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## Fish in chips: an automated microfluidic device to study drug dynamics *in vivo* using zebrafish embryos

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Interference of the Hedgehog (Hh) signaling pathway by cyclopamine leads to abnormal embryonic development. We monitor this dynamical drug effect in zebrafish embryos with highly precise microenvironment control using an integrated microfluidic device. This chip-based platform, which is programmable and automated, greatly facilitates the accuracy and reproducibility of the *in vivo* assays.

The reactions between drug molecules and live organisms are highly dynamic. Modern drug discovery depends greatly on automated screening to objectively evaluate the efficacy of candidates, as well as the dynamics of reactions.<sup>1</sup> Due to the intrinsically low consumption of samples and reagents, micro-fluidic devices have been successfully applied to large scale screening of biomolecules through intermolecular interactions,<sup>2</sup> cell-based bioassays,<sup>3</sup> and phenotyping of multicellular organisms.<sup>4,5</sup> Additionally, microfluidic approaches have been reported to evaluate drug dosage dependence on-chip, to perform single cell assays,<sup>6</sup> create many isolated compartments and droplets for cell-based assays,<sup>7</sup> to test the compound toxicity, and to evaluate cells' dynamical behaviors.<sup>8</sup>

To further study the pharmacological effects of drugs, many experiments have also been applied to whole-animals, using *C. elegans* and zebrafish as model systems.<sup>9,10</sup> These experiments, which include high-throughput image capture,<sup>11</sup> laser surgery,<sup>12</sup> embryo injection,<sup>13</sup> and drug screening,<sup>14</sup> greatly reduce the labor compared with conventional methods. Chip-based approaches have demonstrated many advantages including the ability to create parallel and reproducible microenvironments for experiments.<sup>15</sup> The flexibility and accuracy of liquid manipulation, typically at the picoliter to nanoliter scale, allows experimentation with higher credibility by eliminating handling variations.<sup>16</sup>

Here we report an integrated microfluidic device for drug screening of zebrafish embryos. The zebrafish is a widely used model organism in chemical genetic/genomic screening and studies on embryonic development.<sup>17,18</sup> One of the most important advantages is that zebrafish embryos are easy to visualize and permeable to small molecules. We explore the impact of a small molecule, cyclopamine, on the embryonic development of zebrafish. Cyclopamine as well as its derivatives and analogues have been considered as drug candidates since they can effectively interfere with the Hedgehog (Hh) signaling pathway through the direct inhibition of a seven-pass transmembrane G-protein-coupled receptor-like protein Smoothened (Smo).<sup>19</sup> The Hh pathway is highly related to the embryonic pattern formation and the development of numerous cancers.<sup>20</sup> Hh signaling deficiency will also lead to defects of the primary intersegmental vessel sprouting, causing the characteristic phenotypes such as the U-shaped somites and shorter intersegmental blood vessels (ISV) in zebrafish embryos.<sup>21,22</sup>

We constructed the integrated microfluidic device (Fig. 1a) made up of poly(dimethylsiloxane) (PDMS) through multi-layer soft lithography.<sup>23</sup> This device had two layers of channels: control channels and fluidic channels, and the crossover of these two layers of channels formed monolithic micro-valves. The fluid flow can be pneumatically controlled through valve actuations. An array of openly-accessible culture chambers was punched through a PDMS chip. Each chamber is 4 mm in diameter with a small volume of about 40 µL, suitable for culturing a single embryo of zebrafish. Unlike the most multilayer microfluidic devices, the wells can be easily accessed from the top, making embryo transfer easy using pipettes. The crosssection of fluidic channels is much smaller than the size of embryos, ensuring that the embryos stay inside the wells during the fluidic flow. In each chip we placed 24 chambers, which could be observed simultaneously under a stereoscope. We monitored each embryonic development through phase contrast microscopy until 36 hours post-fertilization (hpf). By comparing the embryos that were cultured using conventional 96-well plates, we found that there was no phenotypical

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**Fig. 1** (a) The zebrafish embryo culture chip, and phase-contrast images of zebrafish cultured in different conditions. Pictures are taken at 36 hpf. (b) The schematic diagram of medium refreshment. (c) Phase-contrast images of zebrafish cultured in microfluidic chip with and without the "withdraw-refill" medium changes. Pictures are taken at 36 hpf.

difference between two culture methods. However, the chipbased experiment provided the flexibility of controlling the microenvironment during the culture period, offering a much better solution for study of dynamic response to drug treatment *in vivo*.

For long-term zebrafish culture and drug treatment, the medium refreshment is critical. We cultured the single zebrafish embryos in the chamber array, and applied "withdrawrefill" cycles to efficiently replace the old medium with the new medium within 10 s (Fig. 1b). When a medium change was needed, we closed the input valves to stop medium supply and opened the output valves to suck the old medium out of the chambers using vacuum. When the chambers were completely withdrawn, we closed the output valves and opened the input valves to refill the chamber with fresh medium under the positive pressure (2 psi). The zebrafish embryos developed normally on-chip, indicating the little impact of fluid flow caused by these "withdraw–refill" cycles.

In the Hh pathway, Smo and Patched (Ptc) are two key transmembrane proteins. In the absence of the Hh ligand, Ptc suppresses the activity of Smo and finally represses the transcription of target genes. While the Hh ligand binds to Ptc to block its suppression on Smo, the release of Smo activates the glioma-associated oncogene homolog (Gli) to its activated form (Gli-A), which could be transported into the nucleus and initiate the expression of downstream target genes (Fig. 2a).<sup>24,25</sup>



**Fig. 2** The Hedgehog (Hh) signaling pathway, with (a) and without (b) cyclopamine binding. (c) The GFP fluorescence intensity of *8xGli:GFP* zebrafish incubated by the medium with different concentrations of cyclopamine from 6 to 36 hpf.

Cyclopamine, as well as many of its derivatives, can interfere with the Hh pathway through suppressing of Smo, and sequentially repress the expression of downstream genes (Fig. 2b).<sup>26,27</sup>

We used a transgenic zebrafish, 8xGli:GFP, to detect the dynamics of the cyclopamine affecting Hh pathway. This zebrafish stably carries constructs with 8 Gli-A binding sites and the green fluorescent protein (GFP) gene. As a reporter, GFP is expressed after Gli-A binding to its binding sites in the presence of Hh signaling.<sup>22</sup> Hence the GFP expression clearly indicates the activation of the Hh pathway. We treated these transgenic zebrafish with cyclopamine at 0  $\mu$ M, 50  $\mu$ M and 100  $\mu$ M from 6 hpf until 36 hpf, and screened the fluorescent images at 36 hpf. In this experiment we did not change the medium. The reason for starting the drug treatment at 6 hpf was to try to eliminate other side effects since Smo starts to express at that time.

The GFP fluorescence intensity of the embryo bodies, especially the somites, reflected the endogenous activity of the Hh signal pathway, which was noticeably changed by the addition of cyclopamine, as shown in Fig. 2c. The response to cyclopamine is clearly dosage dependent. The zebrafish embryos treated with 30-60 µM cyclopamine did not cause a significant change of the GFP fluorescence intensity of the animal, while a cyclopamine concentration higher than 70 µM drastically reduced the expression of the GFP. Conventional experiments performed in larger volumes, using Petri dishes or micro-well plates, typically use 40 µM cyclopamine to treat zebrafish embryos to induce the defects.<sup>22</sup> We hypothesize that this slight difference in the dosage requirement is mainly due to two reasons: the total amount of the medium per culture well is less in the microfluidic platform, and the absorption and penetration of small molecules in PDMS. The detailed mechanism of this dosage difference remains to be further studied.

It has been reported that Hh signaling deficiency leads to defects in ISV sprouting, and the zebrafish embryos treated with cyclopamine will also induce U-shaped somites. Another transgenic zebrafish, *flk:GFP*, whose vascular endothelial cells were labeled using the GFP, was employed to validate the



**Fig. 3** Characterization of cyclopamine caused developmental abnormalities in *flk:GFP* transgenic zebrafish. (a) The bright-field and fluorescence images of the normal V-shaped somites and ISV of zebrafish embryos (36 hpf). (b) The bright-field and fluorescence images of zebrafish that were treated with cyclopamine.

influence of cyclopamine to somites and ISV of zebrafish embryos. We treated the single embryos with 0  $\mu$ M, 70  $\mu$ M and 100  $\mu$ M cyclopamine, respectively. To study the drug effect at different stages of the development and observe the dynamical interactions, we actively control the exposure of medium to the embryos with different periods: 6–36 hpf, 6–17 hpf, 17–36 hpf and 24–36 hpf.

The zebrafish embryos untreated with cyclopamine showed normal V-shaped somites and regular ISV at 36 hpf, as shown in Fig. 3a (white arrows), while treatment with cyclopamine often causes somites from V-shape to U-shape (Fig. 3b). Another typical abnormality caused by cyclopamine is the shortened ISV due to sprouting defects. We found that the occurrence of these abnormalities was related to the time that the embryos were treated with cyclopamine. The embryos treated with 70  $\mu$ M or 100  $\mu$ M cyclopamine at the earlier stage of the development, 6–36 hpf and 6–17 hpf, displayed the U-shaped somites, while treatment at the later stage, *e.g.*, 17–36 hpf and 24–36 hpf, did not have an effect on the somites (Table 1). For each condition, we tested at least 3 individual embryos. We did not observe the heterogeneity at the single embryo level.

Table 1 The somite development of zebrafish embryos treated with cyclopamine

Cyclopamine co	ne concentration			
	0 μΜ	70 µM	100 µM	
6-36 hpf	+	_	_	
6–17 hpf	+	_	_	
17–36 hpf	+	+	+	
24-36 hpf	+	+	+	

Note: "+": normal V-shaped somite, "-": abnormal U-shaped somite.

 Table 2
 The intersegmental blood vessels (ISV) development of zebrafish embryos treated with cyclopamine

	$0 \ \mu M$	70 µM	100 µM
6-36 hpf	+	_	_
6–17 hpf	+	+	+
17–36 hpf	+	_	_
24-36 hpf	+	+	+

The embryos treated with cyclopamine at 6–36 hpf and 17–36 hpf developed abnormally shortened ISV and when treatment time was 6–17 hpf or 24–36 hpf, the embryos had normal ISV (Table 2). We observed that the key period for ISV sprouting was 17–24 hpf, while the important time period for somite formation was 6–17 hpf. After 24 hpf, cyclopamine would not affect the zebrafish embryonic development on either ISV or somites any more. These results were comparable with the previous studies.<sup>21,22</sup>

In conclusion, we have developed an easily-accessible and automated microfluidic device to identify the effect of cyclopamine on ISV and somite development using zebrafish embryos. The whole process eliminated the tedious handling steps of medium changing or treatment variation by hand, and hence removed the barrier for high-throughput and automated drug screening. The design allows us to couple the conventional operations, e.g. pipetting, with this openly accessible device, and better fit the needs of chemical biology studies. We used the Glidependent GFP transgenic zebrafish as the reporter to monitor the effect of cyclopamine on the Hh signal pathway by imaging the characteristic phenotypes of zebrafish embryos. We found that in the microfluidic device cyclopamine could block the Hh signalling pathway at approximately 70 µM. We also confirmed the critical period of the effect of cyclopamine on somites and ISV (17-24 hpf and 6-17 hpf, respectively). Our platform is shown to be well applicable to high throughput zebrafish embryo-based automated drug screening with low consumption and waste at the same time, and may be suited for zebrafish larvae-based assays such as staining and drug screening. We also envision that many functions, such as continuous monitoring of the medium change and metabolic products, can be seamlessly integrated into this device.

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