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Tracking zebrafish larvae in group – Status and perspectives ☆

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ABSTRACT

Video processing is increasingly becoming a standard procedure in zebrafish behavior investigations as it enables higher research throughput and new or better measures. This trend, fostered by the ever increasing performance-to-price ratio of the required recording and processing equipment, should be expected to continue in the foreseeable future, with video-processing based methods permeating more and more experiments and, as a result, expanding the very role of behavioral studies in zebrafish research. To assess whether the routine video tracking of zebrafish larvae directly in the Petri dish is a capability that can be expected in the near future, the key processing concepts are discussed and illustrated on published zebrafish studies when available or other animals when not.

1. Introduction

For years, zebrafish has been a promising system for behavioral sciences. Visually guided behaviors such as the optokinetic reflex, the optomotor response, escape response, and prey-capture, and corresponding retino-tectal and spinal cord circuits have been extensively investigated in zebrafish for more than a decade (reviewed in [1]). In addition to these visual and locomotor behaviors, the neurosciences community has been growing increasingly interested in the use of zebrafish for more sophisticated and complex behaviors involved in psychiatric disorders such as drug addiction and withdrawal [2–6], aggressiveness [7,8], fear and anxiety (reviewed in [9]), social interaction [10,11], learning and memory [12,13], and circadian and sleep-wake behaviors [14–17].

As a vertebrate, zebrafish also has the advantage of sharing with mammals similar central nervous system organization and circuitry underpinning behaviors. The principal neurotransmitters found in mammals are largely conserved in zebrafish, including amino acids (glutamate, GABA, glycine) [18], monoamines (histamine, dopamine, norepinephrine, epinephrine, serotonin, melatonin) [19–21], and acetylcholine [22], consistent with the conserved responses of zebrafish to various drugs targeted to the nervous system [2,23,24]. Importantly, zebrafish harbor most of the neuropeptides present in mammalian nervous systems (e.g. [17,25]). The zebrafish represents an extremely attractive system to understand the molecular and neuronal substrates of behaviors.

Zebrafish behavior assessment methods are relevant in an increasing number of experimental contexts as a result of the growing usability of video processing. Not only does video processing allow for measure automation and increased accuracy, leading to higher research throughput, it also allows the definition of entirely new measures based on features that would not be detectable or countable by manual methods. Our aim is to identify and discuss the key processing components and challenges relevant to the video-based macroscopic observation of free-swimming zebrafish at the larval stage in laboratory conditions. We define the larval stage from 3 dpf to the 4th week; the earlier embryo stage is out of scope here as it usually involves different setups and toolkits.

2. Tracking zebrafish larvae motion

Small size, body transparency, and discontinuous kinematics combine to make tracking individual larvae in a Petri dish notoriously difficult. At the time of this writing, we know of no off-the-shelf system that supports tracking zebrafish larvae under such unrestricted conditions. As a result, a large majority of studies physically segregate fish, typically by placing them in individual wells of a multi-well plate.

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Tracking is an intuitive and pervasive concept. Simply counting zebrafish in a clutch requires tracking: keeping track of individual fish while the count is taken. One could safely assert that all behavioral studies, not simply video-based studies, involve some form of tracking. It is no surprise then that the term is ubiquitous in the video-based behavior assessment literature, even when restricted to zebrafish. This abundance can be illustrated by querying Google Scholar: a query with the three words zebrafish, video, and tracking returned approximately 2850 records in February 2013.

Comparing studies is difficult unless we dive into the processing details and define some basic comparison or performance criteria. Regarding the latter, we will pay particular attention to the reliability of the tracking output (accuracy of kinematic measures and error estimation, integrity of the system vis–à–vis potential swap of fish identity), the theoretical basis of the tracking approach, and the cost, in terms of processing power, at which the tracking data is extracted.

In video-processing, multi-target tracking – the process by which several moving objects are tracked from frame to frame – is usually characterized by three major groups of tasks performed on each frame in two consecutive steps. In a first step, object features are extracted from the image (feature extraction) and are processed in data structures that describe the various detected objects (target representation). In a second step, structures representing objects across consecutive frames are associated so that the identity of each object can be traced and various kinematic calculations can be performed.

In everyday life, video is recorded in color; for describing the tracking process however, we will consider that video is recorded in black and white, producing sequences of gray-level images, as it is the case with most zebrafish studies. The few studies that use color video either process each color channel as separate gray-level images, select the best color channel in each phase of the experiment and process images from that channel only [26], or reduce the color planes into a single gray-level image via linear combination [27].

Before we examine the tracking process, we need to examine tracking's raison-d'être: zebrafish larva motion.

3. Zebrafish larva motion

3.1. Movement

Zebrafish larva motion kinematics have been studied in details [28–31]. These studies are based on the stop-motion technique which, as noted by [31], was pioneered in the mid 19th century by the photographer Eadweard Muybridge to investigate the details of animal locomotion.

These studies have characterized a small set of elementary motion building blocks which can be assembled in sequences expressing more complex locomotory patterns. For example, the familiar larva escape movement (fast startle response in [29]) can be precisely defined as a stereotypical sequence initiated by a C-start and followed by a counter-bend and an episode of cyclic swimming. Table 1 shows seven easy-to-characterize elementary patterns adapted from the list of nine basic maneuvers described in [32], although one should keep in mind that such categorizations are not absolute or problem-free, particularly when analyzing mutants.

3.2. Immobility

From a kinematics point of view, detecting immobility is very important because larva motion shows highly discontinuous acceleration pulses (we will discuss the impact of these acceleration discontinuities when we review the mathematics justifying the various tracking approaches). The most significant of these discontinuities are found at the transition between immobility and movement. By removing all episodes of immobility, however brief, one may hope to extract a set of motion segments during each of which second order kinematics is continuous. Therefore, precise detection of immobility is crucial.

Immobility is important for biological reasons as well; it is used to characterize sleep [9,15,16,33], inactivity, and freezing behavior. However, most methods, including commercial larva tracking systems, assess immobility on the basis of displacement thresholds (in [38], a notable exception, the authors perform spectral analysis of the larva's tail curvature variations to assess immobiltiy more precisely). Using such thresholds does not allow for precise detection of immobility because immobility cannot be defined on the basis of a displacement threshold alone due to the frequent presence of zero or very low displacement amplitude movements. Examples of such movements include very low amplitude scoots (see Table 1 and Fig. 1G) and zero-displacement routine turns (Fig. 1G). Immobility episodes are very frequent themselves in larvae (found in 99.7% of all 267 ms time bins in Fig. 1F) and transitions from motion to immobility are typically very brief due to water viscosity.

Active quasi immobility is another example of an easily observed, potentially significant behavior hard to detect with methods using displacement thresholds. Fig. 1A–C shows an example of a freezing behavior that could not be detected via such threshold: during the episode, the fish have frequent immobility maintenance movements that produce displacement amplitudes larger than some of the in-place prey capture movements exhibited during the highly active feeding phase. While the contrast in overall movement provides clues to recognize the freezing episode of the juvenile fish in Fig. 1B, freezing is much more difficult to characterize at the larval stage.

4. Fish representation

Targets are represented computationally by sets of parameters. In point representations, these parameters are the spatial (three-dimensional) or planar (two-dimensional) coordinates of a single point expressed in a Cartesian, cylindrical, or spherical system. In situations where a mere point is not sufficient to represent the target, richer representations are used; we will refer to them as structured.

Structured representations can contain a substantial number of parameters for each target. For example, to maintain the position and wing posture attitude of a *Drosophila*, [27] maintains a set of 25 parameters per fly. Even though the zebrafish larva is smaller in volume and has a geometrically simpler body envelope, the number of parameters used to represent its body position in macroscopic video studies spans two orders of magnitude, from a single pair of coordinates in high-throughput activity-monitoring studies [34] to near 100 points in mesh representations of the fish's outline for detailed kinematic analysis [31].

Maintaining a more complex, richer structure usually necessitates fine object resolutions, which depend on camera resolution and image magnification. Fish resolution can be expressed in number of pixels per fish, or per body length, and commonly ranges from a few pixels ([35], estimate based on camera resolution and image examples) to over 250 pixels ([31], estimate based on camera resolution and movie example) per body length. However, there is no simple rule that maps the two, as different applications may re-sample (resize) images in either direction. Depending on the chosen representation, tracking will mean very different things.