Zebrafish Behavior: Opportunities and Challenges

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Abstract

A great challenge in neuroscience is understanding how activity in the brain gives rise to behavior. The zebrafish is an ideal vertebrate model to address this challenge, thanks to the capacity, at the larval stage, for precise behavioral measurements, genetic manipulations, and recording and manipulation of neural activity noninvasively and at single-neuron resolution throughout the whole brain. These techniques are being further developed for application in freely moving animals and juvenile stages to study more complex behaviors including learning, decision making, and social interactions. We review some of the approaches that have been used to study the behavior of zebrafish and point to opportunities and challenges that lie ahead.

Contents

1. INTRODUCTION

Behavior determines how an animal interacts with its environment. For an ecologist, this interaction, and its consequences for the survival of the animal, may be the primary focus of their research. A neuroscientist, by contrast, may be interested in behavior as the ultimate function of the brain. This has led to different approaches to studying behavior. An ecologist may choose to study diverse species adapted to particular environments and focus on the richness of natural behavior. Neuroscientists often prefer to take a reductionist approach, focusing on isolated components of behavior in artificial conditions that allow tight control of experimental parameters. By permitting repeatability of conditions across experiments and across labs, this makes possible comparisons between experiments and provides statistical power to relate behavior to neural function (Gomez-Marin et al. 2014). The downside of this approach is that the brain evolved to generate behavior in complex natural environments, which may not be captured under reduced conditions. Ideally, we would want an experimental system where we can study naturalistic behaviors combined with tight control of experimental parameters and comprehensive measurements of behavioral output and physiology (**Figure 1**).

Zebrafish are a promising vertebrate model in which to bridge this gap. Increasingly sophisticated tracking and behavioral analysis tools (e.g., Burgess & Granato 2007b, Martineau &

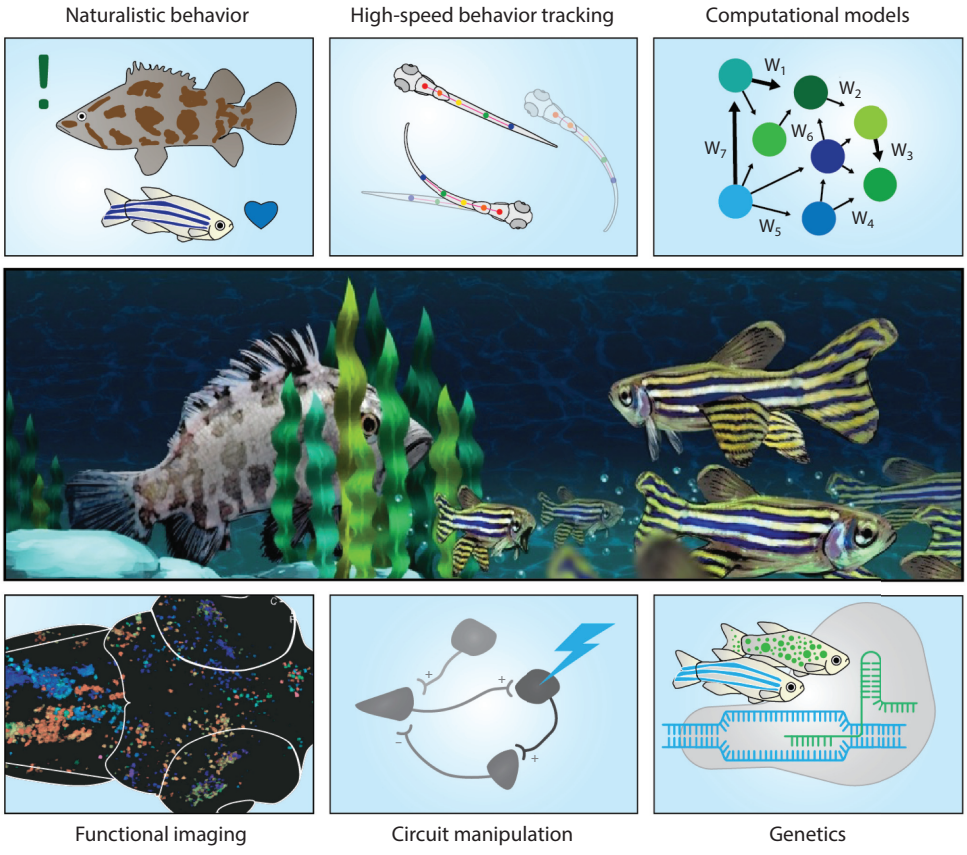

Figure 1

Zebrafish is an ideal species to study the neurobiological basis of natural behavior. This is made possible by a combination of techniques, including new developments in animal tracking, mathematical modeling of behavior, functional imaging of the whole brain, and methods for precise circuit and genetic manipulation.

Mourrain 2013, Mirat et al. 2013, Dell et al. 2014, Pérez-Escudero et al. 2014, Stewart et al. 2015) are bringing the study of behavior in this traditional lab model closer to that of the species typically of interest to behavioral ecologists and are allowing a quantitative understanding of the computations underlying behavioral decisions (Arganda et al. 2012). Technical developments, including the ability to make precise genetic manipulations (Hwang et al. 2013, Auer et al. 2014) and, at the larval stage, to record and manipulate neural activity at cellular resolution throughout the whole brain (Ahrens et al. 2013, Feierstein et al. 2015), now make it possible to address systematically how the brain is implementing these computations. Importantly, more and more, these approaches can even be applied to freely behaving individuals (e.g., Muto et al. 2013). A challenge in the near future will be to combine state-of-the-art optical and genetic methods with the study of more complex behaviors. We discuss here just a selection of behaviors found in early life and some aspects of adult social behavior; more comprehensive reviews of other important aspects of zebrafish behavior are available elsewhere (e.g., Norton & Bally-Cuif 2010, Kalueff & Cachat 2011, Kalueff et al. 2013, Roberts et al. 2013).

2. BEHAVIOR TRACKING

Analyzing behavior presents several technical challenges, both to capture biological movement in all its complexity and to convert it into simple numerical measures to allow for quantitative analysis (Leshner & Pfaff 2011, Dell et al. 2014). This is further complicated when we wish to combine natural behavior with physiological recordings or neural circuit manipulations.

2.1. Video Tracking of Individual Location and Posture

Video tracking is a versatile way to monitor behavior remotely (Dell et al. 2014), and it is an effective approach for zebrafish, whose movements can be captured largely from a single view (**Figure 2*a***). Frequently, fish are placed in shallow water and filmed from above or below, allowing tracking of location and orientation, tail curvature, horizontal eye position, and pectoral

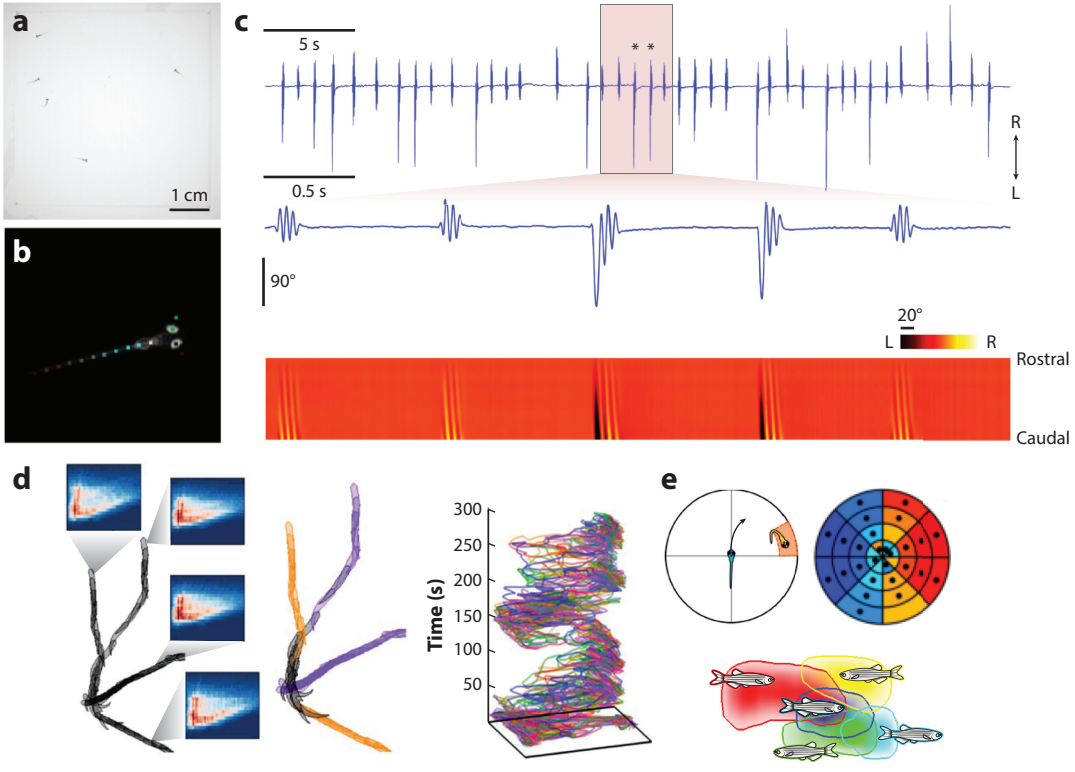

Figure 2

Behavioral tracking, from individual posture to group dynamics. (*a*) Larval fish swimming in shallow water observed from above with a high-speed camera. (*b*) Automated image processing is used to track eye and tail positions. (*c*) Fish swim in discrete bouts. (*Top*) Tail-end angle relative to the head during spontaneous swimming of a seven-day-old larva. (*Middle*) Zoomed view of five swim bouts including two turning maneuvers (*asterisks*). (*Bottom*) Heat map of angle along the tail over time showing the rostrocaudal propagation of bending. (*d*) Tracking of individual zebrafish in groups by recognition of image fingerprints (*left*) allows for an automatic assignment (*middle*, *orange* and *purple*), and resulting trajectories coincide with those performed by humans (*right*, colors in trajectories of 8 fish when there is coincidence with human assignment, corresponding to 99%). Panel adapted with permission from Pérez-Escudero et al. (2014). (*e*) Trajectories from panel *d* are used to compute the interaction, measured as the probability that a fish will accelerate to the right when another fish is in different points in space at 24 days. Dots indicate regions with $P < 0.005$ compared to shuffled video data, and overlapping colored ovals indicate territories for a group of five fish after aggressive encounters.

fin motions (Easter & Nicola 1997, Liu & Fetcho 1999, Budick & O'Malley 2000, Green et al. 2011) (**Figure 2*b*,*c***). Larvae show tail beat frequencies up to 100 Hz and can turn up to 30° in a millisecond, requiring video rates as high as 1,000 Hz. Fortunately, because the postural space is quite limited (Girdhar et al. 2015), the very high-dimensional video data can be compressed to much more compact descriptions, making possible continuous acquisition from many fish. Although many studies have analyzed each movie frame manually (Easter & Nicola 1997, Budick & O'Malley 2000, McClenahan et al. 2012), automation of this process becomes critical for high-throughput methods. Several image-processing algorithms and free software packages allow automatic extraction of kinematic variables from video images (Liu & Fetcho 1999, Burgess & Granato 2007b, Fontaine et al. 2008, Huber-Reggi et al. 2012, Martineau & Mourrain 2013, Mirat et al. 2013, Zhou et al. 2014). Confining the fish to a two-dimensional environment is a useful simplification, but, under more natural conditions, zebrafish of all ages do explore their world in three dimensions (3-D). In larvae, side and head-on views have been used to quantify vertical swimming behavior and vertical and torsional eye movements (Bianco et al. 2012, Fernandes et al. 2012, Ma et al. 2014). Multiple viewpoints can be used to track individuals in 3-D (Zhu & Weng 2007, Cachat et al. 2011, Stewart et al. 2015) and even capture the 3-D posture of larvae (Nair et al. 2015, Voesenek et al. 2016), providing a rich new domain for exploration.

2.2. Categorization of Swimming Patterns in Larvae

Complex behaviors can often be described in terms of sequences of more basic motor patterns (Tinbergen 1951). This can allow a simplified description of behavior and provide an indication of how the underlying neural systems are organized. However, the question of how to divide behavior into discrete units in time is a difficult one that is often settled by subjective analysis based on experienced observation, although, more recently, researchers have made efforts to segment behavior in an unbiased way via unsupervised machine learning methods (Brown et al. 2013, Berman et al. 2014, Wiltschko et al. 2015). A key feature of swimming in the larvae is that it is not continuous but has a burst and glide structure, with brief bouts of tail movement alternating with interbout periods during which the fish does not propel itself actively (**Figure 2*c***). This divides behavior naturally into discrete units. In different situations, such as during fast escapes, hunting, or routine locomotion (Kimmel et al. 1974, Budick & O'Malley 2000, Borla et al. 2002, McElligott & O'Malley 2005), larvae use swim bouts with distinct kinematic properties, and these bouts form distinct clusters in the space of swim kinematics (Burgess & Granato 2007a,b). The simplicity of this motor organization provides an opportunity to develop a complete ethogram of the larva: an exhaustive list of its behavior patterns. So far, researchers have described at least nine different swim bout patterns (Fero et al. 2011). A key question is whether these represent truly distinct movements or whether they are different parts of a continuum. Both stimulus-driven modulation of bout parameters and evidence of clear categorical boundaries have been observed in different contexts (e.g., Burgess & Granato 2007b). In the case of forward locomotion, larvae, when driven to swim with increasing speeds, at first increase bout duration smoothly but eventually show an abrupt change in gait (Severi et al. 2014).

2.3. Tracking Individuals in Groups

It is often necessary to track multiple fish together. Tracking systems developed for single individuals can be used when it is not necessary to follow individual paths, for example, to measure the mean distance between animals. Some systems are more adapted to group settings (Martineau & Mourrain 2013, Mirat et al. 2013), and recently developed machine learning algorithms can

maintain correct identities for indefinite time periods (Pérez-Escudero et al. 2014) (**Figure 2*d***). These algorithms extract an abstract fingerprint in the image for each animal and use it to identify them automatically even after they cross or disappear from view. Automatic analysis of interaction behavior in zebrafish can now be done in naturalistic conditions (**Figure 2*e***). Additionally, machine learning algorithms can be applied that learn to recognize certain behaviors (Kabra et al. 2012). Combining existing technology and new results in artificial intelligence (Mnih et al. 2015) should soon allow for quantitative detailed measurements of the behavior of individual zebrafish in groups in 3-D, including body posture and complex naturalistic environments. Controlled stimulation is now possible for vision but less so for water flow and mechanical stimulation. Control of social stimulation can be done partially with video (Saverino & Gerlai 2008, Neri 2012, Gerlai 2014), but virtual reality and miniaturization of present-day robotic fish (Butail et al. 2014) will allow more realistic controlled interactions.

2.4. Computational Modeling of Behavior

Models attempt to capture relevant structure in behavioral data and can be used to demonstrate a sufficient explanation, understand causal relations, make predictions, and relate levels of description. There is a long tradition of modeling behavior, and principled models exist for many behaviors, for example, foraging, mate choice, and maternal care (Kokko 2007). Although zebrafish offer the opportunity to combine a mathematical modeling approach with manipulations during free behavior, models so far have been limited mainly to locomotion. Some capture the water flow and body dynamics (Li et al. 2012, 2014). Others use hidden Markov models, defining movement segments as states and computing transitions between them, to compare across conditions (Liu et al. 2011, Li et al. 2013). Obtaining the relevant states from raw data can be achieved using dimensionality-reduction techniques, as applied recently to collapse zebrafish postures into a small postural space and chains of postures into a behavioral space (Girdhar et al. 2015). Outside the problem of locomotion, a state-based approach has been used in zebrafish aggression using first-order Markov chains of manually labeled states (Oliveira 2013) and also to model exploratory behavior in larvae (Dunn et al. 2016b). As behavior is produced by neuronal circuit processing coupled to a body, another class of models for behavior is based on the mathematics describing this substrate (Enquist & Ghirlanda 2005). In zebrafish, a simple network model of this type has been used for locomotion (Girdhar et al. 2015), and systems neuroscience studies in zebrafish, including comprehensive maps of population activity and connectivity, will soon enable more realistic versions of this type of model. Hypothesis-driven models of behavior propose an organization principle and derive predictions from it. Well-developed theories that have been applied to behavior are game theory, dynamic programming, control theory, reinforcement learning, and decision-making theory (Shoham & Leyton-Brown 2009). In zebrafish, however, there is little application of such models, with the exception of a locomotion model based on a jump diffusion model applied commonly to financial markets (Mwaffo et al. 2015) and a model of group decision making (Arganda et al. 2012). Application of engineering system analysis methods to behavior will be a helpful tool in building more quantitative models (Beck et al. 2004, Haesemeyer et al. 2015).

2.5. Other Considerations

Laboratory conditions may differ from natural ones in key ways, making it important to obtain better accounts of the behavior of zebrafish in the wild. Currently, more is known about their habitats and diversity of morphological traits (Spence et al. 2008, Arunachalam et al. 2013, Parichy 2015) than about behavior. Recent results show typical group sizes and aggression behavior vary

remarkably depending on region (Suriyampola et al. 2016). The behavioral and genetic variability across different laboratory strains (Brown et al. 2012, Lange et al. 2013) and wild-derived animals (Wright et al. 2006) is potentially problematic for experimental standardization, but it offers interesting opportunities to study the underlying genetics.

3. BEHAVIORS IN EARLY LIFE

Much of the work on zebrafish has focused on early larval stages, in which the animal is still small and transparent and powerful imaging and genetic methods can be most easily applied. Typically, the behaviors that have been studied in larvae are innate, reflexive responses, although many can be modified, for example, by short-term plasticity (Roberts et al. 2013). Larvae are sensitive to a variety of stimulus modalities, including touch, olfaction, chemosensation, audition, vestibular inputs, heat, and vision. Here we present some of the main lines of research in larval behavior; for a more comprehensive listing, see Fero et al. (2011) and Kalueff et al. (2013), and for a review of spinal motor circuits, see McLean & Fetcho (2008).

3.1. Development of Motor Behavior

The first movements of the larva, which consist of spontaneous contractions of the tail, appear at just 17 h postfertilization (Saint-Amant & Drapeau 1998). At this stage, just six types of spinal neurons are active, with a repeating segmental organization (Bernhardt et al. 1990), synaptic transmission is entirely via gap junctions rather than chemical synapses (Saint-Amant & Drapeau 2000), and descending input is absent. All this makes for an attractive system to study the development of spontaneous behavior in simple circuits. Over the next 3–4 h, activity patterns mature from uncorrelated bursting patterns to coordinated side-to-side alternation. Patterned optogenetic inhibition, combined with activity imaging, revealed that this is an activity-dependent process (Warp et al. 2012). Touch-evoked responses, mediated by Rohon-Beard sensory neurons, appear at 21 h (Saint-Amant & Drapeau 1998), and by 1–2 days, the fish are able to swim in a coordinated manner and respond appropriately to stimuli in different locations (Saint-Amant & Drapeau 1998). By 4 days old, although the brain continues to grow, major brain structures and axon tracts, including different clusters of neuromodulatory neurons, are present, and the larvae begin to feed. Most studies of larval behavior have focused either on very early stages, in which the developing circuits are still very simple, or on 5–10-day-old larvae, in which many robust spontaneous and sensory-evoked behaviors can be observed. It will be interesting to follow how functional connectivity and behavior develop through intermediate stages, in which activity can now be tracked through the whole brain (Chhetri et al. 2015).

3.2. Escape and Startle

Escape swimming was one of the first behaviors to be investigated in the zebrafish model (Eaton & Farley 1973, Kimmel et al. 1974). Escapes are at first evoked reliably by touch and later, at about four days old, by acoustic stimuli (Kimmel et al. 1974, Kohashi et al. 2012). These reflexive responses have very short latencies, down to a few milliseconds, but have surprising layers of complexity. Larvae control the magnitude and direction of the escape turn depending on the location of the stimulus, by the pattern of activity in an array of six identified neurons in the brain stem (O'Malley et al. 1996, Liu & Fetcho 1999). Tail-directed stimuli activate the giant Mauthner reticulospinal neuron, while head-directed stimuli also recruit its homologs in adjacent segments, causing a larger initial bend (O'Malley et al. 1996). The relative simplicity of the escape

circuit has made it rich terrain for understanding the circuit mechanisms underlying behavior at a cellular level (Lorent et al. 2001, Satou et al. 2009, LaCoste et al. 2015, Hale et al. 2016, Koyama et al. 2016). Whereas the escape system is highly specialized, recent work, building on the genetic architecture of the hindbrain, has suggested that the circuits mediating different motor behaviors are built from the same canonical set of cell types (Kinkhabwala et al. 2011, Koyama et al. 2011), and the same system may be adapted for rapid visual decision making in the archerfish (Schlegel & Schuster 2008). Intriguingly, the Mauthner system integrates information across many stimulus modalities. For example, fast escapes may also be elicited by water flow, sensed by the lateral line system (McHenry et al. 2009), and by looming visual stimuli (Temizer et al. 2015, Dunn et al. 2016a, Yao et al. 2016). Recent work has begun to explore, in more detail, motor control during escapes, including alternative startle patterns (Liu & Hale 2014), coordination of tail and pectoral fin movements (McClenahan et al. 2012), and 3-D escape trajectories (Nair et al. 2015).

3.3. Illumination and Phototaxis

Ambient light levels exert a strong influence over zebrafish swimming behavior. In 20-h embryos, before the retina is even connected to the brain, light, acting through opsins expressed in spinal cord neurons, inhibits spontaneous activity (Friedmann et al. 2014). By 30 h, a bright flash of light drives a burst of motor activity, via photosensitive cells in the hindbrain (Kokel et al. 2010, 2013). In larvae more than 5 days old, cycling light levels lead to changes in locomotor activity across multiple timescales (Prober et al. 2006, Burgess & Granato 2007a, Emran et al. 2008). For example, a sudden transition from light to dark elicits a stereotyped large-angle O-bend, followed by several minutes of increased locomotion, which gradually reduces to a lower level (Burgess & Granato 2007a, Emran et al. 2008). Larvae show phototactic behavior, seeking out lighter areas (Brockerhoff et al. 1995, Orger & Baier 2005), in contrast to adult fish that tend to prefer darker locations (Serra et al. 1999), although many factors modulate and even reverse these preferences (Gerlai et al. 2000, Burgess et al. 2010). Phototaxis illustrates effectively how complex behavior can emerge from simple behavioral rules. A model in which the retinal OFF pathway drives turning away from the darker side, and the ON pathway stimulates approach, can reproduce phototactic behavior, even with multiple targets (Burgess et al. 2010). Larvae can also navigate without using spatial cues at all. By increasing locomotor activity in the dark, they spend more time in brighter regions, and they can do this even without eyes, because this is another response mediated by deep brain photoreceptors (Fernandes et al. 2012). Curiously, larvae can even stay within the bounds of a virtual circle in a closed-loop swimming assay, where their position in the arena is used to control the whole-field illumination (**Figure 3*a***). This seemingly complex behavior can be explained largely by a model of the fishes swimming that takes into account the sign of illumination change and the direction of the previous swim bout but has no explicit spatial information (Chen & Engert 2014).

3.4. Optic Flow Responses

Self-movements generate reafferent whole-field visual motion patterns (Britten 2008). In most visual animals, these patterns can elicit compensatory movements of the eyes [optokinetic response (OKR)] or the head and body [optomotor response (OMR)] that serve to stabilize the image of the world on the retina. In zebrafish, rotational motion drives the OKR (Clark 1981, Brockerhoff et al. 1995, Easter & Nicola 1997), whereas translational motion, typically in the form of stripes projected on a horizontal screen below the larva, stimulates instead a swimming OMR (Clark 1981, Neuhauss et al. 1999) (**Figure 3*b***). The OKR appears from three days old, about when retinal

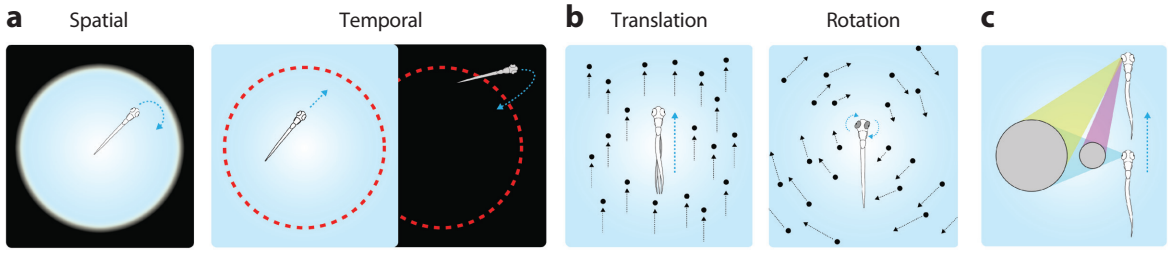

Figure 3

Illustrations of some larval behaviors. (*a*) Phototaxis using spatial and temporal cues. Larvae can stay in a small illuminated area by turning away from a light-dark boundary (*left*). They can also stay inside a virtual circle, where whole-field illumination is toggled when they cross the boundary, by following simple sensorimotor rules. (*b*) Translational motion causes optomotor swimming behavior, which acts to counteract perceived self-motion in the opposite direction. Rotational motion elicits the optokinetic response. (*c*) With monocular vision, objects of different sizes and distances may subtend the same visual angle. However, when the fish swims forward, closer objects move more quickly across the retina.

axons reach their central targets (Easter & Nicola 1997), but the gain increases significantly, particularly for faster stimuli, by 3–4 weeks of age (Beck et al. 2004). The OMR consists of a set of basic motor components, each of which is tuned to motion direction: forward swims driven by forward motion and turns driven by lateral movement (Orger et al. 2008). Although there is an intermittent and stochastic element to the generation of swim bouts (Portugues et al. 2015), larvae can adjust their average speed to match the stimulus quite precisely (Severi et al. 2014) and can adapt their response gain based on visual feedback (Portugues & Engert 2011, Ahrens et al. 2012). Distinguishing between rotation and translation requires integration of motion information across different regions of the visual field. Several clusters of neurons in a pretectal area respond to horizontal motion and integrate inputs from the two eyes in ways that could distinguish rotational and translational flow, suggesting that this region could provide a common input for OMR and OKR behaviors (Kubo et al. 2014). It remains to be determined how responses to other motion directions are organized, although studies from other fish indicate that the same region processes vertical motion (Masseck & Hoffmann 2009). Beyond these compensatory reflexes, it will be interesting to learn how optic flow is integrated with other signals to guide behavior. In mutant fish with reversed wiring in the visual system, eye movements can trigger abnormal looping behavior, suggesting that the larvae integrate motor efference copy and sensory inputs (Huang et al. 2009). Zebrafish also use their lateral line to sense water flow in the absence of visual cues and swim to counteract the current (Olszewski et al. 2012, Suli et al. 2012), although visual cues appear to dominate when available (Olive et al. 2016).

3.5. Hunting and Feeding

The natural prey of the larva are small motile organisms, such as paramecia, and prey capture behavior has two distinct phases: an initial orientation and approach phase, during which the larva converges its eyes and maneuvers the prey into the newly formed binocular zone, and a capture swim that is triggered once the prey is positioned at the correct location in front of the fish (Borla et al. 2002, McElligott & O'Malley 2005, Bianco et al. 2011). Whether binocular vision is required to initiate capture has not been demonstrated. The features that identify a stimulus as potential prey are not completely clear, and there is some disagreement within the published literature (Bianco et al. 2011, Semmelhack et al. 2014, Romano et al. 2015). Researchers agree that size is an important feature, but, because larval vision is monocular and has a fixed focus, retinal size is an ambiguous

cue for the true size of an object. One simple way to resolve this ambiguity is by the movement of the stimulus when the fish itself swims (**Figure 3*c***). How zebrafish integrate object and self-motion is still unknown, but prey capture behavior is unreliable in immobilized fish, and this is improved by the introduction of closed-loop visual feedback (Trivedi & Bollmann 2013). When orienting to prey, the fish decrease the interval between swim bouts and use a characteristic set of movements that includes the J-turn, in which the rostral part of the tail is stiffened, allowing the fish to turn with minimal water disturbance (Gahtan 2005, McElligott & O'Malley 2005). This change in kinematics, together with eye convergence, identifies clearly when the larvae are hunting. How different motivational states are controlled is an important question (Horstick et al. 2016), and a recent study has implicated the hypothalamic-pituitary-interrenal axis and serotonergic systems in control of the feeding state in larvae (Filosa et al. 2016). A key question is whether there exist dedicated neural pathways for particular behaviors. Retinal ganglion cells in zebrafish project to 10 central arborization areas, and one of these receives input that is tuned specifically to prey-like features (Semmelhack et al. 2014), suggestive of a behavior-centered organization.

3.6. High-Throughput Behavior Profiling

Zebrafish larvae can be maintained for days arrayed individually in multiwell plates, which is ideal for high-throughput analysis of behavior, suitable for genetic, pharmacological, and neurotoxicological screens. Assays include circadian variations in activity (Prober et al. 2006) and responses to whole-field illumination changes and acoustic/vibrational stimuli that can been delivered uniformly across many wells, and also more complex stimuli and measurements of behavioral plasticity (Pelkowski et al. 2011, Wolman et al. 2011, Bruni et al. 2016). A very fruitful approach has been to use low-dimensional representations of behavioral phenotypes, so-called behavioral bar codes or fingerprints, as the basis for unsupervised clustering of chemical compounds (Kokel et al. 2010, Rihel et al. 2010, Wang et al. 2014, Woods et al. 2014, Bruni et al. 2016), allowing the identification of novel functional classes of compounds and new drugs for existing targets. Although these approaches can treat behavior as a nonspecific readout of brain function, it is also desirable to target particular human diseases. Many human neurological disorders are characterized by complex cognitive symptoms, and it is not always clear how to model these in zebrafish. In many cases, though, these symptoms may emerge as a consequence of more basic cellular deficits in the function of more complex circuits. Much recent work has focused on identifying endophenotypes, which provide a stable and easily quantifiable readout of the core cellular or circuit deficits in a disorder (Gould & Gottesman 2006) and can be used to assay potential treatments. An example of a promising endophenotype in zebrafish is prepulse inhibition in the startle reflex, which has been associated with schizophrenia (Burgess & Granato 2007b, Bergeron et al. 2014). In a chemical screen described above, Rihel et al. (2010) showed that changes in swimming behavior during a light and dark cycle can provide a sensitive readout of perturbations in different neural systems. Recently, using a mutant fish that reproduced a genetic deficit linked to autism, they described a phenotype in this assay and then used it to identify novel potential therapeutic agents (Hoffman et al. 2016).

3.7. Ontogeny of Complex Behaviors

A potential concern with using week-old zebrafish as a behavioral model is that the nervous system is still at a transitional stage, and therefore their behavior represents a snapshot in development during which important neural systems may be absent or not yet mature. Although week-old larvae have an array of innate reflexive responses and display several forms of nonassociative plasticity, their ability to show robust associative learning is a subject of debate (Roberts et al. 2013), and

they display more limited social interactions. Experimental accessibility decreases with age, but recent work on habenula and brain stem circuits has demonstrated the potential for noninvasive functional imaging out to at least three weeks of age (Jetti et al. 2014, Vendrell-Llopis & Yaksi 2015), and systematic studies of ontogeny will be essential to determine the optimal time window in which to study different behaviors. A careful study of the development of simple classical and operant conditioning assays found that these behaviors attained adult levels at some point between three and six weeks of age (Valente et al. 2012). Interestingly, this same time period may also see major alterations in innate reflexes (Bak-Coleman et al. 2015) and an increase in social interaction (Dreosti et al. 2015). The improvement in learning may represent a maturation of neural circuits, but it may also result from a failure to identify stimuli relevant to the larva, and some studies have described classical conditioning assays applicable to seven-day-old larvae (Aizenberg & Schuman 2011, Hinz et al. 2013) and motor learning (Portugues & Engert 2011, Ahrens et al. 2012).

4. SOCIAL BEHAVIORS

As zebrafish progress from larvae to adults, their behavior becomes increasingly complex, particularly in the realm of social interactions, which include shoaling and schooling, decisions in groups, aggressive encounters, and mating.

4.1. Shoaling

Zebrafish form shoals and the more polarized groups, known as schools, because of attraction to each other and not owing to attraction toward shared environmental features (**Figure 4*a***). The many possible advantages of being in a shoal include better detection of predators, food, and mates (Krause & Ruxton 2002). Groups of zebrafish are dynamic: Distance among animals increases with time after they are placed in a novel tank and decreases in the presence of a predator (Miller & Gerlai 2007). Research on zebrafish groups has focused mainly on what makes a group attractive to an individual. Zebrafish choose to shoal with conspecifics over an empty compartment and show a preference for larger shoal sizes (Arganda et al. 2012) and fish of similar appearance (Snekser et al. 2010), with activity level and sex composition also playing a role (Pritchard et al. 2001, Ruhl & McRobert 2005). Detection of conspecifics requires binding of motion and form, with the head rather than the tail providing the relevant cues (Neri 2012). Lab experiments with shoals are often performed by placing an individual between two compartments and measuring the proportion of time that the animal is in the compartment with other conspecifics (**Figure 4*b***). This laboratory test has the advantage of being simple, but it limits the sensory input, interferes with the natural interaction dynamics, and introduces walls, which may also be intrinsically attractive, thus adding noise to the behavior. Its use in an ontogeny analysis showed that zebrafish were biased to the side with conspecifics from three weeks of age (Dreosti et al. 2015). An ontogeny analysis using more naturalistic conditions could potentially reveal earlier and richer social interactions. Dissection of the circuit mechanisms underlying social processing (O'Connell & Hofmann 2011, Goodson 2005) should be advantageous in this species, given the imaging tools already applicable at three weeks. The importance of reward circuits in building social interactions (Al-Imari & Gerlai 2008), and the roles of dopamine (Buske & Gerlai 2011), oxytocin, and other peptides (Choleris et al. 2013), remain challenging questions.

4.2. Aggression and Mating

Adult zebrafish behaviors can be very complex but still show stereotyped phases that facilitate quantification and modeling. One example is dyadic fighting in male zebrafish, which can be

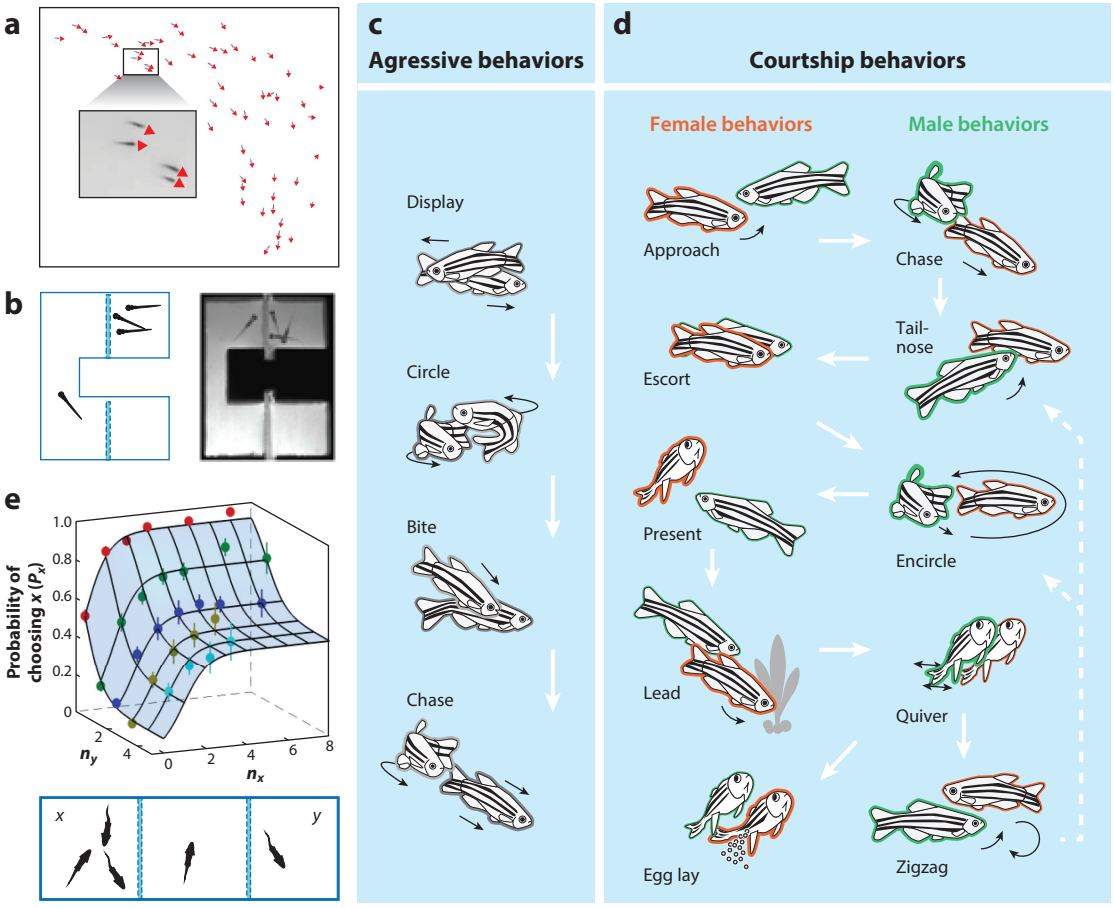

Figure 4

Adult and juvenile behaviors. (*a*) Freely moving groups of four-week-old zebrafish show strong schooling behavior. (*b*) Two-choice setup with transparent divider used to show attraction of three-week-old zebrafish to conspecifics. Panel adapted with permission from Dreosti et al. (2015). (*c*) Aggression between two zebrafish follows a sequence of complex motion patterns. Panel adapted with permission from Oliveira et al. (2011). (*d*) Courtship has an even more complex chain of behaviors. Panel adapted with permission from Darrow & Harris (2004). (*e*) Decision making in adult zebrafish in a social chamber. Plot shows fit of observed probabilities of choosing one of the chambers with a decision-making model based on Bayesian estimation theory. Panel adapted with permission from Arganda et al. (2012).

divided into assessment and postresolution phases (Oliveira et al. 2011). Each phase is structured clearly in different behaviors (**Figure 4*c***). In the assessment phase, fish exhibit display, circle, and bite behaviors. In the resolution phase, agonistic behaviors (bite, chase, strike) are always initiated by the winner. Furthermore, tactics in territorial behavior depend on rank (Gillis & Kramer 1987, Grant & Kramer 1992). Dominant fish defend territory around which food is made to appear and chase other fish, whereas subordinate fish use aggression rarely but sneak in to feed when dominants are feeding or chasing. The structure of the setups can allow for the appearance of satellite fish that can change flexibly from a subordinate role to defending a region distinct from that defended by the dominant fish (Grant & Kramer 1992). Status also influences more subtle behaviors such as eavesdropping (Abril-de Abreu et al. 2015). After observing a fight, only bystander dominant fish became more attentive toward the loser of observed fights. The effect of

winning or losing a fight is long-lasting, with 85.7% and 4.5% of winners and losers, respectively, winning against a naive individual (Oliveira et al. 2011). Fights thus produce very robust output behavioral differences, making this an interesting manipulation for other behaviors and of value for molecular (Oliveira et al. 2011) and neuronal circuit analysis. The highly structured nature of fights and territorial behavior calls for further quantitative analysis combining tracking in 3-D, automatic assignment to behavioral classes, and modeling beyond first-order Markov processes. Similarly, a rich sequence of behaviors during courtship (Darrow & Harris 2004) (**Figure 4*d***) is amenable to quantitative study.

4.3. Decision Making in Groups

Collective animal behavior is studied mainly by behavioral ecologists (Couzin 2009, Sumpter 2011). Fish species have played an important role in these studies, as collective behavior is easy to reproduce in the lab (Ward et al. 2008, Ioannou et al. 2012, Herbert-Read et al. 2013). Zebrafish can play an important role in grounding these studies in neuronal processing. Experiments in zebrafish have led to a model of decision making in collectives according to which animals combine private and social estimations into decisions (Arganda et al. 2012) (**Figure 4*e***). This modeling work shows that zebrafish stands out as a species with particularly simple interactions and has helped researchers to understand aspects of human collective behavior (Grabowicz et al. 2015, Madirolas & de Polavieja 2015). A variety of other possible modeling frameworks for collective motion in zebrafish include reinforcement learning or a more explicit sensory-motor control model. Ultimately, we would want to understand animal interactions by combining these simplified models with realistic and detailed neuronal processing models derived from comprehensive functional studies. An interesting but less explored question is how animals learn in groups (Lindeyer & Reader 2010, Oliveira 2013). Zebrafish can learn from demonstrators, for example, about an escape route (Lindeyer & Reader 2010).

5. CONNECTING NEURAL SYSTEMS AND BEHAVIOR

Zebrafish offer a special opportunity among vertebrate model systems to connect neural population activity with behavior. Recording from behaving animals requires techniques that are robust to movement and do not impede free expression of behavior, and computational methods that can relate the measured activity to behavioral output.

5.1. Combining Functional Imaging and Behavior

The need for stability during high-resolution imaging or electrical recordings is at odds with the fact that fish move very rapidly in 3-D. Larvae can be restrained by embedding them in a gel (Eaton et al. 1984), which is cut away to leave the tail, eyes, or pectoral fins free to move. Such preparations allow for imaging at subcellular resolution throughout the brain or precise optical manipulations and provided some of the first examples of calcium imaging in behaving animals (O'Malley et al. 1996). When greater stability is needed, the fish is paralyzed by blocking neuromuscular transmission genetically or pharmacologically (Buss & Drapeau 2001, Ono et al. 2001) or by isolating the spinal cord (Gabriel et al. 2007). Because this approach leaves electrical signals in motor neurons, or muscles, intact, suction electrodes can be used to measure fictive behavior (Masino & Fetcho 2005, McLean et al. 2007). A drawback of restrained preparations is that the normal sensory feedback from visual, vestibular, and lateral line systems is absent. Algorithms that extract the fish's intended swim vector from its real or fictive movements can

be used to introduce closed-loop visual feedback, which can make behavior more robust and naturalistic and also allows feedback gain to be manipulated (Portugues & Engert 2011, Ahrens et al. 2012, Trivedi & Bollmann 2013). Feedback in other modalities is more complicated and may require alternative means of holding the fish, such as pinning (Masino & Fetcho 2005) or suspension from pipettes (Ahrens et al. 2012).

Two-photon microscopy has been widely applied for functional imaging (Renninger & Orger 2013) but is fundamentally limited in speed, so recording from large volumes requires sequential imaging over time (Portugues et al. 2014). Light-sheet excitation permits the whole-brain volume of a fish larva to be scanned in much less than a second, allowing the near-simultaneous recording of whole-brain activity dynamics (Ahrens et al. 2013, Panier et al. 2013). Although impressive, this acquisition rate is still below what would be needed to capture fast activity dynamics or correct for movement artifacts due to swimming. Methods that allow volume images to be captured in a single exposure (Levoy et al. 2006, Abrahamsson et al. 2012) or remove mechanical limitations (Bouchard et al. 2015, Quirin et al. 2016) therefore show great potential. A concern is that visible excitation light may interfere with behavior. Optomotor swimming can nonetheless be evoked robustly during light-sheet imaging (Vladimirov et al. 2014), and two-photon light-sheet excitation is possible (Wolf et al. 2015). Restrained preparations have limitations for studying natural behavior, particular in cases in which the fish must interact with its environment, so there is a clear imperative to develop imaging in freely swimming animals. Most of the brain of freely moving *Caenorhabditis elegans* can be imaged by canceling their movements using a motorized stage (Nguyen et al. 2016, Venkatachalam et al. 2016), but this approach presents a challenge for zebrafish that move orders of magnitude faster. Muto et al. (2013) imaged visual responses to paramecia in freely swimming larvae using conventional epifluorescence, taking advantage of the fact that larvae remain quite still between bouts of swimming. This approach has potential, especially if used in combination with sparse genetic labeling of neurons (Scott et al. 2007), volume imaging methods, or new approaches such as optoacoustics (Sela et al. 2015). An alternative to live imaging is to integrate neural activity in such a way that it can be measured later at high resolution. Campari is an engineered protein that can be photoswitched from green to red fluorescence in a calcium-dependent manner (Fosque et al. 2015). The light levels necessary to achieve switching in the first generation were extremely high, but subsequent generations are expected to improve the sensitivity and conversion ratio. A recent study took advantage of the fact that phosphorylation of ERK is a natural indication of recent activity within each neuron. Using an antibody selective for the phosphorylated form, researchers could map activity patterns under different behavioral conditions and register this to a reference brain, along with different gene expression patterns (Randlett et al. 2015).

5.2. Circuit Manipulations in Behaving Fish

To relate neural circuit function to behavior directly, it is critical to perturb activity in defined populations and observe the consequences. Neurons can be killed or silenced selectively using genetically expressed toxins, laser ablations, or expression of genes that render cells sensitive to light or chemical treatment (e.g., Lee et al. 2010, Tabor et al. 2014, Koyama et al. 2016, Sternberg et al. 2016). Optogenetics allows activity to be modulated up and down in a reversible manner, reducing potential developmental and compensatory effects, and has been applied widely in zebrafish (Portugues et al. 2013). Spatially restricted illumination can be delivered to head-restrained fish, either using optic fibers or projection of arbitrary patterns using digital micromirror device (DMD)-based systems (Arrenberg et al. 2009, Warp et al. 2012, Zhu et al. 2012, Kimura et al. 2013). A greater challenge is to deliver light to specific brain areas in a moving animal. One study implanted optic fibers into freely swimming adult zebrafish to activate the ventral

habenula during a learning task (Amo et al. 2014). However, this method is not applied easily to fish at earlier life stages. If the expression pattern is specific enough, it is sufficient to expose freely swimming animals to uniform diffuse light (Zhu et al. 2009, Fajardo et al. 2013, Barker & Baier 2015, Cheng et al. 2016). However, this requires intense illumination that is likely itself to alter the fish's behavior. As a promising alternative, transient receptor potential (TRP) channels activated by heat, capsaicin, and menthol are effective in fish larva for modulation of neural activity on timescales of seconds to minutes (Chen et al. 2015).

5.3. Relating Neural Activity to Behavioral Parameters

Now that it is possible to record activity throughout the brain, how should this population activity be related to behavior? Many studies have relied on scaling up standard single-neuron analyses, for example, mapping the distribution and spatial organization of tuning properties and correlating activity traces with behavioral parameters (e.g., Miri et al. 2011b, Kubo et al. 2014, Portugues et al. 2014). In the oculomotor system, population analyses of tuning properties have been used to build realistic network models of the velocity to position integrator (Miri et al. 2011a, Daie et al. 2015). In many cases, population responses are tackled by first reducing the dimensionality of the problem using methods such as principal component analysis (Ahrens et al. 2012, Cunningham & Yu 2014, Cheng et al. 2016, Dunn et al. 2016a). This approach can be enhanced by taking into account behaviorally relevant parameters (Kobak et al. 2016). One feature that may be identified in population recordings is the existence of cell assemblies, collections of neurons that tend to fire together on short timescales. Assemblies have been identified in zebrafish based on correlations with behavioral events (Bianco & Engert 2015) or by cluster analysis based on temporal firing patterns (Jetti et al. 2014, Romano et al. 2015, Thompson et al. 2016), and future work may take advantage of approaches that incorporate realistic features of neural networks (Billeh et al. 2014). A drawback of dimensionality-reduction methods is that control of behavior may depend on high-dimensional features of neural dynamics, and therefore researchers will need to develop ways to identify and describe these features. Advances in topological data analysis, for example, are making possible the analysis of patterns in high dimensions (Lum et al. 2013), and applications to neuronal data are promising (Singh et al. 2008). Nonlinear activity-behavior relationships may be identified using nonparametric methods such as Random Forests (Petreanu et al. 2012).

6. CONCLUSIONS

Advances in stimulus delivery, behavioral tracking, and the measurement and manipulation of brain activity allow increasingly precise control and experimental accessibility of zebrafish behavior in naturalistic conditions. The rapid pace of research in this area should bring more quantitative modeling approaches, a greater understanding of behavior ontogeny, and improved optical tools that will make zebrafish an appealing system not only for studies of genetics and development but also for investigating the neural circuit basis of complex behaviors.

DISCLOSURE STATEMENT

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