to the droplets containing the embryos using the sandwiching method (Figure 3), followed by 24 hours of incubation in the droplets. Afterwards embryos treated with the same CPPos were collected in a 96-well plate to allow eggs to hatch and 72 hours post fertilization zebrafish larvae were imaged using fluorescence microscopy. The results of the screen are summarized in Figure 4a. Tested peptoids were found to be localized in different tissues: skin (Fig. 4b, 3), olfactory pits (Fig. 4b, 1 and 2), otic vesicle (Fig. 4b, 1 and 2) and caudal vein (Fig. 4b). Each CPPo had a unique signature of tissue distribution in the larvae (Fig. 4b and S3), which confirmed the absence of cross-contamination between the droplets during the addition of CPPos and incubation period. To exclude the influence of the platform on the outcome of the experiment we performed the same protocol in a 96-well plate. All 8 CPPos were observed in the same locations in zebrafish larvae, as in the case of the DMA platform (Fig. S3). Introduction of the CPPos to zebrafish embryos in 96-well plates required
Figure 3. Sandwiching method for addition of compounds to individual droplets containing zebrafish embryos. (a) Schematic representation of the sandwiching method. (b) Sandwiching aligner. From left to right: upper holder of the aligner with glass slide preprinted with substances of interest, lower holder of the aligner with a Droplet-Microarray slide containing zebrafish embryos, closed aligner containing Droplet-Microarray with zebrafish embryos sandwiched and glass slide preprinted with substances of interest. (c) Microscope images of Droplet-Microarray containing zebrafish embryos after addition of a fluorescent dye (fluorescently labeled peptoid NlysNlysNpheNlysRhodB, ID number 175) in the checker-board pattern. (d) Microscope images of a Droplet-Microarray slide containing zebrafish embryos after addition of different amounts of the dye and graph showing mean fluorescence intensity of droplets containing different amounts of the dye. (n = 3, error bars represent standard deviations).
13.4 times more of the CPPos than on DMA slides (13.4 nmol vs. 1 nmol of CPPos per embryo, respectively). More than an order of magnitude reduction in compound consumption is critical for increasing the throughput of whole animal screening applications. These results demonstrate the applicability of the DMA platform for parallel introduction of zebrafish embryos to minute amounts of compounds in miniaturized droplets in a one-embryo-per-droplet manner.

Figure 4. Screening of CPPos in zebrafish embryos on Droplet-Microarray platform. (a) The list of CPPos used for the proof-of-concept screening of zebrafish embryos on the DMA platform and their final locations in a zebrafish larva. (b) Representative fluorescent microscope images of different locations the peptoids were detected in zebrafish larvae 72 hours post fertilization. Olfactory pits (green arrows), otic vesicle (blue arrows), skin (red arrows), caudal vein (yellow arrows).

Toxicity screening on zebrafish embryos using DMA platform

The zebrafish is an important and widely used animal model for toxicity testing in the fields of drug discovery and ecotoxicology. Therefore, we evaluated the compatibility of the DMA platform with miniaturized toxicity screenings using single-embryo arrays.

As toxic compounds for this proof-of-concept study we chose ZnCl$_2$ in concentrations ranging from 1 to 10 µM, and AgNO$_3$ in a range of concentrations from 0.001 to 0.5 µM (Fig. 5b and c). Zebrafish embryos 24 hours post fertilization were spread onto a DMA slide and introduced to different concentrations of ZnCl$_2$ and AgNO$_3$ using the sandwiching approach, followed by 24 hours of incubation. Afterwards the entire DMA slide
was imaged using either a microscope (Fig. 5a) or by a mobile phone digital camera (Fig. 5b). Viability of fish embryos was then estimated by visual examination (Fig. 5). In microscope images the morphology of embryos can be observed in detail and coagulated eggs or embryos with developmental abnormalities can be easily detected (Fig. 5a). In addition, microscopic analysis gives the opportunity to examine more complex morphological changes in response to a compound if there is a need for it.

In addition, the viability of embryos could be also easily estimated with naked eye. Individual droplets containing single coagulated/dead embryos could be easily distinguished from the droplets with healthy embryos by the color and transparency of the corresponding droplets. This effect was probably caused by protein release and degradation following chorion disruption due to the toxic effect of the chemicals (Fig. 5b). Thanks to the fact that each droplet contains a single embryo it is possible to quantify viability by simply counting the number of affected droplets from the images made with standard digital camera. This can be done either manually or by using image analysis software (Fig. 5b).

The dose-dependent response to both compounds obtained on DMA platforms was in good agreement with that obtained in 96-well plate. (Fig. 5c), The results indicate that the DMA platform can be used for miniaturized toxicity screenings with an outcome comparable to that obtained in microtiter plates. Comparing to 96-well plates, the DMA platform allows for reduction in compound consumption for a factor of 40 compared to 96-well plate, considering that single embryo is incubated in one well. According to OECD FET guidelines13, 20 embryos per chemical concentration are used with 1 embryo per well in a 96-well plate, a total of 5 concentrations per compound. The volume required to test one concentration with 20 embryos with 1 embryo per well is 100 µl (5 µl droplet x 20) and 4 mL (200 µl per well x 20) for DMA and 96 well plate, respectively. This is 40 times difference in volume, which reflects on compound consumption. In addition, the DMA platform enables screening of single embryos separated in individual droplets in only 5 µl of volume, avoiding unwanted influence of neighboring embryos. Finally, the DMA platform enables a quick imaged-based read-out directly from a DMA chip. On one hand, it enables the estimation of viability in a whole population of treated embryos from a single image by visual examination as opposed to taking multiple images of each well by an automated microscope. On the other hand, it allows for detailed microscopic analysis leaving opportunity for using the DMA platform for even more complex biological read-outs.

Discussion

The whole organism drug screenings are more relevant compared to cell-based assays, however, very costly and challenging. Zebrafish is picking up as a widely used model organism for discovery of new drug candidates and drug toxicity studies due to its high genetic and physiological similarity to humans and, therefore, high predictability of pharmacological effects of the drugs.10 In addition, zebrafish have small size, short reproductive cycle, high fecundity and transparent embryos, all of which makes it a convenient and relevant model for compound screenings. Chemical screens on zebrafish are a growing fraction of phenotypic-based screenings in the fields of drug discovery and toxicology.10, 12 This will inevitably lead to an increase in the number of compounds to be tested on zebrafish in the future.
Here we introduced a Droplet-Microarray (DMA) platform, based on hydrophilic-superhydrophobic micro patterning, as a platform for performing miniaturized high-throughput whole-organism screenings. We chose zebrafish embryos as a model organism and demonstrated pipetting-free spreading and collection of embryos, incubation of single embryos in droplets of as low as 5 µL volume, parallel addition of compounds into individual droplets containing embryos and microscopic analysis directly on the DMA.

Figure 5. Toxicity screening in zebrafish embryos on Droplet-Microarray platform. (a) A microscope image of the whole Droplet-Microarray and representative droplets containing control zebrafish embryos and embryos introduced to ZnCl₂ for 24 hours. (b) Digital camera images of the whole Droplet-Microarray slide containing zebrafish embryos introduced to different concentrations of AgNO₃ for 24 hours. Only representative images of DMA introduced to five different concentrations of AgNO₃ (0.01, 0.025, 0.05, 0.075 and 0.1 µM) are displayed. Red and green arrows show difference in the appearance of droplets containing dead and live
fish embryos, respectively. Viability values (on the right) were calculated using two methods: (A) observation of the embryo appearance using microscopy-generated images (see figures in a) and (B) counting the number of transparent vs. non-transparent droplets from digital camera imaged. (c) Graphs showing concentration dependent effects of ZnCl$_2$ and AgNO$_3$ on viability of zebrafish embryos on Droplet-Microarray platform and in 96 well plate. In total 300 and 200 embryos per concentration were tested on DMA and 96 well plate, respectively.

Currently, every pharmaceutical company regularly performs millions of cell-based experiments generating huge costs, which are one of the reasons for the slow drug development pace and unaffordability of such screenings to regular biology labs. In vivo-like screenings based on whole organisms can be even more costly, time-consuming and technically challenging. Miniaturization and multiplexing of such screenings is a must to reduce the costs and to make in vivo screenings accessible as a tool used in biological laboratories. The DMA platform enables such miniaturization and multiplexing leading to an order of magnitude reduction of consumption of chemicals used per embryo. This will be important in order to reduce the costs of the experiments as well as to enable screenings that might not be possible in MTPs at the moment due to limited compound availability.

Automation of whole-organism screenings has been a challenging problem due to the incompatibility of standard pipetting robotics with big sizes and irregular shapes of animals. Automated commercially available solutions for transfer of embryos to MTP wells are limited and often expensive. Therefore, most of the performed whole organism screenings are based on semi-automated in-house developed workflows. The possibility of pipetting-free spreading of zebrafish embryos on DMA slides is beneficial compared to multiple pipetting steps that are needed to transfer individual animals to MTPs. In the current study the spreading of zebrafish embryos was performed manually, which leads to a certain variability in droplet volumes (Fig. S1d). The main goal of this proof-of-concept study was to demonstrate the possibility of creating low-volume single-embryo arrays for high-throughput in vivo screenings. Such manual set up is useful for middle to high throughput screenings without the need for expensive robotics and automation, which makes it attractive for smaller research laboratories without access to screening centers. However, for higher throughputs, industrial applications and further reduction of volume variability, the DMA platform can be potentially automated and integrated into the usual high throughput screenings pipelines established, for example, in pharmaceutical industry. The dimensions and format of DMA slides are flexible and can be manufactured in dimensions of standard microtiter plate formats in order to fit into existing liquid dispensers, plate robotics, or plate readers.

It is important to be able to collect embryos from particular compartments after a screen for further analysis. Embryos can be easily collected from desired locations on DMA by rolling the droplet of water (Video S3) without the need to pipette each individual embryo. We used this principle for screening zebrafish embryos with a library of cell penetrating peptoids (CPPos). Zebrafish embryos were introduced to CPPos at their early developmental stages while on the DMA platform and afterwards collected for further cultivation and analysis of the CPPos’ distribution on later developmental stages. Finally the distribution of CPPos in zebrafish larvae introduced to them on the DMA and in 96-well plates was identical, further
confirming suitability of the DMA platform for introduction of zebrafish embryos to chemical compounds.

Compartmentalization of single embryos is possible in MTPs, however, in the majority of screenings performed in MTPs several embryos are located in one well in order to reduce the consumption of compound and reagents. However, screenings performed on single isolated embryos can represent the phenotypical change of each individual animal in response to a compound more accurately. In this case the possible influence of affected animals on its neighbors can be avoided. For example, it is well known, that by rupture of the chorion the hatching enzymes are released into the medium, promoting the hatching of neighboring embryos. This results in the fact that embryos incubated together hatch synchronously compared to embryos incubated separately. Such influence might interfere with the assessment of a compound’s toxicity and estimation of its direct effect on each individual embryo. Separation of individual animals is also beneficial for studies based on behavioral read-outs. For example, chemical screenings based on Photomotor Response (PMR) read-outs, where zebrafish embryos react to a light flash with an increase in motor activity, are typically performed on multiple embryos located in one well. In this case the movement of an embryo can be influenced by movements of neighboring eggs or hatched larvae. The DMA platform permits formation of a single-embryo-per-droplet array where both cross-contamination and cross-talk between the individual droplets are excluded.

Having single animals per compartment in defined locations is also convenient for microscopy. Microscopic analysis of animal models is challenging not only due to the three-dimensional complexity of the whole-organism, but also due to the fact that the animals can have different orientations toward an objective, can appear on top of each other, be randomly distributed in the well, and can sporadically move during the imaging. There are several methods for immobilization of zebrafish in the larval stage for imaging applications, such as systems based on agarose, cellulose or plasma-thrombin. On the DMA platform each embryo is fixed in a defined position due to water surface tension, which simplifies its tracking and microscopic analysis. The size and geometry of HH spots on the DMA platform can be easily adapted depending on size and developmental stage of the animal.

In addition to complex microscopic read-outs that are possible to perform directly on the array, the DMA platform enables estimation of toxicity by quantification of viability using a digital camera image of a single-embryo-per-droplet array. In such images the droplets containing coagulated embryos could be easily distinguished from the droplets with healthy embryos by the discoloration of the medium caused by protein release and degradation followed by chorion disruption. Such read-outs allow for fast and easy estimation of toxicity in a miniaturized array format from a single image of an array without the need for sophisticated microscopy and image analysis. This process could be automated and performed, for example, directly on a smart phone in several seconds time, introducing a completely new concept of functional biological read-out of whole-organism toxicity screenings.

In the current study we have demonstrated for the first time a whole-organism screening in miniaturized array format on a Droplet-Microarray platform. Using zebrafish embryos as a model for our proof-of-principle study, we have optimized pipetting-free spreading and collection of embryos, incubation of embryos in volumes as low as 5 µl, parallel compound addition and microscopic analysis directly on the platform. Thus, DMA technology enables (1)
miniaturization; (2) pipetting-free handling; (3) single-animal screening; (4) compartmentalization and immobilization of animals in defined locations; (5) image-based read-out directly on the platform. Due to its universality and flexibility of the DMA platform, it could be adapted for a wider spectrum of whole-organism studies, where it can be used for introduction of minute amount of compounds, immobilization of animals for imaging as well as for more sophisticated screening workflows. We believe that the DMA technology can make whole-organism screenings more cost- and time-efficient, as well as enable screenings that were not conducted before due to limited compound availability or complex handling.

**Experimental Section**

*Preparation of Droplet-Microarray slides*

“Fabrication of superhydrophilic-superhydrophobic patterned surfaces of Droplet-Microarray (DMA) slides was presented previously 30, 44. Glass slides (Schott Nexterion) were first incubated with 1M NaOH (Carl Roth GmbH + Co. KG, Karsruhe, Germany) for 1 hour and neutralized with 1M HCl (Merck KGaA, Darmstadt, Germany) for 30 minutes. Activated glass slides were modified with 20% v/v solution of 3-(trimethoxysilyl)propyl methacrylate* in ethanol for 30 minutes at room temperature. Polymer layer was introduced by first by applying 25 μL of polymerization mixture (24 wt% 2-hydroxyethyl methacrylate (HEMA)*, 16 wt% ethylene dimethacrylate (EDMA)*, 12 wt% 1-decanol, 48 wt% cyclohexanol* and 0,4 wt% 2,2-dimethoxy-2-phenylacetophenone* onto an inert glass slide, then, covering it with modified glass slide and, finally, cross-linking the polymer by UV irradiation with 5 mW/cm² intensity and 260 nm wavelength for 20 minutes. Inert glass slides were fluorinated in trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane* vapor under 50mbar vacuum overnight. The polymer layer was modified with alkyne groups by incubating the slides in solution containing 45 mL of dichloromethane (Merck KGaA, Darmstadt, Germany), 56 mg of 4-(dimethylamino)pyridine (Novabiochem, Merck KGaA, Darmstadt, Germany) and 180 µl of N,N′-diisopropylcarbodiimine (Alfa Aesar) for 4 hours under stirring at room temperature. Superhydrophobic background was created by applying 5% v/v solution of 1H,1H,2H,2H-Perfluorodecanethiol* in acetone onto the polymer surface and irradiating the slide through a photomask (Rose fotomasken, Bergisch Gladbach, Germany) with 260 nm UV light at 5 mW/cm² for 1 min. Following that superhydrophilic spots were formed by applying 10% v/v ß-mercaptoethanol (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) solution in 1:1 water:ethanol onto the patterned surface and irradiating the slide with 260 nm UV light at 5 mW/cm² for 1 min. * chemicals were purchased in Sigma-Aldrich Chemie, Munich, Germany.”

*Spreading and incubation of zebrafish embryos on Droplet-Microarray*

Zebrafish embryos 24 hours post fertilization were cleaned from coagulated eggs and spread over the dry Droplet-Microarray slide using Pasteur pipette with opening of x mm (Video S2). Embryos were taken up with a Pasteur pipette with an opening of 3 mm and aligned inside the pipette in one row. The pipette was moved with constant speed along the rows of hydrophilic spots to gradually push single embryos from the pipette one after another. Each
embryo comes to the tip of the pipette by gravity and is pushed out onto a hydrophilic spot with liquid around it. The droplet breaks at the edge of the hydrophilic spot while the pipette is moved over the superhydrophobic border leaving the embryo trapped in a droplet on the hydrophilic spot (Fig. 1a, Video S2). Obtained Droplet-Microarray containing zebrafish embryos was placed inside a 10 cm Petri dish, which was placed inside a 15 cm Petri dish containing wet tissues to ensure fast equilibration of the humidity (Fig. S1c). This setup prevented fast evaporation of the droplets during the incubation. Zebrafish embryos were incubated in incubator with constant temperature of 29℃ for 24 hours. Images of zebrafish embryos in individual droplets were taken by Keyence BZ-9000 microscope (Keyence, Japan)

**Measuring the volume of the droplets**

Zebrafish embryos were spread onto dry Droplet-Microarray slide using Pasteur pipette (Video S2). The full volume of a droplet was aspirated with automated pipette to estimate the exact volume of spontaneously formed droplets. In total 108 droplets were measured.

**Sandwiching method for addition of substances**

For addition of CPPos and toxic substances into droplets containing zebrafish embryos the sandwiching method described earlier was applied\(^9\). The substances of interest (CPPos, ZnCl\(_2\), AgNO\(_3\)) were printed onto fluorinated glass slide in geometry corresponding to geometry of Droplet-Microarray using ultra-low volume non-contact dispenser sciFLEXARRAY S11 (Scienion). Glass slides (Schott) used for printing were fluorinated by incubating overnight in desiccator containing open vial with 30 µl of trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane under 50 mbar vacuum. The amount of substance printed per droplet was calculated for desired final concentration in droplet with assumption that each droplet has a volume of 5 µl. Pre-printed slides were dried overnight in darkness in a dry box containing drying silica gel. For parallel addition of substances to zebrafish embryos Droplet-Microarray containing zebrafish embryos was fixed in lower holder of the aligner (Fig. S1b), the glass slide with pre-printed substances was fixed in the upper holder of the aligner (Fig. S1a). Afterwards two holders were sandwiched together (Fig. S1c) precisely aligning both Droplet-Microarray and pre-printed slides. Four micrometer screws on top of the upper holder (Fig. S1c) were turned clockwise till the end ensuring maximum distance between the slides. Two slides were brought together by turning the micrometer screws (Fig. S1c, red arrows) counterclockwise till the droplets touched the upper slide, which could be monitored through the window (Fig. S1d). In this position aligner was placed in incubator with constant temperature of 29℃ for 15 minutes. To avoid evaporation of droplets during the substances transfer the aligner was covered with tissues wetted with fish water. After 15 minutes the aligner was opened and Droplet-Microarray containing zebrafish embryos was placed back to double Petri dish and in incubator with constant temperature of 29℃ for 24 hours.

To evaluate sandwiching approach for transfer of precise concentrations of substances to the droplets zebrafish embryos on DMA was introduced to 6 different concentrations of fluorescent dye (fluorescently labeled peptoid NlysNlysNpheNlysRhodB, ID number 175). Fluorescent dye was printed using ultra-low volume non-contact dispenser sciFLEXARRAY S11 (Scienion) in amounts 1, 2, 4, 6, 8, 10 nmol in three repeats onto fluorinated glass slides and dried. The printing and transfer of preprinted dye to DMA was
performed as described above. To compare concentrations of dye in the droplets mean fluorescence intensity of droplets after dye transfer was calculated using ImageJ software. The images were first converted to 8 bit format. Afterwards each square droplet was selected manually and mean gray value was measured. Droplets were not distinguished according to the volume and all repeats were taken into analysis. Mean values of mean fluorescent intensities obtained from three repeats of each concentration of dye were plotted and error bars represent standard deviation of three repeats.

**Synthesis of fluorescently labeled cell penetrating peptoids (CPPos)**

The synthesis of the cell penetrating peptoid library was carried out as reported by Kölmel et al. using permutation of four different side chains (\(N\)-2-prop-2-yn-1-ylglycine (Nprg), \(N\)-(p-chlorobenzyl)glycine (Npcb), \(N\)-4-aminobutylglycine (Nlys) and \(N\)-benzylglycine (Nphe)). All CPPos were labeled with Rhodamine B.

Table 1. The list of CPPos used for the proof-of-concept screening of zebrafish embryos on DMA platform and their final locations in zebrafish larvae.

<table>
<thead>
<tr>
<th>CPPo ID Nr</th>
<th>CPPo sequence</th>
<th>Location in zebrafish larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>Nprg/Npcb/Npcb/NprgRhodB</td>
<td>Skin</td>
</tr>
<tr>
<td>68</td>
<td>Nprg/Npcb/Npcb/NprgRhodB</td>
<td>Skin</td>
</tr>
<tr>
<td>111</td>
<td>Npcb/Nprg/Nprg/NpheRhodB</td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Olfactory system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Otic vesicle</td>
</tr>
<tr>
<td>175</td>
<td>Nlys/Nlys/Nphe/NlysRhodB</td>
<td>Olfactory system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Otic vesicle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caudal Vein</td>
</tr>
<tr>
<td>183</td>
<td>Nlys/Nphe/Npcb/NlysRhodB</td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Olfactory system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Otic vesicle</td>
</tr>
<tr>
<td>208</td>
<td>Nphe/Nprg/Nlys/NlysRhodB</td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Olfactory system</td>
</tr>
<tr>
<td>214</td>
<td>Nphe/Npcb/Npcb/NpcbRhodB</td>
<td>Skin</td>
</tr>
</tbody>
</table>
The 2mM solution of CPPos was printed onto fluorinated glass slides (for details see “Sandwiching method for addition of substances”) in amount of 500 nL per each spot. Pre-printed slides were dried overnight in darkness in a dry box containing drying silica gel. Zebrafish embryos were exposed to CPPos at 24 hours post fertilization by sandwiching method (for details see “Sandwiching method for addition of substances”) and incubated in droplets on Droplet-Microarray for 24 hours. Six embryos were exposed to each CPPo per experiment, the experiment was repeated three times. Afterwards, embryos exposed to one CPPo were washed together in one Petri dish with Pasteur pipette (Video S3) and incubated for 24 hours to let embryos hatch. At 72 hours post fertilization zebrafish larvae were plated into 96-well plate and imaging was performed at 10x magnification in bright field and with RFP filter using Olympus IX81 microscope (Olympus, Tokyo, Japan).

**Screening of CPPos in 96-well plate**

Zebrafish embryos 24 hours post fertilization were plated in 96-well plate in amount of 3 embryos per well, which contained 100 µl of fish water and 20 µl of 2mM CPPo. Embryos were incubated with CPPos for 24 hours. Afterwards, embryos exposed to one CPP were placed in Petri dish together and incubated for 24 hours to let them hatch. At 72 hours post fertilization zebrafish larvae were plated into 96-well plate and imaging was performed at 10x magnification in bright field and with RFP filter using Olympus IX81 microscope (Olympus, Tokyo, Japan).

**Toxicity screening on Droplet-Microarray**

Water solutions of ZnCl2 and AgNO3 were printed onto fluorinated glass slides (for details see “Sandwiching method for addition of substances”) in appropriate amounts to achieve the following final concentrations in droplets: 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µM for ZnCl2 and 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.05, 0.1 µM for AgNO3. For each concentration three Droplet-Microarray slides containing 108 droplets in 18 columns and 6 rows were used (Fig. 1a). Each of those slides contained 12 control untreated embryos (2 columns x 6 droplets) and 96 embryos (16 columns x 6 droplets) exposed to one concentration of the toxicant. In total 288 embryos were exposed to each concentration of both toxicants. The addition of compounds and incubation was performed as described in “Sandwiching method for addition of substances”. Zebrafish embryos were exposed to compounds at 24 hours post fertilization for 24 hours. To analyze the effect of ZnCl2 and AgNO3 the viability of embryos was estimated. For this purpose, the full Droplet-Microarray slide was imaged using Keyence microscope in bright field at 2x magnification (Fig. 5a) and viability of embryos was estimated by eye. The embryo was considered alive if it had normal morphology and a heartbeat, and the embryo was
considered dead if it had abnormal morphology and no heartbeat (Fig. 5a). The viability was plotted against the concentration of the toxicant using GraphPad Prism 7. The sigmoidal curves were created based on nonlinear regression technique. IC50 values of the compounds were calculated in the program.

**Toxicity screening in 96-well plate**

Water solutions of ZnCl$_2$ and AgNO$_3$ were pipetted into 96-well plate in appropriate amounts to achieve the following final concentrations in a well: 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µM for ZnCl$_2$ and 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.05, 0.1 µM for AgNO$_3$. Afterwards the plates were dried on air. Zebrafish embryos were pipetted into each well containing dried toxicant in amount of 5 embryos in 200 µl of fish water per well. In total 180 embryos were analyzed for each concentration of both toxicants. Zebrafish embryos were exposed to compounds at 24 hours post fertilization for 24 hours. To analyze the effect of ZnCl$_2$ and AgNO$_3$ the viability of embryos was estimated. For this purpose, embryos were imaged in bright field at 2x magnification using automated Olympus IX81 microscope (Olympus, Tokyo, Japan). Viability of embryos was estimated by eye. The embryo was considered alive if it had normal morphology and a heartbeat, and the embryo was considered dead if it had abnormal morphology and no heartbeat (Fig. 5a). The viability was plotted against the concentration of the toxicant using GraphPad Prism 7. The sigmoidal curves were created based on nonlinear regression technique. IC50 values of the compounds were calculated in the program.

**Acknowledgements**

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**Declaration.**

All experiments used zebrafish larvae between 1 and 2 dpf for which no special animal licensing is required and was carried out following European and national regulations. Husbandry is performed in accordance with the German law on Animal Protection and the Directive 2010/63/EU. Our animal facility is supervised and regularly inspected by the competent authority “Regierungspräsidium Karlsruhe” (#Az.35-9185.64).

**References**


