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Zebrafish Entrapment By Restriction Array (ZEBRA) device: a low-cost, agarose-free zebrafish mounting technique for automated imaging†

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Abstract

The zebrafish has emerged as a useful model system for a variety of studies, including the investigation of inflammation and immunity. However, current zebrafish imaging techniques, such as agarose mounting, can be time-consuming and detrimental for long-term imaging.

Alternatively, automated sorting and imaging systems can be costly and/or complicated to assemble. Here we describe the Zebrafish Entrapment by Restriction Array (ZEBRA) device, a microfluidic device that can be used to quickly and repeatably position zebrafish embryos in a predictable array using only a pipette. This technique is well suited for use with automated microscope stages leading to decreased imaging time and increased throughput compared to traditional methods. The addition of access ports above the embryo can be used to administer treatments, and potentially wounding or injections. We demonstrate the effectiveness of this device for a neutrophil migration screening application using larvae 3 days post fertilization (dpf) Tg(mpx:dendra2). Larvae were loaded into ZEBRA devices and treated with a neutrophil attractant (LTB₄) or LTB₄ with and without a PI3K inhibitor, LY294002. Treatment with LY294002 impaired neutrophil motility into the fin induced by LTB₄ treatment. The findings report the development of ZEBRA a device that can be used to screen for small molecules that affect leukocyte motility and inflammation using live zebrafish.

Introduction

The zebrafish has emerged as a useful model system for a variety of studies due to its optical transparency and amenability to genetic manipulation and chemical screens.^{1–3} Because the immune systems are largely conserved between zebrafish and mammals, the zebrafish is a good model for studying inflammation and immunity.^{4–6} Current imaging techniques

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typically involve positioning embryos in agarose,^{7,8} which can be tedious and time-consuming for larger scale screening applications using traditional microscopy. These methods can lead to imperfect alignment of the embryos with different parts of the embryos in different imaging planes and irregular spacing between embryos. Both of these issues limit the use of traditional automated imaging systems involving an automated stage with programmable movements. Additionally, embedding embryos in agarose is not ideal for long-term imaging as it can result in developmental defects.⁹

To overcome the limitations inherent in the traditional agarose mounting and imaging process, several commercial and non-commercial automated systems for sorting, handling and imaging zebrafish have been developed.^{10,11} However, these systems can be costly and/or complicated to assemble, which limits their accessibility and wide-spread usage. Agarose molds for use in high-throughput histology studies were designed to place embryos in wells that are aligned in predictable arrays,¹² and an extension of this device to position live zebrafish embryos in a predictable array for use with an automated stage could be imagined. However, while this system is low-cost, manipulation of embryos to correctly position embryos is still required. Others have developed microfluidic chips that can be used to trap and/or treat zebrafish embryos for a variety of studies but these systems can be complicated for everyday use as they require networks of syringe pumps and tubing interfaced with a polydimethyl-siloxane (PDMS) chip.^{13–15}

Here we present the Zebrafish Entrapment by Restriction Array (ZEBRA) device, a microfluidic device operated with a simple pipette using surface-tension driven pumping (aka “passive pumping”)¹⁶ to quickly position zebrafish embryos in a predictable array. The device can be used to position embryos for imaging the lateral or dorsal view. Automated imaging can be performed with the use of an automated stage and without the requirement of specialized detection equipment. Because agarose is not needed to embed the embryo, long-term live imaging can be performed without risking the developmental integrity of the embryo. Access ports can be added above the embryo in a location that can be adapted for a variety of experiments that require treatments, wounding or injection. We demonstrate the effectiveness of this device for use in a neutrophil migration screening application to identify small molecules that affect inflammation.

Materials and methods

Zebrafish maintenance

All protocols that used zebrafish in this study were approved by the University of Wisconsin-Madison Research Animals Resource Center. Adult zebrafish and embryos were maintained according to standard protocols¹⁷ and raised at 28.5 °C.

Device fabrication

For ZEBRA devices made in polystyrene, designs were created in SolidWorks 2010 and converted to SprutCam 7. The channels were milled using a Tormach Personal CNC 770 into 1.2 mm thick polystyrene using the Mach3 CNC controller program. The channels are then chemically bonded to 0.19 mm thick polystyrene using acetonitrile to create a closed

channel with two open ports. To fabricate PDMS ZEBRA devices that could be bonded to glass for high-resolution imaging, PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) base and crosslinker were mixed at a 10 : 1 ratio and degassed for 45–60 min under vacuum at room temperature. The degassed PDMS was then poured over SU-8 master molds which were generated using standard soft lithography methods.¹⁸ The PDMS was cured at 80 °C for 4 h, and allowed to cool to room temperature prior to removal from the master mold. Devices were then oxygen-plasma-treated to bond the PDMS channels to a glass surface and to render the inside of the chambers hydrophilic.

Device loading and characterization

The ZEBRA device was initially filled by adding water to the output port and aspirating from the input port until water was drawn into the entire channel. Following anesthetization in E3 containing 0.2 mg ml⁻¹ tricaine (ethyl 3-aminobenzoate, Sigma Aldrich), passive pumping¹⁶ is used to load an embryo into the channel. To position the embryo on its side it must be loaded head-first into the ZEBRA device which is accomplished by taking the embryo into the pipette tail-first and adding it to the input port of the ZEBRA device (Fig. 1B, ESI Video 1[†]). To position the embryo vertically it must be loaded tail-first into the ZEBRA device, by first taking the embryo into the pipette head-first and then adding it to the input port of the ZEBRA device (Fig. 1C, ESI Video 2[†]).

For characterization of optimal dimensions for the ZEBRA device for zebrafish at 3–5 days post fertilization (dpf), channels were designed with a range of heights between 0.25 mm and 1.0 mm, widths between 0.4 mm and 1.2 mm, and constrictions with widths between 0.3 mm and 0.6 mm. The optimal channel design consists of an oval input port 6 mm by 3 mm and circular output port with 8 mm diameter, both 1.2 mm tall. A straight channel 0.8 mm wide and 0.5 mm tall connects the two ports and has a constriction 0.3 mm wide towards the end. The total length of the device is 30 mm long. To characterize the repeatability of trapping zebrafish embryos in different orientations, twenty-five to thirty-five 3, 4, or 5 dpf zebrafish were loaded into the device head-first and tail-first. The occurrences of zebrafish embryos being trapped on their side or with their dorsal side facing upwards were counted.

To facilitate the addition of treatments to the zebrafish, ZEBRA devices with small access ports placed above the embryos were developed. Access ports were elliptical in shape with major axes of 1 mm and minor axes of 0.6 mm. To characterize the addition of treatments, 5 µL of a blue food dye was added to the access port above a 4 dpf embryo loaded into the channel. The dye filled the channel due to passive pumping (Fig. 2D, ESI Video 3[†]). The embryo was then rinsed by adding 5 µL of E3 water to the access port (Fig. 2E, ESI Video 4[†]).

The ZEBRA devices with access ports (Fig. 2) and multi-ZEBRA devices (Fig. 3) were initially filled with water using similar techniques as described for the single ZEBRA devices. Embryos were loaded into the ZEBRA devices with access ports in the same manner as with the normal ZEBRA devices. To load embryos into the multi-ZEBRA device, passive pumping¹⁶ was used to load four embryos, following anesthetization by tricaine, one

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by one into each of the four channels (Fig. 3). After an embryo is loaded into one of the channels, flow is restricted causing the next embryo to flow into one of the remaining channels, similar to the “worm clamp” device presented by Hulme *et al.*¹⁹ For long-term trapping of embryos, the devices were kept in a humidified bioassay tray containing water for the 24 h duration to minimize evaporation as described in Berthier *et al.*²⁰ These precautions were not taken during shorter-term experiments (45–60 min), but no observable evaporation or effects were seen.

Treatment and screening

Drug or staining treatments can be applied to the embryos loaded in the ZEBRA device *via* passive pumping¹⁶ by micropipetting the treatment to the small access port above the embryo (Fig. 2). Similarly, the treatment can then be rinsed away by micropipetting water into the access port (Fig. 3). For drug treatment with LTB₄, 3 dpf Tg(mpx:dendra2)⁵ embryos were placed into the channels, and 5 µl of 150 nM LTB₄ was added to the access port. To inhibit migration of neutrophils from the caudal hematopoietic tissue (CHT) 3 dpf embryos were also treated with 5 µl of 65 µM LY294002. To view neutrophil migration, embryos were imaged every 30 s for 45 min intervals following drug treatment. Neutrophils that migrated from the CHT to the fin were counted before and after treatment (Fig. 4).

Image acquisition

Images and videos of zebrafish loaded into the ZEBRA device were taken using an Olympus SZX12 stereoscope and PixeLINK Capture acquisition software. Time-lapse fluorescence images were taken on a Nikon SMZ-1500 zoom microscope (Nikon, Melville, NY, USA). Fluorescent confocal images were acquired with a light-scanning confocal microscope (FluoView FV1000, Olympus, Center Valley, PA, USA) using a numeric aperture 0.75/20× objective.

Results and discussion

The ZEBRA device was designed and optimized to immobilize 3–5 dpf zebrafish embryos in a predictable array without the use of agarose. The device consists of an enclosed channel with a restriction width that is accessed *via* an input and an output port (Fig. 1A). The device is first filled with water as described above. To speed the filling process, the devices can be oxygen-plasma treated to render the inside of the channels hydrophilic. Other possible methods to fill the device include adding a droplet of fluid to the input port and applying a vacuum to the output port, or using vacuum filling.²¹ After initial filling, a pipette can be used to add a droplet of water containing a selected zebrafish embryo to the input port of the device. Due to differences in surface tension water is driven from the smaller input port (with higher surface tension) to the larger output port (with lower surface tension); this is known as passive pumping.¹⁶ Upon reaching the channel restriction the zebrafish is trapped in place. Users can choose whether to load the zebrafish into the device head-first or tail-first depending on the desired application (Fig. 1).

The preferred orientation of 3–5 dpf embryos is to lie horizontally. When an embryo is loaded into the ZEBRA device and passive pumping is used to apply flow, the embryo is

move horizontally through the channel. When an embryo is loaded head first into the device, the head enters the constriction and is pinned with the embryo remaining horizontal (Fig. 1B, ESI Video 1[†]). When an embryo is loaded into the device tail first, the embryo still flows through the channel horizontally but upon encountering the constriction flow continues to drive the embryo into the constriction (Fig. 1C, ESI Video 2[†]). Unlike the head of the channel, the tail is more pliable so as the embryo is driven tail-first into the channel the embryo is turned (starting at the tail) to a vertical position. When the yolk sack (which is larger in diameter than the constriction) of the embryo encounters the constriction, the embryo is then pinned in the channel dorsal side up.

In practice, choosing which orientation to load zebrafish into the ZEBRA device is similar to and potentially easier than choosing and orienting individual embryos with traditional mounting techniques (*i.e.* agarose mounting). Using traditional agarose embedding methods, it could take close to 5 min or longer, depending on the user, to position each embryo in a predictable array for imaging. Alternatively, the ZEBRA device allows users to position each embryo in 10 s or less. Additionally, preparing and cooling the agarose adds up to 15 min to the immobilization process, whereas the initial filling the ZEBRA device takes less than 1 min.

Using channels with the optimized dimensions described above, it was found that when zebrafish were loaded into the device 100% of 3, 4, or 5 dpf zebrafish were trapped on their side. When 15 embryos were trapped horizontally in the device, we found that the tail fins were positioned with the tip of the tail fin between 0 and 150 μm from the edge with an angle between -3 and 12 degrees from the center-line of the device. For these measurements, all embryos had their ventral side facing the bottom of the channel. However, in practice 50% of embryos were trapped facing this direction, while 50% faced the other direction so this will cause some variation in the exact position of the tail fins. This variation may not be an issue at lower magnification as more of the tail fin and device can be seen in one field of view; for higher magnification it may be necessary to scan several fields of view when using automated imaging systems to completely capture the desired area. Using confocal imaging we found that the tail fins were within a vertical range of 50 μm or less. However, this range may be affected based on the specific imaging system in use.

When zebrafish were loaded into the device tail-first, 77% of 3 dpf zebrafish were trapped vertically with the dorsal side facing upward, while 23% were trapped with the ventral side facing upward. We observed that 100% of 4 or 5 dpf were trapped with their dorsal side facing upward. Because the device parameters and operation remained consistent, the inconsistencies in trapping 3 dpf embryos in the vertical position is likely due to the fact that during development, some 3 dpf embryos may be less “bottom heavy” compared to further developed 4–5 dpf embryos. Therefore, as this subset of 3 dpf embryos flows horizontally through the channel, there is a chance that the embryo will turn with their ventral side facing upward when the tail encounters the constriction.

While most of the ZEBRA devices used in this study were fabricated from polystyrene, it is possible to use a variety of materials for different applications. For example, ZEBRA

devices for use with high-resolution confocal imaging were fabricated from PDMS with a glass slide on the bottom (Fig. 2A and B). Finally, it was found that zebrafish embryos could be trapped in the ZEBRA device for at least 24 h without adverse effects, indicating that this device may be a suitable option for long-term imaging applications. After 24 h there was no observable evaporation so it is predicted that the length of experiment can be increased further.

ZEBRA devices with small access ports above the zebrafish trapping region were designed to enable the delivery of treatments using a smaller volume of reagents than would be required if treating through the input port. Fig. 2C–E (and ESI Videos 3 and 4[†]) demonstrate the addition of 5 μ L of a blue dye to the access port and then rinsing with 5 μ L of water. Whether adding treatments through the input port or the access port, the predictable placement of the ports is amenable for use with automated liquid handling systems for increased throughput. Additionally, the access ports could potentially be used for wounding experiments or to provide injections while the zebrafish is immobilized in the channel.

Multiple ZEBRA devices controlled by a single set of input and output ports were developed to trap multiple zebrafish embryos (Fig. 3). The multi-ZEBRA device enables the positioning of four zebrafish in a smaller area compared to 4 single ZEBRA devices. The device works similarly to a device described in Hulme *et al.*¹⁹ to trap *C. elegans*. After a zebrafish is loaded into one of the channels in the multi-ZEBRA device, flow is restricted causing the next embryo to flow into one of the remaining channels. This is repeated until all of the channels are filled. The multi-ZEBRA device could potentially be expanded to contain a greater number of trapping regions.

To test if the ZEBRA device would be amenable for screening, single ZEBRA devices with access ports were used and zebrafish were treated with LTB₄, which attracts neutrophils.²² 3 dpf Tg(mpx:dendra2)⁵ embryos were placed into the channels, and 5 μ L of 150 nM LTB₄ was added to the access port. To view neutrophil migration embryos were imaged for 45 min intervals following drug treatment. Neutrophils that migrated from the CHT to the fin were counted before and after treatment (Fig. 4C and D). To inhibit migration of neutrophils from the CHT 3 dpf embryos were treated with 5 μ L of 65 μ M LY294002, a PI3K inhibitor.²³ Neutrophils that migrated into the fin were counted and it was confirmed that inhibition of PI3K reduced neutrophil migration from the CHT to the fin with LTB₄ treatment (Fig. 4E and F). This data demonstrates that the ZEBRA device can be used for zebrafish drug screens without the negative effects that are seen with typical agarose embedding.

Conclusions

We have developed and characterized a method to quickly and repeatably position zebrafish embryos in a predictable array using the ZEBRA device. The device can be used to position embryos horizontally or vertically, and can be designed with access ports to enable the administration of treatments. We demonstrated the use of this device in a screening application by observing neutrophil migration in response to drug treatment. Due to its low

cost and ease-of-use, the ZEBRA device is a promising alternative zebrafish mounting technique for medium-throughput applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A) Image of ZEBRA device fabricated in polystyrene. B) Schematic and image of 4 dpf zebrafish loaded into the device on its side. The zebrafish is first taken into a pipette tail-first and then loaded into the device head-first *via* passive pumping. C) Schematic and image of 4 dpf zebrafish loaded into the device vertically. The zebrafish is taken into a pipette head-first and then loaded into the device tail-first. Images in B and C are in devices fabricated in PDMS. Scale bars represent 0.5 mm.

Fig. 2.

A–B) ZEBRA devices were fabricated out of PDMS and bonded to glass to facilitate high-resolution confocal imaging. Images of the tail region (A) and head region (B) of a 3 dpf Tg(mpx:dendra2) zebrafish embryo trapped in a ZEBRA device fabricated from PDMS and a glass slide on the bottom. Neutrophils are shown in green. Scale bar represents 50 μ m. C–E) ZEBRA devices with access ports were developed to facilitate treatments and screening. C) Image of a 4 dpf zebrafish embryo in a single ZEBRA device with access port. D) After the addition of 5 μ L of blue dye to the access port the embryo is completely covered. E) The embryo is dye-free again after rinsing with 5 μ L of water through the access port. Devices shown in C–E were fabricated in polystyrene. Scale bars represent 0.5 mm.

Fig. 3. Image of the multi-ZEBRA device. Four embryos were sequentially loaded head-first into the device. Scale bar represents 5 mm (top) and 0.5 mm (bottom).

Fig. 4.

A neutrophil migration screening assay was performed on 4 dpf Tg(mpx:dendra2) zebrafish embryos. A–B) Images of an untreated embryo tail at 0 min and 45 min. C–D) Images of an embryo treated with LTB4 at 0 min and 45 min after treatment. E–F) Images of an embryo treated with LTB4 and the neutrophil migration inhibitor LY294002 at 0 min and 45 min following treatment. G–H) 10 embryos were treated with either LTB4 or LTB4 and LY294002 and neutrophils that migrated from the CHT into the fin were counted before and after treatment. Our results indicate that migration was reduced when the inhibitor LY294002 was applied. Scale bar represents 100 μ m.