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A Modular, Low-cost Robot for Zebrafish Handling*

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Abstract— **The zebrafish (danio rerio) is one of the most important model organisms in modern drug discovery and disease modeling. Handling and analyzing large numbers of zebrafish larvae require an immense manpower and involve time-consuming manual processes. A novel modular, robotic platform for high-throughput screening is being developed at BioRobotLab (KIT). In this article the fish sorter, which is a robotic device for the automation of a manual process in bio analysis, is presented. The fish sorter detects randomly spread zebrafish eggs and larvae up to an age of 120 hours post fertilization (hpf) in Petri dishes and transfers them to standard 96- or 384- well plates. The robot is controlled by an advanced algorithm with sensor-based process control. Fast and precise hardware components lead to a high working speed and success rate >= 95%.**

I. Introduction

For years, the zebrafish (danio rerio) has been an important model organism in biotechnology for addressing questions of vertebrate embryo development [2, 13]. Assays for embryonic development, investigations of toxicological issues, and various kinds of genetic screenings have been performed. High-throughput processes are needed to test thousands of chemicals. Small molecule screens to identify potential drugs also require a large number of samples [6]. Even European guidelines like REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) create a demand for testing thousands and thousands of samples during the approval process of new chemicals [12].

Zebrafish screening involves numerous manual steps, such as mating, filling into well plates, microscopy, and data evaluation. Increasing sample quantity therefore means more manual work. A major aspect of increasing screening capacity is substituting manual work by automatic processes [5]. Several steps in bio analytics have already been automated. Semi-automated microscopes and liquid handling devices are commercially available.

In the process chain of a zebrafish assay the placement of zebrafish into well plates (WP) is essential for the individual treatment of embryos with certain compounds. As fish larvae have been filled manually into well plates so far, preparation of hundreds of thousands of zebrafish embryos is a very time-consuming and exhausting work step. In order to reduce human labor and increase success rates by improving reproducibility, automated embryo handling is needed.

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This paper focuses on a new approach to an automatic dispensing device for transferring zebrafish eggs and larvae from Petri dishes into well plates (WP).

Only a few robotic systems to handle fish larvae have been developed so far. Recent work by Zhang et al. describes a parallel process of fish egg sorting [14]. Fish eggs are immobilized by vacuum in a pattern similar to a quarter of a 96-WP and then transferred all together. Another approach to transferring zebrafish to WP was made by Graf et al. (CSEM) [7].The zebrafactor separates fish eggs by circular fluid flow and dispenses them via a tube into the WP. Another system is described by Pardo et al. [8]. Using a fluidic handling system, larvae can be sorted by fluorescence and even laser surgery can be applied. The commercially available bio sorter "COPAS" by Union Biometrica can fill WP and, at the same time, evaluate fish embryos by fluorescence scanning [10]. Passing the scanning area, three color lasers excite the fluorescent parts in the fish and the emitted light intensity is measured.

None of these systems can be used to fill wells with defined small volumes. Wells always have to be full, which is problematic in toxicity screenings and small molecule screens, where precise dilutions are required. The vacuum immobilization method does not allow for the handling of hatched fish larvae. The COPAS system as well as the Pardo system combine various functions like dispensing, screening, and even laser surgery, as a result of which they are very expensive. If dispensing of embryos is needed only, these two devices provide functions that go far beyond the objective of many assays.

II. NEW CONCEPT FOR MODULAR LAB ROBOTS

Modular and inexpensive systems are needed, which ensure reliable performance and easy handling. Our system offers the possibility to increase throughput by having several robots working in parallel. Due to the modular cube concept, the system can be adapted to various tasks in bio analytics. Highly compatible interacting robots can be used to automate a complete zebrafish screening process. Recently, an intelligent microscope was developed. This device automatically detects zebrafish in WP and acquires detailed images of the beating heart. Automatic recording of video sequences of every fish´s heart in a 96- well plate is possible. Furthermore, a parallel microscope and a first version of the fish sorter function were realized [9]. In this paper the new fish sorter is presented. This advanced fish sorter is controlled by a sorting algorithm that allows for reacting to several events. A fast actuator driving the z-axle reduces process duration. With a specially developed sensor a quality control step is implemented to enhance the success rate.

III. ZEBRAFISH SORTING SYSTEM

The fish sorter is based on a three-axis robot which moves a pipette in three dimensions. A camera is mounted next to the z actuator which moves the pipette tip. A target object is picked up into the tube by aspirating a certain amount of fluid. Using this configuration, the process of transferring embryos to the well plate is similar to the manual way of doing it. The system sorts zebrafish eggs and larvae up to an age of 120 hpf into 96- and 384- standard well plates. During the sorting process coagulated and vital fish eggs can be differentiated.

Apart from the three-axis robot with a digital camera and a pipetting system, the main elements to perform a quick and reliable sorting process are the sorting algorithm, the fish detection algorithm and the aspiration control sensor. The main features of the fish sorter are described below.

A. *Fish Sorter Hardware*

The hardware configuration can be seen in figure 1. The robot consists of two linear axles driven by stepper motors at a moving speed of up to 0.5 m/s and a position accuracy of about 0.1 mm. With this x-y system, a third actuator and a camera are moved in parallel. The third actuator is a linear motor and serves to move the pipetting tip in vertical direction. High-power LEDs with aluminum reflectors illuminate the Petri dish to obtain high optical contrast between fish and Petri dish, thus establishing best conditions for image-based fish detection. A custom-made special linear pump is connected to the pipetting tip via a flexible tube made of PTFE (Teflon). This special linear pump hardly causes any pulsation of fluid flow due to its hardware concept. Aspiration and pumping are achieved by movement of a linear axle which is operated by a stepper motor. In this way, the volume can be adjusted precisely.

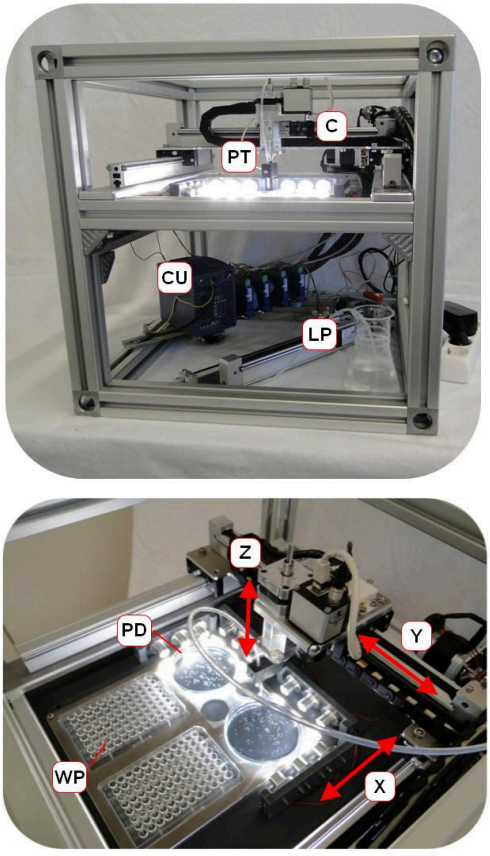

Fig. 1. Fishsorter: PT= pipetting tip with aspiration sensor, C= camera, CU= control unit, LP= linear pump, PD= petri dish, WP= well plate, X.Y,Z= three axis robot

Due to the hardware configuration with the pipette tip, the system can also be used for toxins. Contaminated tubing can be replaced easily. Furthermore, the pick and place principle allows filling wells up to a predefined volume. The volume to pick up fish can be adjusted in a range from 20 µl to 50 µl. To improve the success rate of the process, we developed a sensor which checks the aspirated content. Only if there is a fish in the tube, is the fluid transferred to the well plate. By evaluating the signal from the sensor during the sorting process, closed loop control is ensured. If no fish is picked up, the aspirated fluid is pumped back into the Petri dish. Coagulated eggs are sorted into a special container.

B. *Sorting Process*

A schematic overview of the sorting process can be found in figure 2. First, the camera is positioned above the Petri dish and a picture is taken. By a specially developed image processing algorithm (see section C), every embryo in the Petri dish is detected. All the embryo positions detected in the picture are converted into robot coordinates. Having chosen one embryo as a target by considering the distances to surrounding fish, the pipette tip is moved to the evaluated position. When the pipette tip is positioned above the embryo, the linear pump is activated and the embryo is aspirated into the tube. While aspirating, the sensor checks the content. The acquired signal shows whether the fish was aspirated correctly. If not, the amount of fluid is pipetted back into the well plate. If it was aspirated successfully, the next step is pipetting the embryo into the well plate by

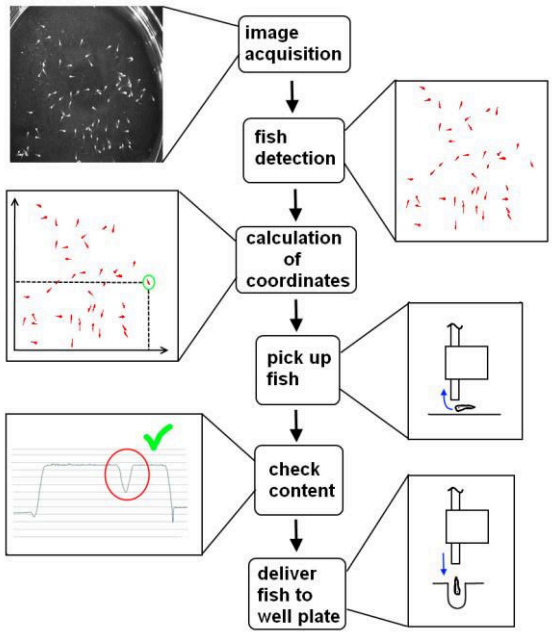

Fig. 2. Sorting process

moving the pipette tip to the designated well. To increase throughput and reduce the time required for moving, several fish eggs can be picked up and stored in the tube before they are pipetted into the well plate.

C. Fish Detection

To achieve best results with image detection, the illumination is an essential element. The 12 LEDs illuminate the Petri dish, thus obtaining high contrast between fish and background. Based on zebrafish image analysis tools [1, 4], we developed a special fish detection algorithm (see fig. 3) in cooperation with the bio signal analysis group at KIT. Two versions of this algorithm are used to either detect eggs or hatched larvae. Based on the acquired image of the Petri dish, fish are separated from the background by applying a threshold. Subsequently various morphological operators are applied to detect fish. To minimize detection errors artefacts resulting from light reflections and threshold are eliminated. During the sorting process, fish in the Petri dish may agglomerate to clusters. These clusters can cause errors in the pipetting process, as fish that are too close to each other could be aspirated together. Cluster coordinates are also detected and labeled as no-go areas in order to prevent picking up more than one fish. The result of image detection is the creation of two lists with coordinates for single fish and for cluster positions. These coordinates are converted into global robot coordinates represented in mm.

D. Cluster Spreading

Single fish cannot be picked up individually, when they are gathered in clusters. If all fish were in clusters, the process would have to be stopped, just as if the Petri dish was empty, although there are many fish left in the Petri dish. To enable the system to separate close neighbors, a cluster spreading procedure was implemented. By pipetting single drops of water into the cluster, fish float away from the impact position. In this way, fish groups are separated and the sorting process can be continued by detecting and pipetting single embryos.

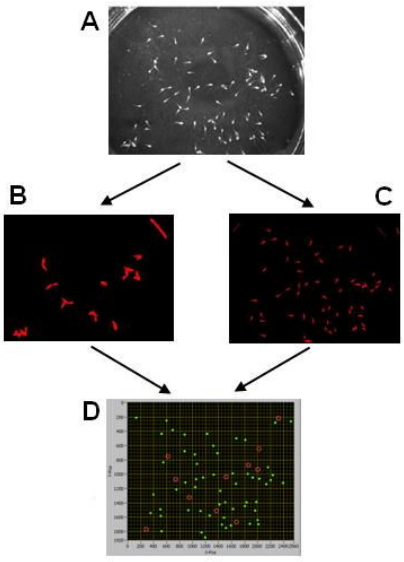

Fig. 3. Image-based fish detection: A: original image, B: detected cluster, C: detected fish, D: calculated coordinates

E. Quality Control by the Aspiration Sensor

To ensure transfer of the fish identified to the well plate, we developed a sensor to check the aspirated content. Occasionally, fish are not picked up, although the pipetting tip was placed right above them. The designated well would be classified as empty, because there would be no fish in it. To avoid empty wells or wells filled with more than one fish, a sensor was developed which detects whether there is a fish in the tube or not. Every time the pump is activated, the sensor checks the ingoing content. The acquired signal can be seen in figure 4. A software interprets the signal to decide whether the embryo was picked up correctly by analyzing several parameters of the signal. The amplitude and the width of the characteristic peak of an embryo allows for distinguishing even between living eggs and coagulated eggs.

IV. Evaluation

A. Vitality of Transferred Embryos

When automating the handling of whole living organisms, it is essential not to harm the organism. Working with entire living organisms is associated with special handling requirements. To obtain information about how zebrafish react to mechanical treatment and abiotic stress, various influences have been investigated [11]. As demonstrated in [3], zebrafish are not damaged by placement in a tube. To prove that the system does not harm the embryos, the system was compared to a manual disposable single-use pipette. Two 96-well plates were filled with the manual pipette and two with the robotic system placing single eggs (24 hpf) into every well. After 24 h of incubation at 28° C, the rate of coagulated eggs in both groups was evaluated. As there was no difference in the survival rate (manual: WP1 9% and WP2 13% coagulated; fish sorter: WP3 9% and WP4 13% coagulated), it may be stated that the system is as harmless as the manual method.

Furthermore, we tested whether hatched larvae are affected

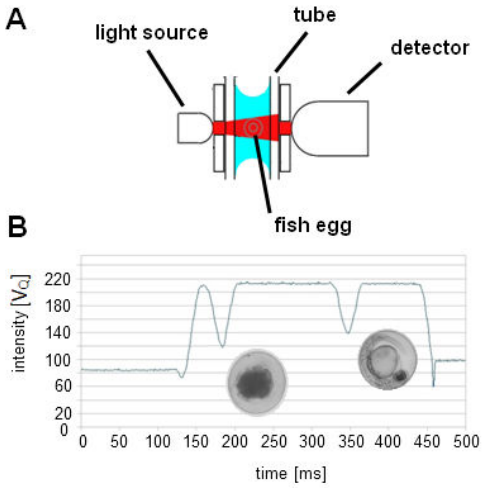

Fig. 4. Aspiration sensor, A: work principle, B: acquired signal for coagulated (left) and living fish eggs (right)

TABLE I
SORTING PERFORMANCE

Type	Well plate	Time	Success rate
fish egg	96	19 min	98%
	384	80 min	
hatched larva	96	25 min	95%
	384	105 min	

by the pipetting process. We used the sorter to put 50 larvae (120 hpf) into a 384-well plate. As the larvae were genetically modified to identify injuries via fluorescence, it was possible to check for injuries caused by the pipetting process. It turned out that there were no injuries or other negative influences on the larvae.

B. Liquid Handling Precision

Liquid handling has to be precise, because desired dilutions shall be exactly as planned for every well. To evaluate the precision of the transferred amount of fluid, the fish sorter was compared to the commercial standard pipette of the type *Gilson Pipetman 200*. We weighed several droplets pipetted with both systems and fixed volume calibration. The differences of the masses of the droplets were determined. For the manual pipette as well as for the fish sorter, the deviation was in the range of up to 5%. Hence, it can be stated that the accuracy of the fish sorter is comparable to that of the manual pipette by Gilson, which is an established and reliable tool for bio analysis.

Due to the small volume pipetted, we applied a second method to determine the value of the pipetting accuracy. With a capillary glass tube, we measured the aspirated volume for discrete amounts of fluid. Pipetting liquid amounts between 12 to 35 µl showed pipetting deviations of less than 5%.

C. Duration of Sorting Process

To determine the success rate and the time it takes to fill a plate, long-term experiments were performed. We filled eight 96- WP with fish eggs and sixteen 96- WP with hatched larvae. The average values can be seen in table 1. Filling a 96- WP with fish eggs takes about 19 minutes, which is comparable to the time needed for manual filling. The average time to fill a 96- WP with hatched larvae is about 25 minutes. Filling of 384- WP can be performed in about four times the duration of 96- WP. The success rate, defined as the percentage of wells that contain exactly one single embryo, was determined to be 98% for fish eggs and 95% for hatched fish.

V. CONCLUSION

A robotic tool that automatically sorts fish eggs and hatched larvae into standard well plates was reported. It was demonstrated that the system does not harm the fish. The process speed is comparable to the manual sorting performance. Hence, the system has the potential to substitute the manual process. The quantity of liquid transferred to the well plate can be adjusted precisely. The process can be executed without any intervention of lab staff. Consequently, the throughput can be augmented by parallelizing the process. Operating various robots in parallel may result in several times the output a single person could achieve manually. Due to low-cost hardware setup, parallel sorting is affordable. As the system is specialized in one task and can be complemented by other robotic modules, custom-made screening processes can be set up.

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