Methods

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A fully automated touch-response behavior inspection pipeline on zebrafish larvae

Eine vollautomatisierte Inspektionspipeline für Berührungsreizreaktionen der Larven des Zebrabärblings

Abstract: A touch-evoked response of zebrafish larvae provides information on the mechanism of the gene functional expressions. Recently, an automated system has been developed for precise and repeated touch-response experimentation with minor human intervention. To quantify the collected data, we propose a fully automated multi-larvae touch-response behavior inspection pipeline based on larva tracking and segmentation. Experimental data with different treatments is analyzed by using the proposed inspection platform for demonstration, and the result proves that this platform can generate comparable touch-response behavior inspection readouts efficiently and automatically. The initial results were published in 31. Workshop Computational Intelligence, and this paper summarizes and extends the main work of the respective article.

Keywords: experiments; full automation; object tracking; touch response

Zusammenfassung: Eine durch mechanische Berührungsreize ausgelöste Reaktion der Larven des Zebrabärblings liefert Informationen über den Mechanismus der genetischen funktionellen Expression. Kürzlich wurde ein automatisiertes System entwickelt, um genaue und wiederholte Berührungsreizreaktionsexperimente mit geringer menschlicher Assistenz durchzuführen. Zur Quantifizierung der gesammelten Daten schlagen wir eine vollautomatisierte Inspektionspipeline für Berührungsreizreaktionen von mehreren Larven vor, die auf Larventracking und Segmentierung basiert. Experimentelle Daten mit unterschiedlichen chemischen Behandlungen werden durch die vorgeschlagene Inspektionsplattform analysiert. Die Auswertung bestätigt, dass die Plattform vergleichbare Inspektionsanzeigen der Berührungsreizreaktionen effizient und automatisch erzeugen kann. Die ursprünglichen Ergebnisse wurden in einem Konferenzbeitrag beim 31. Workshop Computational Intelligence veröffentlicht und werden in diesem Artikel resümiert und erweitert.

Schlagwörter: Vollautomation; Berührungsreizreaktionen; Objektverfolgung; Experimente

1 Introduction

Zebrafish larvae are commonly used animal models for organism-based screenings due to their small size, high fecundity, and short reproductive cycle [1]. Their specific (repeatedly and obvious) behaviors indicate certain functional mechanisms of mutants by the treatments [2, 3], making it possible to do the large-scale high-throughput screening of chemicals or drugs. Automated experimental systems to acquire the data of these behaviors have been developed so far [3–7], so the automated high-throughput inspection of the data from the systems is also becoming in higher demand, as visual inspection is time-consuming and not statistically comparable. In particular, the automated touch-response experimental system on zebrafish larvae has been developed to conduct the experiment by controlling a blunt needle to touch the larvae at a specific position and with a predefined force (Figure 1). The touch-evoked response of zebrafish larva consists of three components, including a C-Bend (the larva bends the body as a C-Shape,

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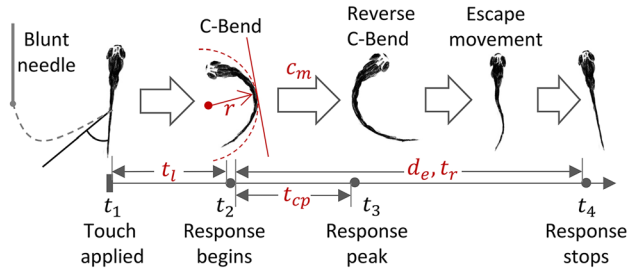
Figure 1: The diagram of conducting touch-response experiment and the corresponding response.

shortened as C-Bend), reverse C-Bends, and escape movement (changing the position). The touch-response experimental data (videos) are in a high frame rate [8, 9], so the automated inspection is essential in this case. During the touch-evoked response of zebrafish larvae, four time points of importance are the time when touch applied (t_1), response begins (t_2), response peak (t_3), and response stops (t_4). Four criteria are to be quantified, including the latency time (t_l), C-Bend curvature maximum (c_m), C-Bend peak time (t_{cp}), response time (t_r), and escape distance (d_e). However, it is challenging to generate a precise number of C-Bend curvatures and escape distance manually [7]. Furthermore, the operators cannot keep the same criteria all the time for each video, as the video has more than ten thousand frames on average. Thus, we proposed a touch-response quantification pipeline for single zebrafish larva in [8], but as for the multi-larvae case, we face more challenges: (i) multiple larvae need to be tracked and segmented at the same time; (ii) the larva that is touched shall be defined; (iii) the quantification of multiple larvae has higher computational costs. To solve these problems, we proposed an AI-based Multi-larvae Touch-response behavior Inspection Pipeline (AMTIP) in the work published in 31. Workshop Computational Intelligence [10] with the main work summarized and extended in this paper.

In AMTIP, the tracking procedure plays a vital role, especially in the tracking of multiple larvae [9]. Recently, machine learning or deep learning based tracking methods have emerged to promote the accuracy of the tracking procedure [11, 12], and much previous work focused on the tracking and segmentation of single or multiple adult zebrafish [13–16]. To make the best of the deep learning methods, we use a U-Net [17] based segmentation method for the initialization of tracking. However, those high-computational methods are difficult to be used in the tracking procedure of our high-frame-rate videos. In order to make the inspection pipeline less complex, we propose an optical flow based needle tracking procedure and a particle filter based larvae tracking procedure. Besides, the

segmentation for each larva is also of importance to the analysis of the movements. In [18], a Gaussian Mixture Model (GMM) based segmentation is used to detect the moving objects, and the noise is filtered according to the region size by using a global Otsu (a conventional automated thresholding) method. However, considering global information in our platform makes the procedure more computationally expensive. Therefore, a local region growing based segmentation method is used for each larva according to the result of tracking procedure. Based on the tracking and segmentation results, we propose AMTIP to find the touched larvae and generate the behavior quantification according to the proposed experiment criteria. In order to test the performance of the proposed platform, we conduct six sets of experiments with different drugs and analyze the experiment criteria and detected errors (failure cases). With the verification of the experiment results, AMTIP shows a high efficiency for analyzing the touch-response experimental data and reduces the efforts for the operators involved in the experiments. The methods used in AMTIP can make contributions to the optimization of object tracking methods for analyzing videos with expensive computation. As well, AMTIP can also be transformed into the inspection pipeline of other organisms (like medaka) and can also be added with more quantification criteria.

The organization of the article is as follows. Section 2 describes the design of the proposed AMTIP. Section 3 provides the setup of the experiments, the quantification criteria and results as well as the discussion. According to the above results, conclusions are drawn in Section 4.

2 Multi-larvae touch-response inspection pipeline

The touch-response inspection procedure transfers the raw data collected by the acquisition platform into variables (criteria) that make sense to humans. Figure 2 visualizes the architecture of AMTIP that transfers the videos collected by the data acquisition system [8, 9] and generates the quantification criteria of the touch-response behaviors, including latency time t_l, C-Bend curvature maximum c_m, C-Bend curvature peak time t_{cp}, response time t_r, and escape distance d_e. Four time points are vital to the quantification of touch response, including t_1 (touch applied), t_2 (response begins), t_3 (response peak), and t_4 (response stops). The AMTIP contains three essential parts: initialization, tracking and segmentation procedure, and quantification.
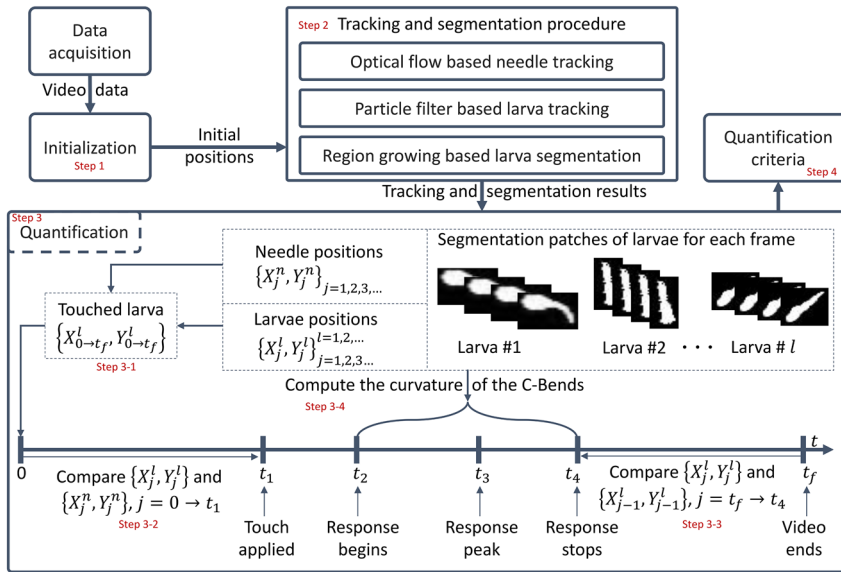
Figure 2: The diagram of the AI-based multi-larvae touch-response behavior inspection pipeline (AMTIP), including the initialization, tracking and segmentation procedure, quantification, and quantification criteria. The steps are marked in red.

The initial positions (initialization, Step 1 in Figure 2) of the needle and larvae obtained from the first frame are vital to the accuracy of the tracking procedure in AMTIP. Thus, a U-Net is used to segment the needle and larvae, which can be used directly as the initial positions for the tracking procedure.

Given the initial positions, each following frame of the video is processed by the tracking and segmentation procedure (Step 2 in Figure 2), including an optical flow based needle tracking [19, 20], a particle filter based larva tracking [11], and a region growing based larva segmentation. As the needle moves slowly, an optical flow is sufficient to estimate the needle position for each video frame (with coordinates in the frame denoted as $\left\{ X_j^n, Y_j^n, t_j \right\}$ where n indicates the needle and t_j indicates the frame j). The optical flow, however, cannot be applied to track the larvae that move rapidly, so a particle filter based larvae tracking is considered to generate the positions of the larvae for each frame ($\left\{ X_j^l, Y_j^l, t_j \right\}$ where l indicates the larva). To fulfill the detailed analysis of the touch-response behaviors of the larvae, a region growing based larva segmentation is used. The seed points of the region growing are chosen by the larva positions generated by the larvae tracking procedure, as described in [10]. The outputs of the tracking and segmentation contain the image patches of all larvae, as well as the positions of the larvae and needle for each frame.

With the results of the tracking and segmentation procedure, the quantification criteria (latency time t_l, C-Bend curvature maximum c_m, C-Bend curvature peak time t_{cp},

response time t_r, and escape distance d_e) are generated by the following three steps,

1. Each video contains multiple larvae (Larva #1, Larva #2, Larva #l, etc.), so the larva that is actually touched by the needle needs to be firstly distinguished among the larvae in the video by AMTIP for the following quantification (Step 3–1 in Figure 2). The needle stops at the larva position after touch is applied, so the initial position of the touched larva (X_0^l at $t = 0$) is the closest to the final position of the needle ($X_{t_f}^n$ at $t = t_f$).

2. As for the latency time t_l and response time t_r, the essential time points, including t_1 (touch applied), t_2 (response begins), and t_4 (response stops), need to be computed as detailed in Figure 2. As shown in Step 3–2 in Figure 2, the distance between the needle and larva for each frame is computed from $t = 0$ until the time point with the distance lower than a heuristic threshold T_{nl}, as t_1 (when touch is applied). The t_2 is obtained as the time point when the larva moves and the response begins (with a heuristic threshold T_{mq}: the percentage of the particles used in particle filter in Step 2). Similarly, as shown in Step 3–3 in Figure 2, the t_4 is searched from t_f reversely until the time point when the response stops. Consequently, the latency time is computed as $t_l = t_2 - t_1$, and the response time is computed as $t_r = t_4 - t_2$. The escape distance is computed by the sum of the distances between the larva positions in the frames from t_2 to t_4, namely $d_e = \Sigma_{j=t_2}^{j=t_4-1} \sqrt{\left(X_j^l - X_{j+1}^l \right)^2 + \left(Y_j^l - Y_{j+1}^l \right)^2}$.

3. To quantify the amplitude of the touch-response behaviors, the curvature of the C-Bend for each frame is to be analyzed according to the skeleton of the larvae. Besides, the C-Bend curvature maximum c_m can be computed, as well as the time point of c_m (t_3, response peak), generating $t_{cp} = t_3 - t_2$.

3 Experiment

3.1 Experiment setup

Different chemicals can have different influences on the touch-evoked behaviors of the zebrafish larvae. Thus, the experiments on long-term treatments (denoted as E_{lt}) with different chemicals are conducted on the zebrafish larvae to analyze the difference between various chemicals, with the protocol visualized in Figure 3 on a timeline, and the experimental setup is outlined in Table 1.

In Experiment E_{lt}, the larvae at 73 h post-fertilization (hpf) are put in the well plate to conduct the touching on the body. The details of the
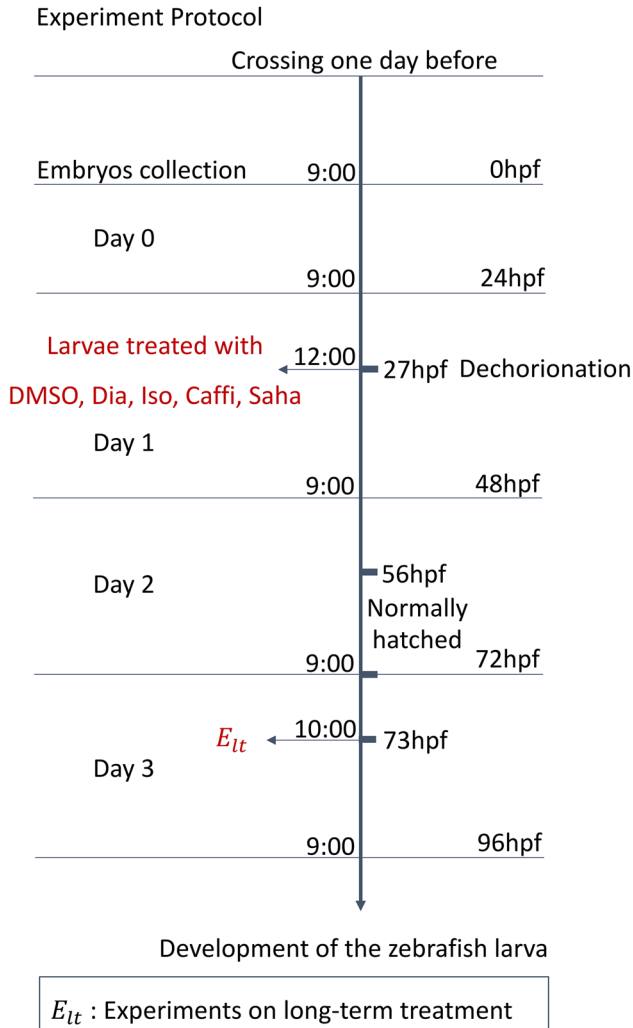
Table 1: The experiment setup of experiment E_{lt}. *Wild*: larvae in fish water. *DMSO*: larvae in dimethyl sulfoxide. *Dia*: larvae treated by diazepam. *Iso*: larvae treated by isoprenaline hydrochloride. *Caffi*: larvae treated by caffeine. *Saha*: larvae treated by suberoylanilide hydroxamic acid.

Parameter	Quantity
T_{nl} (used in AMTIP, Section 2)	10 pixels
T_{mq} (used in AMTIP, Section 2)	50 %
Volume of water	4 mL
Age of larvae	73 hpf
Dechorionation	27 hpf
Touching positions	Body
Number of larvae per well	4
Type of larvae	*Wild* and treated by *DMSO (1 %)*, *Dia*, *Iso*, *Caffi*, *Saha*
Concentration of chemicals	100 μmol/mL
Total number of larvae	173

treatments are outlined in Table 1. Six experiments are conducted: *wild* type (without treatment), larvae with Dimethyl sulfoxide (*DMSO*),[1] as well as larvae treated by Diazepam (*Dia*) to reduce the movements [21], Isoproterenol hydrochloride (*Iso*) with unknown effects, Caffeine (*Caffi*) for also reduction of movements [21], and Suberoylanilide hydroxamic acid (*Saha*) with unknown effects, respectively. Each treatment is in a concentration of 100 μmol/mL for the demonstration. The larvae are dechorionated and treated at 27 hpf for long-term treatment. The proposed AMTIP is used to quantify the data collected in Experiment E_{lt} for verifying that AMTIP can generate different touch-response behavior criteria with different chemical treatments on zebrafish larvae in the long term (assumption). Even though the proposed AMTIP is designed for the multi-larvae case, it can be used to quantify the data in the single-larva case.

3.2 Experiment results

The experiment on long-term treatment (E_{lt}) is conducted to collect 173 videos (24 videos for *Wild*, 27 videos for *DMSO*, 38 videos for *Dia*, 30 videos for *Iso*, 24 videos for *Caffi*, 30 videos for *Saha*, with the dataset denoted as DA-Elt in Table 2) and the quantification is run via AMTIP with the results visualized in Figure 4, including latency time t_l, C-Bend

Table 2: The datasets of experiment E_{lt}.

Dataset denotation	Chemical	Number
DA-Elt	*Wild* type	24 videos
	DMSO	27 videos
	Dia	38 videos
	Iso	30 videos
	Caffi	24 videos
	Saha	30 videos
Total		173 videos

Experiment Protocol

Figure 3: Protocol of the quantification experiment E_{lt} which is marked in red.

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1 As each treatment is prepared with *DMSO*, the experiments on the larvae with only *DMSO* (1 %) are also conducted as controls.
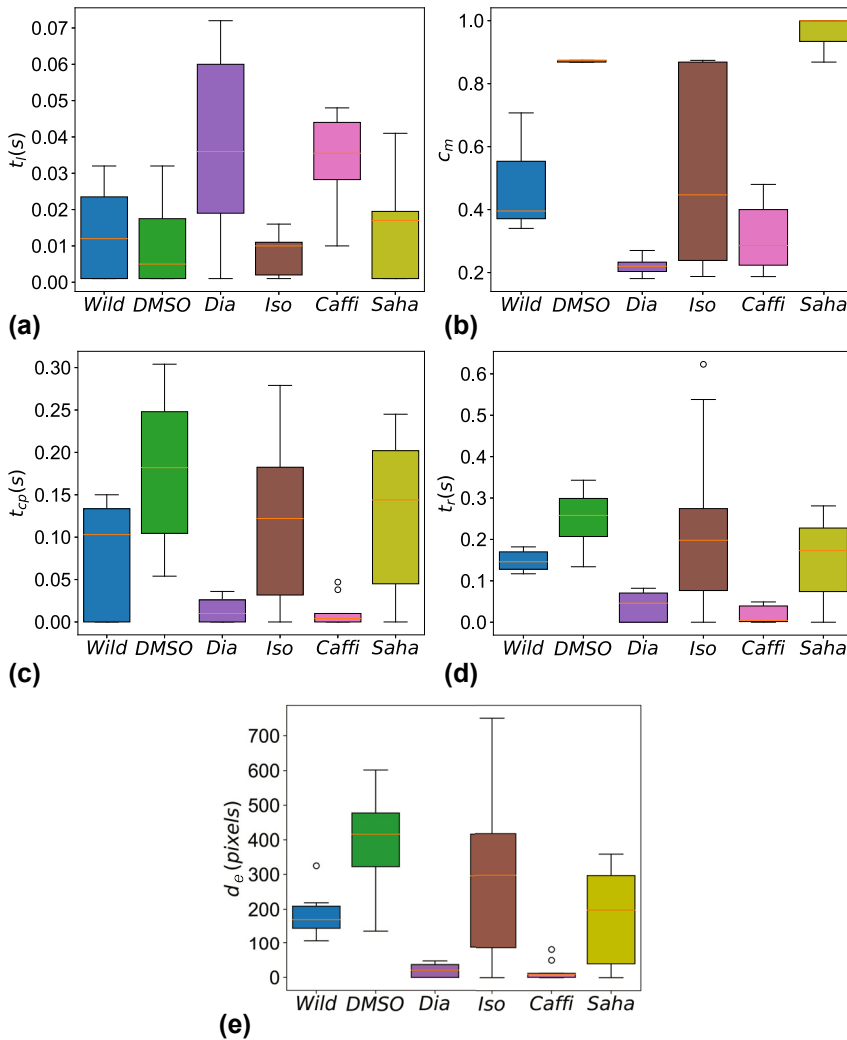
Figure 4: Five quantification indices on DA-Elt with six experiment cases (*wild*, *DMSO*, *Dia*, *Iso*, *Caffi*, and *Saha*) generated by AMTIP, including latency time t_l, C-Bend curvature maximum c_m, C-Bend curvature peak time t_{cp}, response time t_r, and escape distance d_e. (a) Latency time. (b) C-Bend curvature maximum. (c) C-Bend curvature peak. (d) Response time. (e) Escape distance.

curvature maximum c_m, C-Bend curvature peak time t_{cp}, response time t_r, and escape distance d_e. The results in Figure 4 verify the assumption of Experiment E_{lt} in Section 3.1 that the proposed AMTIP can generate different quantification results (the chemical effects) for different chemicals. In detail, the larvae with a longer latency time have lower response strength (lower c_m), shorter time to shape the C-Bend peak (lower t_{cp}), and less response duration (lower t_r and d_e), examples seen from the cases of *Dia* and *Caffi*. This result also proves that the larvae under the treatments of *Dia* and *Caffi* respond less compared with the *wild* and *DMSO*. Additionally, the treatments of *Iso* and *Saha* cannot change the touch-response behaviors of the larvae significantly with similar results to the *wild* and *DMSO*.

3.3 Evaluation of AMTIP

The proposed AMTIP can fail in the touch-response quantification owing to the inaccuracy of the segmentation method and missing objects by the tracking procedure used in the inspection pipeline. Thus, the detected errors (failure cases) are to be analyzed to evaluate the proposed AMTIP. The collected video data contain some unquantifiable ones, such as the larvae are not touched, and the larvae or needle cannot be detected. The dataset DA-Elt collected in the experiment on long-term treatment E_{lt} is used to analyze the detected errors: including

the number of videos with no larvae touched (#*NT*) as well as those with failures of quantification (#*QF*). Among the videos collected (#*C*), shown in Table 3, the ground-truth numbers of the videos with no larvae touched (#NT_g, generated by visual screening) are compared with the numbers output from AMTIP (#NT_p), with the false positive rate (*FPR*) and false negative rate (*FNR*). As well, the numbers of failures of quantification (#*QF*) are given with the percentage (E_{QF} = #*QF*/|#*C* − #NT_g|). The AMTIP can generate #NT_p and #*QF* automatically and find more than 90 % videos without any larvae touched on average (1 − *FNR*). Besides, around 10 % of valid videos (#*C* − #NT_g) cannot be quantified by AMTIP (failure cases). In addition, the larvae under the treatment of *Dia* are assumed to have a response scarcely. Thus, the output of latency time is expected to be infinite, and the other quantification criteria (C-Bend curvature maximum, C-Bend curvature peak time, response time, and escape distance) are expected to be 0. However, AMTIP can only generate finite numbers less than the duration of videos (15 s in our case), but from Figure 4a, the latency time of *Dia* is still useful to be compared with the controls as it shows a much longer latency time than those of *wild* and *DMSO*. Furthermore, the results in Figure 4b–e are over zero (negative outputs), caused by the following reasons: (i) some larvae still have a slight response; (ii) the movements of the needle can push the larvae away (fake response); (iii) the tracking procedure generates the movements of the larvae because

Table 3: The analysis of the detected errors (failure cases) of the proposed AMTIP. *#C*: the number of the collected videos. *#NT$_g$*: the ground-truth number of the videos with no larvae touched, generated by visual inspection. *#NT$_p$*: the predicted number of the videos with no larvae touched, generated by AMTIP. *FPR*: false positive rate of the videos without larvae touched. *FNR*: false negative rate of the videos without larvae touched. *#QF*: the number of failures of quantification. E_{QF}: the percentage of failure of quantification. $E_{QF} = \#QF/|\#C - \#NT_g|$.

Type	#C	#NT$_g$	#NT$_p$	FPR	FNR	#QF	E_{QF}
Wild	24	4	7	16.7 %	4.2 %	1	5 %
DMSO	27	3	8	22.2 %	3.7 %	0	0 %
Dia	38	4	1	0 %	7.9 %	8	23.5 %
Iso	30	6	6	6.7 %	6.7 %	2	8.3 %
Caffi	24	7	5	4.2 %	12.5 %	3	17.6 %
Saha	30	5	6	13.3 %	10 %	2	8 %
Average	–	–	–	10.5 %	7.5 %	–	10.4 %

of the slight environment changes or other inaccuracy. Nonetheless, the results of *Dia* in Figure 4b–e are much lower than those of *wild* and *DMSO*. In other words, even with slight variance, the proposed AMTIP verifies our assumption on treatment *Dia* that reduces the touch response of zebrafish larvae. Finally, AMTIP can achieve the quantification in higher efficiency (frame rate: on average 63 ms per frame on CPU) via the proposed efficient tracking and segmentation procedure compared with the U-Net (frame rate: on average 2.60 s per frame on CPU).

3.4 Discussions

The results verify that the proposed inspection pipeline AMTIP can work as an automated quantification tool for the touch-response data in a high frame rate. The AMTIP has the following advantageous strategies:

- The time point when the touch is applied (t_1), as well as the actually touched larva, is obtained by the final position of the needle and the initialized positions of the larvae, as the local segmentation during the tracking procedure is not as accurate as the initialized segmentation by the U-Net.
- The response of the larvae is defined by the particles used in the particle filter based larva tracking (details in Section 2) instead of the change of the larva center, as the centers of the larvae can change slightly but constantly during the tracking procedure, even if the larvae do not move.
- The time point when the touch response stops (t_4) is computed from the last frame reversely to the previous frames, since the larva can move slowly (no significant changes of pixels) for a moment and start moving strongly again.
- The quantification is achieved after the tracking and segmentation of all frames in the video, making it possible to consider the global information of the video.

However, some drawbacks still need to be considered carefully when the users apply AMTIP to the customized data. The tracking procedure and local segmentation of the larvae are the keys to AMTIP, but they may fail in the following cases: (i) the larvae overlap with each other when moving; (ii) the well edge area has similar brightness to the

larvae; (iii) the needle overlaps with the larvae. It is essential to conduct the touch-response experiments on a large scale, so the proposed AMTIP is vital in such cases.

4 Conclusions

In this work, we introduce an AI-based inspection platform for the touch response of zebrafish larvae, which can generate five quantification indices (latency time, C-Bend curvature maximum, C-Bend curvature peak time, response time, and escape distance) automatically without human intervention. This platform uses an automated inspection pipeline based on a multi-larvae tracking procedure, with a U-Net for initialization of the tracking procedure, optical flow and particle filter for tracking, and region growing for local segmentation of larvae. Six sets of experiments (two controls and four treatments) are conducted, and the results generated from this platform as well as the analysis of the detected errors verify the effectiveness of the platform. The AMTIP can generate the expected conclusions as the assumption according to the corresponding experimental results. A high efficiency is also guaranteed with on average 63 ms per frame for the inspection pipeline on CPU. The AMTIP can be applied to the inspection of animal behaviors and systems that are required to analyze position changes in videos and to quantify the movements into criteria.

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