HIGH-THROUGHPUT CELLULAR-RESOLUTION *IN VIVO* VERTEBRATE SCREENING

Carlos Pardo-Martin[1, 2,*], Tsung-Yao Chang[1,*], Amin Allalou[3,*], Carolina Wählby[3, 4] and Mehmet Fatih Yanik[1, 4]

[1]*Massachusetts Institute of Technology, USA*
[2]*Harvard University, USA*
[3]*Uppsala University, Sweden, and*
[4]*Broad Institute, USA*
[*]*These authors contributed equally to the work presented here*

ABSTRACT

Due to its small size and optical transparency, zebrafish larvae are excellent vertebrate models to study human diseases *in vivo*. We previously developed a high throughput screening platform capable of handling, cellular-resolution imaging and optically manipulating zebrafish larvae. Here, we present new novel technologies to significantly increase the throughput of our screening platform by multi-threading the loading and imaging processes and high-speed algorithms to automatically manipulate the larvae.

KEYWORDS: High-throughput, vertebrate screening, zebrafish

INTRODUCTION

Vertebrate models such as zebrafish allow study of complex processes on a large scale that cannot be replicated *in vitro*. Several desirable attributes of zebrafish have fueled its popularity, including the animal's small size, optical transparency, aquatic habitat, and simplicity of culture[1], and it is also currently used by pharmaceutical industry.

Zebrafish studies have been commonly done manually. Cellular resolution assays require optical access to a specific region of the specimen for imaging or manipulation, which is often impeded by intervening organs such as the eyes, heart and skin pigmentations. Furthermore, visualization of most of the regions requires precisely orienting the zebrafish. Current specimen orientation methods require embedding the sample in agar and/or manually rotating the fish with forceps; both processes are too slow and unreliable for high-throughput screens. Therefore, high-content zebrafish screens have been limited to a few thousand compounds per week.

To dramatically speed up the throughput of zebrafish assays, we recently developed a vertebrate automated screening technology (VAST), a platform capable of performing cellular-resolution imaging and optical manipulations on zebrafish larvae[2]. VAST loads larvae from multiwell plates and places them inside ultrathin capillaries under a dual upward-inverted high-resolution high-speed confocal microscope (*Figure 1a*). A set of stepper motors rotate the capillary for rapid orientation of the larvae, enabling cellular and subcellular structures in zebrafish to be visualized at all angles. Monitoring scattered light from the capillary by a system of light-emitting-diodes (LEDs) and a photodetector allows tracking of the fish as it moves from the multiwell plate into the capillary at high flow speeds.

Here, we demonstrate a new technology to significantly increase the throughput of our system, and algorithms to fully automate zebrafish manipulation.

METHODS

A single cycle of the VAST system can be divided into three independent processes; loading, imaging, and unloading. We have previously reported that these steps together take an average of 20 seconds per fish[2]. To increase the throughput of our system we have mechanically separated the fluidic sections of the VAST system into three regions by adding multiple pumps and valves (*Figure 1b*). The three regions are those that control the loading, positioning of the fish under the field-of-view (FOV) and the unloading into a different multiwell plate. By pinching the valves and operating the syringes separately, the flow across each region can be controlled independently from each other. This allows the system to handle different larva in each region and thus load, position, image, and unload three different larvae simultaneously. Multi-threading these three processes significantly reduces the average time per fish which is limited by the longest process and thus significantly increase the throughput. Multi-threading the platform results in an average of 10 seconds per animal and thus a two-fold increase in the platform's throughput (*Figure 1c*).

A key step in the design of a high-throughput screening system is the automation of the fine positioning of the region of interest (ROI) under the FOV. To accurately and consistently image the ROI across samples there is a need to control both its position and rotational orientation under the FOV. Phenotypic variations usually occur in large genetic and chemical screens. Therefore the robustness of the algorithms is crucial.
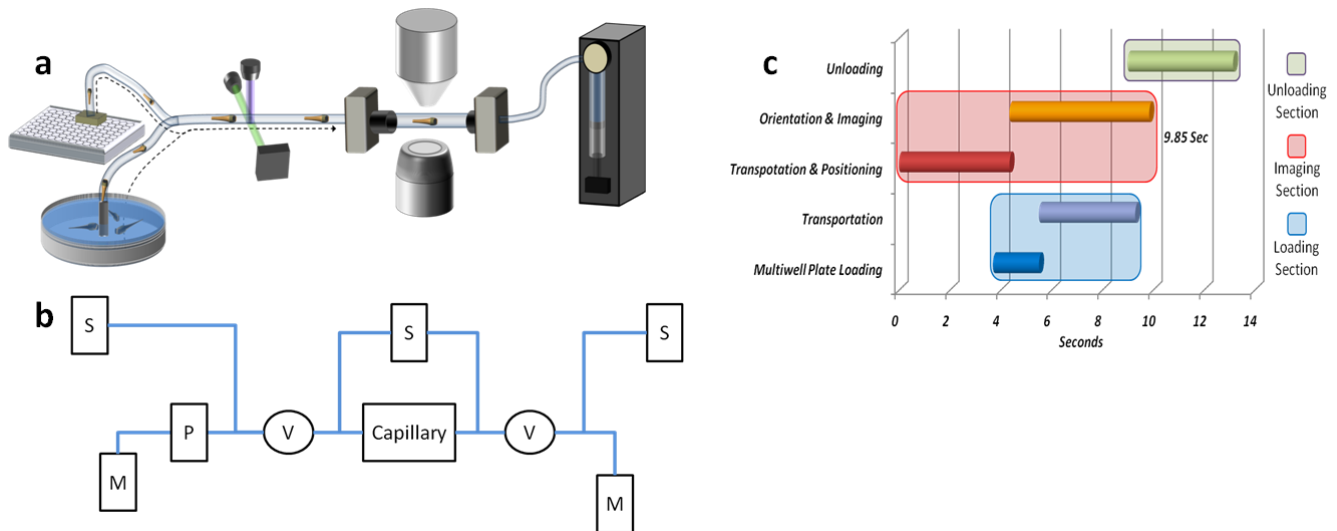
Figure 1: Multi-threading. (a) Schematic representation of the original VAST system. Fish are loaded from multiwell plates or reservoirs into a capillary under the FOV of a dual upright-inverted microscope through a computerized syringe pump. The capillary is then rotated by two stepper motors to precisely orient the larvae. (b) Schematic of pipelining to form three independent regions to allow multi-threading. "S" represent syringes, "V" fluidic valves, "P" the photodetector system and "M" multiwell plates. The blue lines indicate fluidic connections (c) Time lines for each step in the VAST cycle.

Our algorithm works through a four-step process; detecting the direction of entry of the larva in the tube, identifying the larva's rotational orientation, rotating it into the desired position, and fine positioning the ROI in the FOV of the high power objective. The rough positioning of the larvae in the center of the FOV is done through a closed loop feedback between the CCD imaging the FOV and the computer-controlled syringe pump.

The larvae can enter the tube with either the head or tail first, therefore, before further image processing the direction of the larvae has to be determined. We first compute a 1D profile of the fish by summing the pixels across the tube. There is a large intensity difference between the tip of the head and the empty tube surrounding the fish. The tip of the head is identified by finding the maximum of the derivative of the 1D profile. The center of mass of the 1D profile is then computed. The center of mass falls in the upper part of the abdomen, and we find the direction of the larvae by comparing the position of the tip of the head and the center of mass of the fish.

To estimate the rotational orientation of the larvae inside the capillary we rotate it 360 degrees and acquire images at a constant frame rate (*Figure 2a*). Through the frame rate and rotational speed, we then assign a rotation angle for each acquired frame. We then compute the cross correlation between each frame and predefined templates of an age-matched larva imaged in the coronal and sagittal planes. The cross-correlation with the coronal templates yield two strong peaks at the dorsal and ventral orientations (*Figure 2b*). Due to the anatomical symmetry and optical transparency of the larvae, it is difficult to differentiate the ventral and dorsal orientations (*Figure 2c*). The cross-correlation with the sagittal template yields softer peak but robustly detects the ventral portion of the fish is located. This and the direction of rotation are then used to differentiate which peaks from the coronal template are the ventral and dorsal orientation. To handle variations in phenotypes across larvae, we have constructed a collection of templates for larvae under the typical morphological abnormalities (e.g. pericardial effusion). If the maximum cross correlation between the templates and the video of the rotating sample does not reach a user defined minimum our algorithm searches through this collection of templates and computes the orientation using a template with higher correlation. Once the dorsal, ventral and sagittal orientations have been detected the larva is rotated to a predefined angle specified by the user (e.g. dorsally to image the brain, ventrally to image the heart and brachial arches, obliquely to image the pancreas).

We have also incorporated a cell identification algorithm to locate and quantify individual neurons. 3D images of the neuron cells are acquired with a confocal microscope. The GFP-labeled neurons are small and appear as blurred point-source signals. The detection of the neurons is done with a combination of the multiscale product (MP) and three-dimensional stable wave detector (3DSWD) algorithms [3, 4]. The MP is used as a detector, enhancing regions with point-like source signals, and the verifier/separator from the 3DSWD is used to examine the direction of the slope around a point and verify if a high value from the detector is a true signal. In addition, the verifier/separator separates neurons that are in close proximity, such as those forming neuronal clusters.
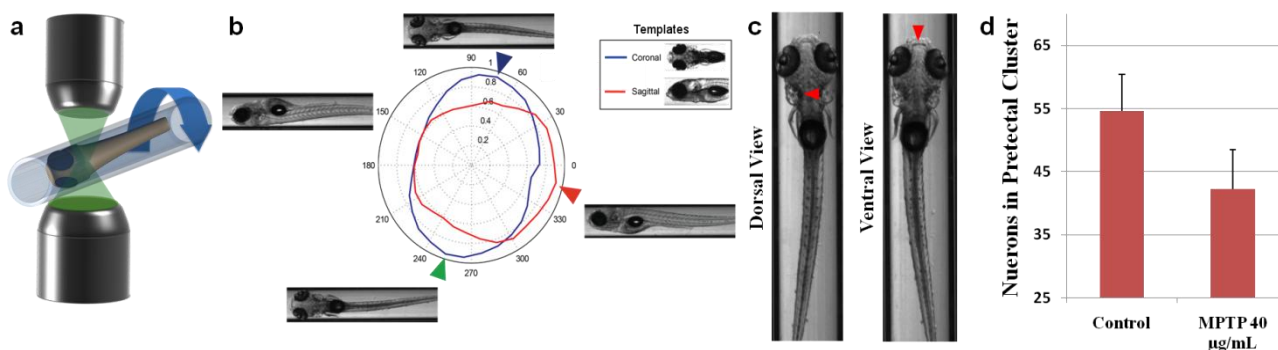
Figure 2: Automatic identification of orientation and cell count. (a) The larva is rotated 360 degrees while the imaging system continuously acquires images. (b) Schematic representation of the automated determination of orientation. Each frame acquired while the fish is rotating is correlated with the coronal and sagittal templates (blue and red curves respectively). The maximum correlations determine the dorsal (blue triangle), sagittal (red triangle) and the ventral (green triangle) orientations. (c) Dorsal and ventral views of a larva. Red arrows indicate some features for distinguishing between the two views. (d) Number of neurons in the pretectal cluster of 7 days post fertilization ET(vmat2:GFP) zebrafish transgenic larvae with and without MPTP treatment as quantified by automated cell identification.

CONCLUSION

We have multi-threaded the fluidic system of VAST to allow handling of multiple larvae simultaneously, thus improving the throughput to 10 seconds per animal. We have also developed image processing algorithms to completely automate the fine manipulation of the larvae inside the thin capillary to allow for completely automated screening. We also developed image processing algorithms to quantify the number of cells present in a region of interest, which allows us to directly quantify the number of neurons in fluorescently labeled neuronal clusters deep in the brain of the larvae using the VAST system. This can be used to screen chemicals to treat a variety of cellular degenerative diseases. We have used these methods to quantify the reduction in number of dopaminergic neurons[5] after treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (*Figure 2d*), a neurotoxin that induces Parkinson's disease in humans and animal models such as zebrafish[6].

Both the algorithms described here and multi-threading the platform significantly increase the throughput and utility of the VAST system and thus allows larger and more complex screens.

ACKNOWLEDGEMENTS

REFERENCES

[1] Zon, L.I. and R.T. Peterson, *In vivo drug discovery in the zebrafish.* Nat Rev Drug Discov, 2005. **4**(1): p. 35-44.
[2] Pardo-Martin, C., et al., *High-throughput in vivo vertebrate screening.* Nat Methods, 2010. **7**(8): p. 634-6.
[3] Vermolen, B.J., et al., *Segmentation and analysis of the three-dimensional redistribution of nuclear components in human mesenchymal stem cells.* Cytometry Part A, 2008. **73A**(9): p. 816-824.
[4] Allalou, A., A. Pinidiyaarachchi, and C. Wählby, *Robust signal detection in 3D fluorescence microscopy.* Cytometry A, 2010. **77**(1): p. 86-96.
[5] Wen, L., et al., *Visualization of monoaminergic neurons and neurotoxicity of MPTP in live transgenic zebrafish.* Developmental Biology, 2008. **314**(1): p. 84-92.
[6] Sallinen, V.J., *Zebrafish as a model of Parkinson's disease*, in *Institute of Biomedicine*. 2009, University of Helsinki: Helsinki.

CONTACT

M.F.Yanik, yanik@mit.edu