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Research report

Automated analysis of behavior in zebrafish larvae

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ABSTRACT

Zebrafish larvae have become a popular model system to examine genetic and environmental factors that affect behavior. However, studying complex behavior in large numbers of fish larvae can be challenging. The present study describes a novel high-resolution imaging system that is unique in its ability to automatically analyze the location and orientation of zebrafish larvae in multiwell plates. The system revealed behaviors in zebrafish larvae that would have been missed by more manual approaches, including a preference to face a threatening stimulus from a distance and a clockwise orientation in a two-fish assay. The clockwise orientation of the larvae correlates with a clockwise orientation of molecular structures during early development. Larvae with reversed embryonic asymmetries display a counter-clockwise orientation in the two-fish assay, suggesting that embryonic asymmetry and chiral behavior are regulated by the same developmental mechanisms. The developed imaging techniques may be used in large-scale screens to identify genes, pharmaceuticals, and environmental toxicants that influence complex behaviors.

1. Introduction

Although there is no substitute for looking at behavior with the naked eye, automated imaging systems have become valuable tools for the analysis of behavior in a variety of organisms. Large data sets can be obtained while avoiding observer bias or fatigue. In addition, automated imaging systems have been developed to facilitate high-throughput genetic, pharmacological, and environmental screens [14,27,38]. The images in a high-throughput screen can contain a significant amount of information and image-based screens are often referred to as 'high-content screens'. Large-scale screens and particularly high-content behavioral screens are impractical in most vertebrates due to the number of animals that need to be examined. A notable exception is zebrafish, which has become a powerful model system for medium to high-throughput applications [7,9,10,12,13,19,20,24,28,39,46]. Zebrafish are small, maintenance costs are low compared to other vertebrates, and a modest colony of fish can produce hundreds or even thousands of embryos on a daily basis. These embryos develop rapidly into free-swimming fish. At 24 h of development, the embryos have a beating hart, moving tail, eyes, and a primitive brain [29,45]. Embryos hatch from their chorion between 2 and 3 days of development. After hatching, the free-swimming 3–7-day-old zebrafish larvae display a range of behaviors that are important for finding food and avoiding predators. Some of these behaviors are robust and suitable for large-scale screening. For example, two robust behaviors of zebrafish larvae that have been examined by mutagenesis screens are the optokinetic and optomotor response [10,11,36]. The optokinetic response is an eye movement in response to a moving object. The optomotor response is a swimming behavior in the same direction as a moving pattern of stripes. While the optokinetic response needs to be evaluated in individual zebrafish larvae, the optomotor response can be examined in groups of larvae. For example, Muto et al. imaged groups of 10–40 larvae in special 'racetracks' [36]. Twelve racetracks were imaged at once, which greatly accelerated the analysis of the optomotor response. In the course of 3 years, more than 500,000 F_3 zebrafish larvae were examined for either the optokinetic or optomotor response [36]. The analysis of behavior in groups of larvae may be particularly advantageous if larvae could be imaged in multiwell plates. These plates are ideally suited for large-scale pharmacological or environmental studies, since one can make use of robotic fluid handlers. In addition, libraries of small molecules can be conveniently added to the culture medium. By imaging larger numbers of individual larvae in multiwell plates, it may be possible to expand the types of behaviors that can be examined in high-throughput screens. However, imaging zebrafish behavior in multiwell plates remains challenging. First, the optics of a multiwell plate is sub-optimal, in particular along the walls of the wells. Second, it is difficult to provide visual stimuli to individual wells of a multiwell plate. Third, it is difficult to measure behaviors other than activity in a multiwell plate. Driven by our own interests in measuring spontaneous and stimuli-induced activity, asymmetric behavior, social behavior, learning, and memory, we developed a novel high-resolution imaging system that is well suited for imaging zebrafish larvae in large culture dishes and multiwell plates.

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The system measures the location as well as the orientation of zebrafish larvae and visual stimuli can be presented to the larvae via a LCD screen. The system was tested by imaging the optomotor response and by imaging asymmetric behavior in a two-fish assay.

2. Materials and methods

2.1. Zebrafish larvae

Adult wild type zebrafish were obtained from Carolina Biological. The fish were maintained in a mixed population on a 14 h light/10 h dark cycle and were fed with a combination of brine shrimp and flake food. Embryos were collected from the tanks at 'dawn' and were raised at 28 °C in a culture medium, containing 60 mg/l sea salt (Instant Ocean) in deionized water and 0.25 mg/l methylene blue as a mold inhibitor. Embryos were grown at a density of 25 embryos per 50 ml culture medium in plastic 8.5 cm culture dishes. Unfertilized eggs were removed from the culture dish at 1 day post-fertilization (dpf). We examined behavior at 6 and 7 dpf when the larvae display a range of hunting and predator avoidance behaviors, but have not yet depleted their yolk sac. The larvae are 4–5 mm long at this time. Twenty minutes before the onset of an imaging experiment, zebrafish larvae were transferred to a new 8.5 cm culture dish (Corning no. 430591) or flat bottom 12-well plate (Corning Costar no. 3513) with fresh culture medium.

2.2. Zebrafish larvae with laterality defects

Thapsigargin, an inhibitor of the endoplasmic reticulum calcium pump, randomizes left–right asymmetry in the heart and brain [31,43]. Embryos were exposed to 0.5 μM thapsigargin from 4 to 6 h post-fertilization (hpf). At 30 hpf, embryos were examined on a Zeiss dissection microscope to determine the location of the heart, which jogs to the left in normal development. Embryos with left-sided hearts and right-sided hearts were transferred to separate culture dishes and were examined for behavioral defects at 7 days post-fertilization. To avoid direct effects on neural physiology, left–right asymmetry defects were also induced by injecting *no-tail* (*ntl*) antisense morpholinos (Gene Tools) in the yolk of mid-blastula stage embryos at 3–4 hpf. We used the same morpholino that successfully knocked down *ntl* gene function in whole embryos [37] and induced a randomization of left–right asymmetry when injected in the yolk at 3–4 hpf [4], i.e. 5′-GACTTGAGGCAGGCATATTTCCGAT-3′. The sequence complimentary to the start codon is underlined. We injected 0.5 nl of a 0.8 mM morpholino stock containing 0.5% (w/v) phenol red to visualize the injection volume. Embryos with left-sided and right-sided hearts were transferred to separate dishes at 30 hpf and were examined for behavioral defects at 7 days post-fertilization.

2.3. The zebrafish imaging system

The zebrafish imaging system was built inside a tall wooden cabinet measuring 55 cm × 35 cm × 180 cm (Fig. 1). Two configurations were tested: an upright

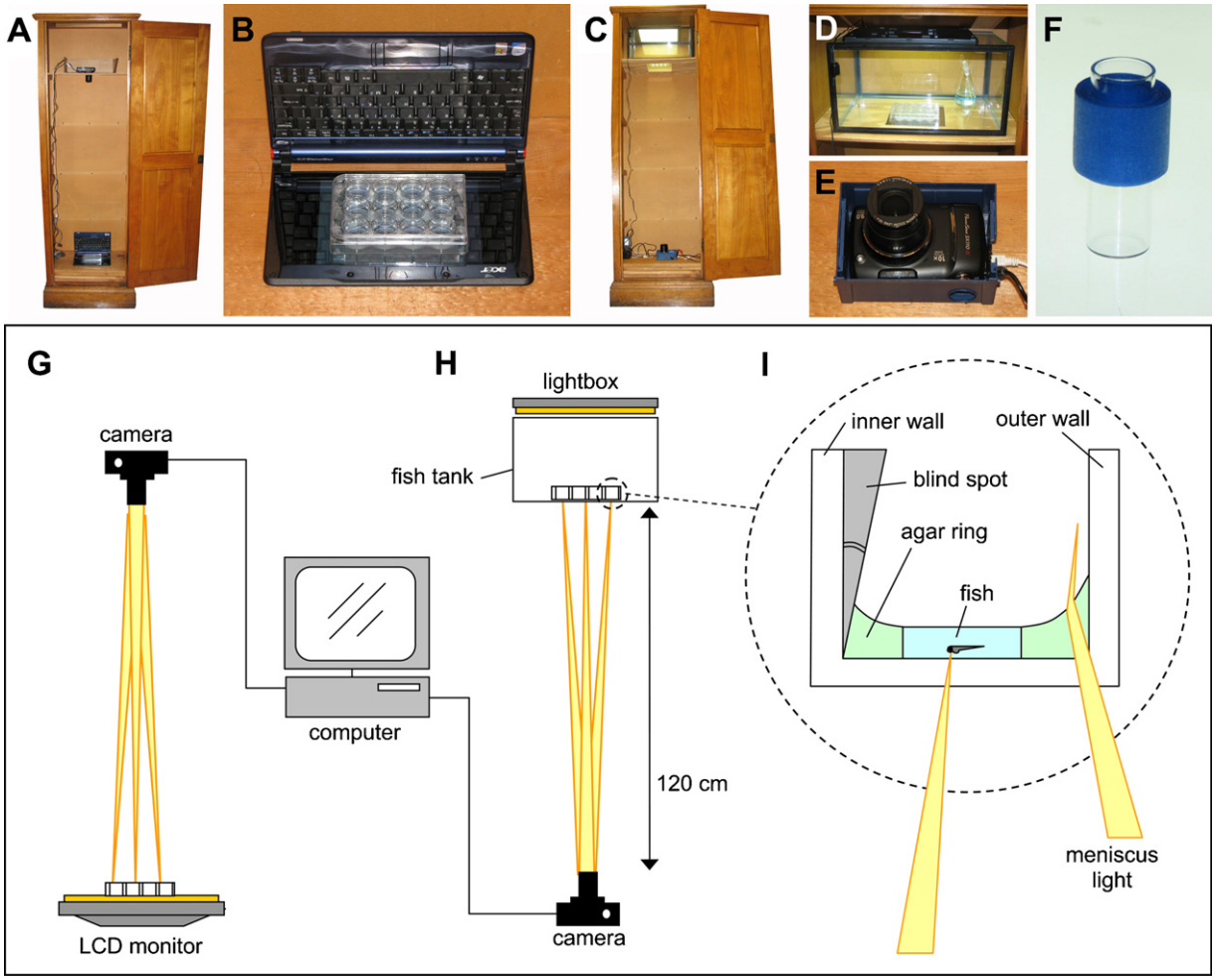

Fig. 1. The zebrafish imaging system. (A) Upright configuration with the camera on the top shelf. The LCD screen of a laptop functions as the light source. PowerPoint presentations with optical stimuli can be presented to the larvae. (B) Laptop with a multiwell plate. (C) Inverted configuration with the camera on the bottom shelf. The culture dish or multiwell plate is illuminated using a thin light box. The culture dish or multiwell plate can be covered with a lid, which stays free of condensation because of the top-lighting. This configuration allows for long-term timelapse recordings of behavior without external stimuli. (D) A multiwell plate is placed inside a fish tank under the light box. (E) Camera located on the bottom shelf. (F) Tool for punching a hole in a layer of agarose. These holes provide a swimming area for fish larvae with excellent optics. (G) Diagram of the upright configuration. (H) Diagram of the inverted configuration. (I) Imaging the outer wells in a multiwell plate can be challenging. The meniscus of the culture medium creates a shadow along the wall of the well, in particular along the outer walls of the well, away from the center of the plate. The meniscus and shadows may be avoided by filling the wells to the rim. However, the fish can then hide in the well's blind spot along the inner wall of the well. The angle of the blind spot increases with an increasing viewing angle. It is thus advantageous to maximize the distance between the camera and multiwell plate. We found that good optics can be attained with a camera-to-plate distance of 120 cm. In this case, the angle of the blind spot is 2.4° in outer wells. The optics can be further improved by using multiwell plates with agarose rings, which limit the swimming area to the center of the well.