CHAPTER 8

Physiological Recordings from Zebrafish Lateral-Line Hair Cells and Afferent Neurons

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Abstract

Sensory signal transduction, the process by which the features of external stimuli are encoded into action potentials, is a complex process that is not fully understood. In fish and amphibia, the lateral-line organ detects water movement and vibration and is critical for schooling behavior and the detection of predators and prey. The

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lateral-line system in zebrafish serves as an ideal platform to examine encoding of stimuli by sensory hair cells. Here, we describe methods for recording hair-cell microphonics and activity of afferent neurons using intact zebrafish larvae. The recordings are performed by immobilizing and mounting larvae for optimal stimulation of lateral-line hair cells. Hair cells are stimulated with a pressure-controlled water jet and a recording electrode is positioned next to the site of mechanotransduction in order to record microphonics—extracellular voltage changes due to currents through hair-cell mechanotransduction channels. Another readout of the hair-cell activity is obtained by recording action currents from single afferent neurons in response to water-jet stimulation of innervated hair cells. When combined, these techniques make it possible to probe the function of the lateral-line sensory system in an intact zebrafish using controlled, repeatable, physiological stimuli.

I. Introduction

The zebrafish has many features that make it a versatile model system for physiological studies. Larvae are optically transparent and genetically tractable, enabling one to express transgenic fluorescent proteins in target cells and subsequently visualize them in the intact organism. Furthermore, they are prolific egg layers, lines are simple to maintain, and large-scale mutagenesis screens are feasible. Finally, zebrafish larvae are well suited for electrophysiology, which provides a key method for answering fundamental questions in neurobiology (Ono *et al*., 2001; Ribera and Nüsslein-Volhard, 1998; Smear *et al*., 2007; Westerfield *et al*., 1990). Researchers have used these advantages to advance fields ranging from development to cell biology and neuroscience (Feldman *et al*., 1998; Patton and Zon, 2001; Söllner *et al*., 2004b; Sumbre *et al*., 2008; Whitfield *et al*., 2002).

An important sensory feature of the larval zebrafish is its lateral-line system. Unlike mammals, in addition to auditory and vestibular organs, all fish and amphibia possess a lateral-line organ that is composed of sensory hair cells (Freeman, 1928; Ryder, 1884). Similar to auditory and vestibular sensory detection, the lateral line detects and encodes water motion through hair-cell mechanotransduction (Hoagland, 1933; Suckling and Suckling, 1950). Lateral-line hair cells are arranged together with support cells into rosette-like structures termed neuromast organs. These neuromasts are located along the surface of the animal (superficial neuromasts) and in fluid-filled subepidermal canals in adults (canal neuromasts). The accessibility of zebrafish neuromasts makes them an ideal platform for studying the molecular, cellular, and physiological features of sensory hair cells.

Here we describe in detail our method for recording activity from the lateral line of larval zebrafish. To date, we have recorded extracellular potentials from individual neuromasts and extracellular action currents from single afferent neurons. This preparation is highly suited for performing physiological studies because it utilizes intact animals and biologically relevant stimuli.

II. Zebrafish Mounting and Immobilizing

The lateral-line system of larvae at 120 hours post fertilization (hpf) is composed of superficial neuromasts arranged around the head to form the anterior lateral line and along the trunk to form the posterior lateral line (Dambly-Chaudière *et al*., 2003). At this stage, all primary neuromasts are present along with a few immature secondary neuromasts still forming (Grant *et al*., 2005; Nuñez *et al*., 2009). Studies with primary neuromasts of the posterior lateral line are ideal because their planar polarity results in hair-cell activation with simple anterior–posterior deflections (López-Schier *et al*., 2004; Nicolson *et al*., 1998).

In order for routine, stereotyped access to neuromasts and afferent cell bodies of the posterior lateral line, larvae are mounted to a recording chamber with a circular opening (PC-R; Siskiyou, Inc. Grants Pass, OR, USA). This opening is covered by a square cover glass and coated with a 2 mm layer of silicone elastomer, Sylgard (#184; Dow Corning, Midland, MI, USA). Coating the chamber with Sylgard is ideal because of its elastic properties and relative optical clarity, which can be maintained with frequent recoating. The recording chamber fits within an adapter plate (PC-A; Siskiyou, Inc.), which allows the mounted larvae to be rotated 360°. Free rotation alleviates the need to mount the larva at a precise position relative to the recording chamber and pipette holders. Free rotation of the recording chamber also facilitates the proper alignment of all electrodes (Fig. 1A).

Prior to establishing a recording, an individual larva must be immobilized and mounted to the recording chamber. This is accomplished in two steps: first, the larva

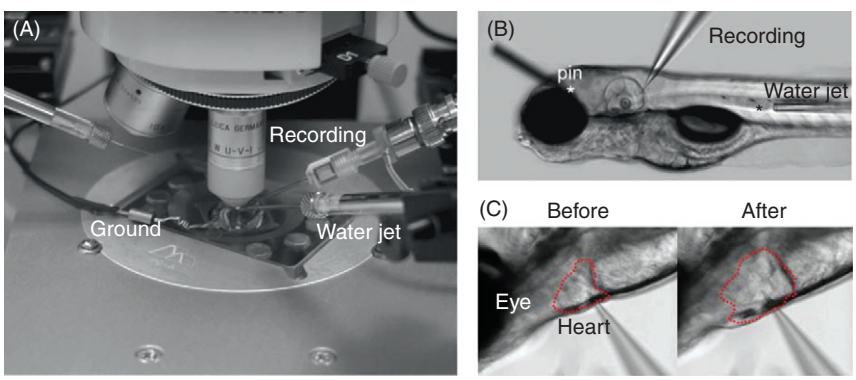

Fig. 1 Setup for mounting and immobilizing zebrafish larva. (A) Photograph illustrating the position of the recording electrode and water-jet pipettes. A third pipette holder is shown on the left side of the image and can be used for dual recordings or as a stimulating electrode. Note the bath ground attaches to the recording chamber, which fits into the circular adapter plate allowing for 360° rotation of the preparation. (B) A 120 hpf larva is pinned to the Sylgard-lined chamber. In this image (10× objective), the recording electrode is positioned for action-current recordings from the posterior lateral-line ganglion. The water jet is positioned just posterior to a neuromast (black asterisk). Note the insertion point of the anterior pin (white asterisk). (C) Before and after α-bungarotoxin injection, which expanded the heart cavity (red dashed outline).

is anesthetized with tricaine and pinned to the Sylgard-lined chamber. Second, the larva is injected with a paralytic (α-bungarotoxin) into its heart and the anesthetic is removed before recording.

A. Anesthesia and Mounting of Larvae

An individual larva is anesthetized in embryo media containing 0.02% tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) for 30 s. Then, using a transfer pipette, the larva and a dropper full of solution are expelled onto the Sylgard-lined chamber. Next, with a dissecting microscope and two watchmaker's forceps (No. 5 Dumont), the larva is positioned on its right side in the center of the chamber and pinned with two tungsten pins. One pin is inserted just posterior to the eye and anterior to the ear (Fig. 1B). The other pin is inserted into the notochord near the end of the tail of the larva.

Pins are fabricated from tungsten rod (0.002 × 3 in., A-M Systems, Sequim, WA, USA). To aid in puncturing the skin, we first electrolytically sharpen one end of the rod using a 1 N NaOH solution and a 9 V battery. Then, a 90° bend is made 1 mm up from the tip end and the rod is cut to form a small, sharpened "L."

Using these techniques, the larva remains immobilized for the length of a given recording (2–30 min), although careful monitoring and readjustments of electrode position may be necessary for long-duration recordings. Conveniently, since larvae do not have gills at this stage, a perfusion setup is not necessary. During recordings, the heartbeat should remain regular, as hair cells are very sensitive to oxygen depletion.

B. Immobilizing Larvae with α-Bungarotoxin

Electrophysiological recordings are precluded by the presence of the anesthetic tricaine, which blocks both neuronal activity and mechanotransduction channels. Therefore, to record lateral-line activity, larvae are immobilized with a paralytic so that the tricaine can be washed away. The paralytic, α-bungarotoxin, blocks the acetylcholine receptor at the zebrafish neuromuscular junction (Westerfield *et al.*, 1990). This toxin is a suitable paralytic for lateral-line recordings since immunohistochemistry with α-bungarotoxin antibodies confirmed labeling of muscle and did not label hair cells of the ear or neuromasts (unpublished observations).

The anesthetized larva is injected with α-bungarotoxin (125 μM) into the heart using a patch pipette with a tip diameter of 1–3 μm. The larva is positioned, and the heart injection is visualized, using the wide-field upright microscope used for our recordings. The α-bungarotoxin pipette is then mounted to the pipette holder that is used for water-jet stimulation; the output tubing of the pipette holder is temporarily switched to a pressure injector (Pressure System IIe, Toohey Company, Fairfield, NJ, USA). Once mounted, the α-bungarotoxin pipette is aligned perpendicular to the heart under a 10× microscope objective. Next, the pipette is advanced toward the heart, pressed against the skin, and then advanced further until the skin is penetrated. After the pipette is inside the heart cavity, a bolus of α-bungarotoxin is injected. Successful injection will result in an obvious expansion of the heart cavity (Fig. 1C). If necessary, inclusion of

phenol red (10%) can increase visualization of the expelled α-bungarotoxin. Following injection, the pipette is retracted and the pinned larva is rinsed several times with standard extracellular solution [in mM: 130 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 290 mOsm; pH 7.8]. The larva is left in 0.5–1 mL of solution for several minutes prior to recording to allow for effective tricaine removal.

III. Microphonics

The term "microphonics" was first used (Adrian, 1931) to describe the cochlear AC potentials that Wever and Bray (1930) observed in recordings from cats. Microphonic potentials have since been found to represent the changes in extracellular potential that result from the inward flow of cations during gating of mechanotransduction channels located at the tips of hair-cell stereocilia (Beurg *et al*., 2009; Corey and Hudspeth, 1983; Jaramillo and Hudspeth, 1991). As a measure of the activity of mechanotransduction channels, microphonic recordings are useful for determining the amplitude and frequency of the transduction response in hair cells. The following methods can be used for recording and analyzing microphonics from individual neuromasts of the larval lateral line.

A. Stimulation of Neuromast Hair Cells

At 120 hpf, larval neuromasts contain 9–14 hair cells. At the apical end of each hair cell is the stereociliary hair bundle. Each hair bundle is connected to a single kinocilium that extends roughly 20 μm into the aqueous medium. A gelatinous cupula covers and encompasses all of the kinocilia. Deflection of the cupula results in the concerted deflection of all kinocilia, which in turn deflects attached stereocilia (Netten and Kroese, 1987). Shearing of hair bundles ultimately opens mechanotransduction channels located at their tips (Beurg *et al*., 2009; Jaramillo and Hudspeth, 1991).

Stimulation of neuromast hair cells is performed with a pipette filled with extracellular solution that is driven by a pressure clamp (HSPC-1, ALA Scientific). This pipette, termed a water jet, should have a circular opening with a diameter of approximately 30 μm. To achieve this pipette shape, thick-walled (1.5 mm OD and 0.86 mm ID) borosilicate glass is fabricated from a single, hard pull (micropipette puller P-97; Sutter Instruments) that results in two pipettes with long, tapering ends. These two long ends are rubbed against each other until one end scores and cleanly breaks the other. Note that this process requires non-filament glass in order to have a clean tip break and laminar fluid flow.

The water-jet pipette is positioned approximately 100 μm from a given neuromast cupula. The height of the water jet should be slightly above the cupula and deflection by the water jet should be equal in forward and reverse directions.

B. Microphonic Recordings from Neuromast Hair Cells

Electrodes for microphonic recordings are pulled to resemble standard patch electrodes using filament glass that is otherwise the same as the water-jet pipettes. Electrode outer tip diameters are of 1–3 μm and have resistances of approximately 3 MΩ when filled with extracellular solution. The recording electrode is mounted at a 45° angle from the water-jet pipette and positioned (using a 40× objective) at a height even with the stereocilia (Fig. 2A). Since mechanotransduction channels are located at the tips of stereocilia, the position of the recording electrode is critical for obtaining the largest possible microphonic signal. Proper position is important because lateral-line stereocilia are very short and difficult to visualize even with a 40× objective, where they appear as a dark spot at the apical surface of each hair cell (white-dashed circle in Fig. 2A).

On day 5, in addition to the primary posterior lateral-line neuromasts, secondary neuromasts are also forming (Grant *et al.*, 2005). Care should be taken to record exclusively from primary neuromasts, as secondary neuromasts activate with deflections of different polarities, which may give confounding results. Also, note that all solutions (water jet, electrode, and bath) should be identical to avoid junction potential errors.

C. Signal Collection and Analysis

Microphonic potentials are recorded in current-clamp mode, sampled at 10 kHz, and filtered at 1 kHz. The voltage signal is then further amplified (10,000× final) and

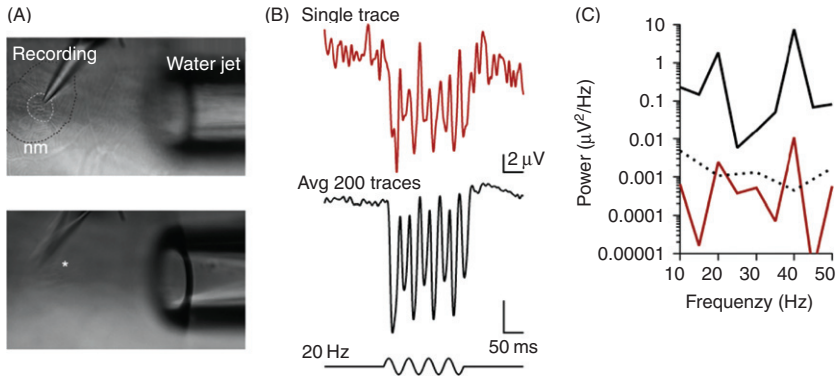

Fig. 2 Microphonic recordings from posterior lateral-line neuromasts. (A) Images (40× objective) of the location of the recording electrode (top panel) and water-jet pipette (bottom panel) relative to a neuromast (nm; black dashed circle). The recording electrode is at the level of the hair-cell stereocilia, which are dark spots under DIC differential interference contrast (white dashed circle). The water-jet pipette is positioned approximately 100 μm from the neuromast. Note that the water-jet height is even with the top of the neuromast cupula (white asterisk). (B) An individual trace (red trace) from a microphonic recording, compared to an average of 200 consecutive traces (black trace), which illustrates the increase in signal-to-noise ratio with averaging. (C) Power spectrum from the individual trace (red line) and the average trace (black line) during the 200 ms sine wave stimulation. Note that the individual-trace power is barely distinguished from the power spectrum of the noise portion of the average trace (black dashed line).

filtered (50–100 Hz; eight-pole Bessel) by an additional amplifier (Model 440, Brownlee Precision). In order to drive the pressure clamp and record the microphonic potential simultaneously, a sinusoidal voltage command is delivered to the pressure clamp via an analog output from the recording amplifier. The pressure of the water jet can also be monitored by a feedback sensor on the pressure-clamp headstage and recorded simultaneously with acquisition software.

As microphonic signals are typically 10 μV, one should take care to effectively remove all electrical noise from the recording setup. Under ideal conditions, recording noise can be held to under 5 μV peak to peak, and microphonic signals can be seen without averaging traces (Fig. 2B). To overcome the variability of neuromast structure and the position of the recording electrode, one can quantify microphonic signals by determining the power of the total signal per unit frequency. The stimulus portion of the signal is analyzed with a power spectral density function (Fig. 2C). Power spectra will contain primary peaks at the stimulus frequency (1*f*) and at twice the frequency (2*f*); for a 20 Hz stimulus, this would result in peaks at 20 and 40 Hz, respectively (see Fig. 2C). These frequency components result from the presence of two groups of hair cells of different orientation that respond to stimuli of opposite polarity (Flock and Wersall, 1962; Ghysen and Dambly-Chaudière, 2007).

D. Confirming a Biologically Relevant Signal

There are several qualifications and tests that help to determine whether a recorded microphonic signal is biologically relevant. If bidirectional stimuli (e.g., sine waves) are used, then the signal should contain the 2*f* frequency component. In addition, a biologically relevant signal should not be detected with stimulation perpendicular to the excitation axis of a neuromast. Furthermore, the microphonic signal should be blocked by mechanotransduction channel antagonists such as dihydrostreptomycin and amiloride (Farris *et al*., 2004). Microphonics should also be blocked by disruption of stereocilia tip links—components necessary for transduction. For example, chelating extracellular calcium with 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N*',*N*'-tetraacetic acid (BAPTA) or ethylene glycol tetraacetic acid (EGTA), both of which break tip links, abolishes microphonics (Assad *et al*., 1991). In addition, microphonics should be absent in larvae lacking mechanotransduction such as *cdh23* or *pcdh15* mutants (Nicolson *et al*., 1998; Seiler *et al*., 2005; Söllner *et al*., 2004a).

IV. Action Currents

The neurotransmitter output of hair cells, which encodes the features of sensory stimuli, results from a unique process of graded transduction. Briefly, gating of mechanotransduction channels results in graded changes in hair-cell membrane potential, which drives graded synaptic vesicle fusion at specialized ribbon synapses. These graded signals are ultimately encoded as sequences of all-or-none action potentials (spikes) in afferent neurons.

When recorded in an extracellular loose-patch configuration, action potentials are measured as action currents. Typically, action-current recordings are less invasive and easier to establish than whole-cell recordings. Furthermore, lateral-line afferent neurons are ideal for this type of recording, as their dendrites are myelinated and spiking is absent without hair-cell neurotransmission (Obholzer *et al*., 2008). These qualities imply that afferent spiking is evoked from spontaneous and stimulation-dependent release of neurotransmitter from hair cells. Thus, action-current recordings are sufficient for examining the temporal sequence of spikes, which provides a direct readout of the activity of innervated hair cells.

A. Electrode and Recording Details

Extracellular recording electrodes are pulled in a similar fashion as microphonic electrodes except with longer shafts and outer tip diameters slightly smaller than 1 μm. Recording electrode resistances should range between 5 and 10 MΩ in extracellular solution. The recording electrode is positioned at an approximately 45° angle to the dorsal side of the pinned larva (Fig. 1B). The electrode should be positioned in the same optical plane as the middle of the posterior lateral line ganglion (PLLg). In a manner similar to α-bungarotoxin injection, the electrode is moved ventrally with increasing pressure against the fish skin until it advances through it. Often following the skin puncture, the pipette will move rapidly past the ganglion. If this occurs, then the electrode should be slowly retracted until it is within the membranous sack that encompasses the cell bodies. The electrode's position within the PLLg is confirmed by inflation of this sack with positive pipette pressure delivered via the electrode holder. Note that if necessary, the PLLg can be visualized using transgenic animals with fluorescently labeled lateral-line neurons (see both Nagiel *et al*., 2008 and Obholzer *et al*., 2008 for constructs).

To record from an individual lateral-line neuron, the electrode is moved against a desired cell and negative pipette pressure is applied (Fig. 3A).Recordings are made with the amplifier in voltage-clamp mode (command voltage = 0) with a 10 kHz sample rate and filtered at 1 kHz. For typical action-current recordings, the series resistance lies within a range of 30–80 MΩ. Confirmation of an established recording is seen first by the appearance of spontaneous spiking, after which the water jet is used to scan for the innervated neuromast, resulting in the conversion of spontaneous to phase-locked spiking.

Occasional application of positive pipette pressure is useful for both increasing the clarity of the ganglion and freeing any skin, cell, or tissue debris that may be obstructing the pipette tip. Also, on occasion, electrodes will rapidly form gigaohm seals, which predicts that whole-cell recordings of afferent neurons will be easily obtained.

B. Analysis of Action Currents

In response to bidirectional stimulation of neuromast hair cells (e.g., sine waves), action-current recordings reveal phase-locked spiking to one direction of stimulation

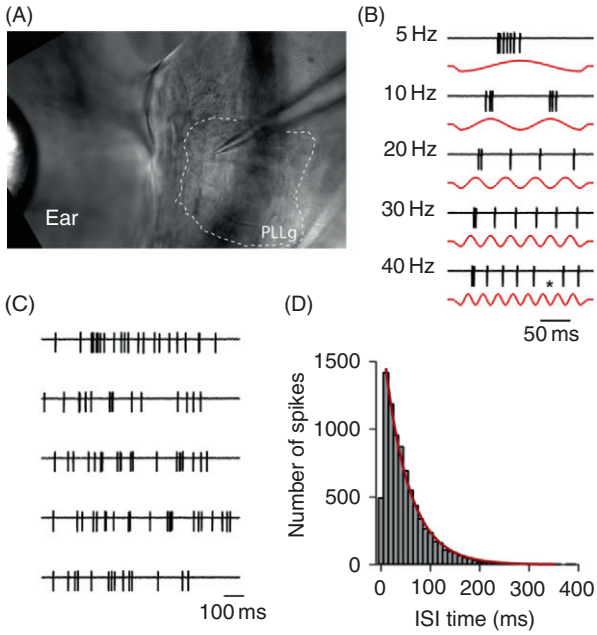

Fig. 3 Action-current recordings from individual posterior lateral-line neurons. (A) Under DIC differential interference contrast optics with a 40× objective, the posterior lateral-line ganglion (PLLg; dashed-with line) is visible just posterior to the ear. The recording electrode is positioned in the anterior-dorsal portion of the PLLg, where the majority of cell bodies for the primary neuromasts are located. Note the otolith in the anterior side of the image. (B) Single traces of action currents in response to hair-cell stimulation at various frequencies. Note the spikes are phase-locked to just one direction of deflection. (C) Five consecutive 1 s traces of spontaneous spiking. The mean interspike interval (ISI) for 400 s of spiking was 50 ms. (D) Histogram representing the distribution of ISIs (10 ms bins) from 400 s of spiking by the cell shown in C. The histogram is best fit by a single-phase exponential decay equation (red line) with a tau of 50 ms.

(Obholzer *et al*., 2008). This occurs because an individual afferent neuron innervates only one of the two populations of hair cells within a neuromast that all display the same hair-bundle polarity (Faucherre *et al*., 2009; Nagiel *et al*., 2008; Obholzer *et al*., 2008). Spiking remains phase-locked with rates of stimulation approaching 100 Hz. At lower frequencies, spiking occurs with each deflection, while at higher frequencies, spikes do not occur with every deflection. In both cases, there is no loss of phase locking to the water-jet stimulus (Fig. 3B). Spike trains in response to continuous stimulation can be visualized with period histograms where individual spike times are binned and plotted relative to the unit period for a given stimulus frequency. The resulting series of unit spike times can be further transformed into unit vectors with corresponding, specific phase angles. Thus, the collection of spikes, now represented by a series of vectors, can be quantified for the degree of synchrony between the stimulus and response by calculating the vector strength (r) of the series (Goldberg and Brown, 1969). We recently utilized this method of analysis to quantify phase-locked activity in larvae with disrupted hair-cell endocytosis (Trapani *et al*., 2009).

In the absence of sensory stimuli, spontaneous spiking is observed in afferent neurons of the auditory, vestibular, and lateral-line systems across species. This spontaneous spiking apparently results from neurotransmitter release from innervated hair cells (Annoni *et al*., 1984; Flock and Russell, 1976; Furukawa and Ishii, 1967; Siegel and Dallos, 1986; Starr and Sewell, 1991; Zimmerman, 1979). The most meaningful feature of spontaneous spiking is the time interval between two consecutive spikes, which is termed the interspike interval (ISI). The temporal pattern of the spontaneous activity can be analyzed using ISI histograms, where features of the histogram describe the nature of the activity. For example, if spikes are generated by a Poisson process, then the ISI histogram will be best fit by a single-phase exponential decay equation with the time constant (tau) corresponding to the recording's mean ISI time. Recording spontaneous action currents from a PLLg neuron results in a diverse range of ISI times (Fig. 3C). The ISI histogram of spontaneous spiking in Fig. 3D is best fit by a single exponential decay equation.

V. Summary

In this chapter, we have described our methods for recording activity from the posterior lateral-line system of the larval zebrafish. While some of the details are specific for this particular preparation, a majority of the techniques are translatable to physiology in other areas of the fish. The mounting and immobilization of larvae, as well as the basic principles of the recording, will benefit any investigator looking to establish physiological recordings in live, intact larvae.

VI. Discussion

The zebrafish is a genetically tractable vertebrate and a powerful platform for electrophysiological studies. The above preparation is extremely valuable in assessing sensory hair-cell function in various auditory/vestibular mutants. The ability to record both microphonics from hair cells and action currents from lateral-line neurons can help to pinpoint the nature of the defect in mutants and reveal the biological role of the protein of interest. Using these techniques provides important information about the impact of mutations affecting genes that are conserved in function from fish to humans. In addition, the preparation allows one to mechanically stimulate hair cells in an undissected animal with intact circuitry that receives sensory input for higher order processing. Such recordings are currently not possible with mammalian model systems.

Future experiments using the lateral-line system may include whole-cell patch clamp recordings from the afferent neuron and potentially from individual hair cells, which will further increase the capability of this preparation. Furthermore, the afferent neuron forms an unmyelinated basket beneath a given, innervated neuromast, and this presents the potential for postsynaptic recordings similar to those achieved in mice (Glowatzki and Fuchs, 2002). Experiments with a double patch-clamp amplifier, where one could

simultaneously record activity at both the neuromast and the afferent neuron, would be extremely powerful for examining sensory signal encoding.

Over the past 10 years, the zebrafish community has developed a large number of mutant and transgenic zebrafish lines. Using either endogenous regulatory sequences or Gal4/UAS targeting allows for spatially and temporally restricted expression of proteins of interest and has created a large set of optogenetic tools: photoconvertible fluorescent proteins to visualize cells, light-gated ion channels to activate cells, genetically encoded voltage and calcium sensors to examine cellular activity, and genetically encoded proteins that poison synaptic transmission to deactivate cells. The combination of transgenic lines with hair cell and afferent-specific expression of these reporters together with the electrophysiology techniques described in this chapter presents the potential for experiments aimed at understanding the fundamental aspects of hair-cell processing and sensory signaling.

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