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**TITLE** (maximum 150 characters)

Mapping synaptic pathways for sensorimotor reflexes in zebrafish larvae using calcium imaging.

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**Short Abstract:** should include a general description of the article (10 words minimum, 50 words maximum)

This paper describes the use of intracellular calcium imaging to study synaptic connectivity within sensorimotor pathways in zebrafish larvae. Calcium imaging utilizes fluorescent proteins that become brighter when neurons fire action potentials. Optical physiology is noninvasive and can record multiple neurons simultaneous, offering advantages for studying neural network dynamics.

**Long Abstract:** (150 words minimum, 400 words maximum)

Zebrafish are an important model species in biology and offer particular advantages for studying neural circuit structure and function. Neural circuits mediating sensorimotor reflexes develop early in zebrafish. By 5 days post fertilization, zebrafish larvae use tactile, visual and other senses to guide their movements. Because larvae are transparent, sensorimotor pathways are accessible to observation and manipulation. Another key advantage is the presence of individually identifiable neurons, including many sensory neurons, central premotor neurons, and spinal cord neurons. Studying ‘the same’ neuron across animals allows detailed information to be compiled about the synaptic connectivity and functional properties of neurons within sensorimotor pathways. This information is critical to understanding how the pathway transforms sensory input into adaptive motor responses.

Hindbrain reticulospinal (RS) neurons, present in zebrafish and all other vertebrates, form the core of a sensorimotor pathway. They receive multimodal sensory inputs and project long axons that descend in the spinal cord. RS neurons are implicated in sensorimotor transformations, but the mechanisms of their operation are not well understood, in part because their synaptic connectivity has not been well characterized. Zebrafish larvae have only about 300 RS neurons, most of which are individually identifiable (Kimmel et al., 1982; Metcalf et al., 1986; Gahtan & O’Malley, 2003), so they present an excellent model for studying the synaptic architecture of sensorimotor pathways. This paper describes how intracellular calcium imaging can be used to study synaptic connectivity in the lateral line sensory projection to RS neurons. Calcium imaging utilizes fluorescent proteins that become brighter when neurons fire action potentials. The basic technique was originally presented in a study of spinal neurons mediating touch-evoked escape turns (Fetcho and O’Malley, 1995), but has been adapted to study other behaviors and sensory systems (Orger et al., 2008; Sankrithi and O'Malley, 2010). All of these studies exploit the presence of identifiable neurons and the optical transparency of larvae.

In the lateral line sensory system, hair cells on the body surface respond to water currents, activating sensory neurons that ascend to the hindbrain. Each sensory neuron can be distinguished by its origin on the body surface (Kucenas et al., 2006; Metcalfe et al., 1985; Ghysen et al., 2007; Raible and Kruse, 2000). Because both lateral line sensory neurons and postsynaptic RS neurons in this pathway are identifiable, the pattern of synaptic connections between these groups can be worked out at the cellular level.

**Protocol Text:**

1. Recording sensory-evoked calcium responses from identified RS neurons

1. Backfilling of RS neurons with fluorescent calcium indicator.

RS neurons are labeled by injecting a small volume (<1.0nl) of fluorescent calcium indicator dye (75% solution w/v solution of BAPTA-conjugated Alexa Dextran 488, Invitrogen) into the spinal cord of living 3-6 day old larvae. The dye is pressure ejected through a sharpened glass micropipette. The glass pipette tip breaks axonal processes of descending neurons in the spinal cord, allowing dye to enter those neurons. The dye diffuses within the cell and fills RS cell bodies in the brain after about 4 hours.

* 1. Prepare a surface of hardened agar gel (1.2% w/v) that the anesthetized larvae will lay on during the injection by pouring melted agar into a 35mm petri dish and placing it on a level surface to cool.
  2. As the agar cools, prepare the glass injection pipette. Using a pipette puller and 1.5mm outer diameter borosilicate capillary glass, pull an injection needle approximately 2µm in diameter at the tip. The tip should then be sharpened using a beveler (ideally) or by breaking it at the end with fine surgical forceps to reach final tip diameter of about 10µm. After fabrication, load the injection needle with the fluorescent calcium indicator dye (Alexa 488-BAPTA, Invitrogen), and mount it in a pipette holder attached to a micromanipulator. A volume of dye sufficient to fill just the tapered end of the needle is sufficient for injecting >30 larvae (Figure 1). Center the injection tip in the view of a standard dissecting microscope, with the needle angled ~30 (degrees?) relative to the microscope stage.
  3. Anesthetize a larva with a brief (less than 20 seconds) exposure to an egg water solution containing MS-222 (0.03% w/v, pH to 7.1). When spontaneous swimming activity ceases, remove the larvae from MS-222 with a transfer pipette and rinse once by resuspending in normal egg water to avoid toxic overexposure to MS-222. The larva will remain anesthetized and immobile for several minutes. Transfer the larva in a small drop of egg water onto the agar surface and, using a Kimwipe, absorb excess water to prevent the larva from sliding on the agar. Larva will naturally lie on one side of their body on the agar plate. Turn the agar plate so that the needle is pointing toward the anterior-ventral surface of the larva (Figure 1B).
  4. Injections are made at approximately the level of the 20th myotome, or halfway between the anal pore and the tip of the tail. Insert the tip through the dorsal axial muscle into the ventral spinal cord, where most RS axons descend. The notochord is visible under white light using a dissecting microscope and is a useful landmark, as most RS axons course parallel and just dorsal to the notochord. Working at about 100x magnification allows easy visualization of dye ejected into the spinal cord, which is useful for targeting the injection. A single 5ms pressure pulse at 20psi usually ejects a sufficient volume of dye. Each injection must be completed within about 3 minutes to ensure that the larvae will recover. After injecting, rinse the larva off the agar bed into regular egg water and place it an incubator at 28° C for at least 12 hours to allow the larva to recover and the dye to diffuse. The dye enters spinal axons that are broken by the pipette tip at the injection site and after about 5 hrs diffuses into RS cell bodies in the brain (Figure 2). Multiple RS neurons are usually labeled by this procedure, most with sufficient frequency and anatomical detail that they can be identified and studied across different preparations.

2. Mounting larvae for confocal imaging

After RS neurons have been backfilled, prepare a larva for confocal calcium imaging. Resuspend the larva in low melting point agar (maintained at 28° C) and transfer it in a small drop of agar onto a new microscope slide such that the stimulus (water pulses) can be applied to the targeted area. RS neurons are easiest to image when focusing through the dorsal surface of the skin, so the larva should be orientated on the slide appropriately depending on whether an upright or inverted microscope is used. When the agar has cooled and solidified (about 1 minute at room temperature), add a drop of regular egg water to keep the larva hydrated within the porous agar.

3. Neuromuscular blockade to prevent movement artifacts in physiological scans.

Although larvae are embedded in agar during imaging, small movements may still be visible in the highly magnified confocal scans and may briefly move the imaged cell out of the field of focus. To prevent data loss due to these movements, use a neuromuscular blocking drug such as tubocurarine (Sigma), which may be dissolved into the normal egg water medium (3mg/ml). Because the neuromuscular blocker acts on muscle, it will paralyze the larva without altering physiological activity of neurons recorded in the brain.

4. Sensory stimulation of lateral line neuromasts during confocal imaging

After determining the section of the body surface to be stimulated, a wedge of agar is dissected away from that area using a scalpel. Agar is removed to expose a small area of skin (1-2 square millimeters) and allow a path for steering the stimulator tip to within about 1 mm of the exposed area while still leaving sufficient agar around the rest of the larva to keep it firmly embedded. The stimulator is a capillary glass electrode with a blunt tip of approximately 15µm in diameter. The electrode is filled with regular egg water medium and attached to the air pressure pulse delivery device (Picospritzer). Air pressure pulses eject a controlled volume of egg water, which produces water currents in the medium surrounding the targeted area of the skin. Hair cells on the surface of the skin detect minute water current produced by this stimulus, and relay neural signals to primary sensory neurons. Primary sensory neurons ascend to the hindbrain and activate RS neurons. Since different parts of the body can be stimulated discretely using this method, it is possible to determine whether different RS neurons in the brain respond to stimulation of different parts of the body surface (Figure 3A). The stimulator (AM-systems 2100 Isolated Pulse Stimulator) and confocal imaging system must be equipped with TTL triggering capability so the stimulus onset can be synchronized with the calcium scan (Figure 3B). The stimulus should be programmed to occur after a stable baseline of fluorescence intensity (at least 250 ms) has been acquired from the cell. This is essential for assessing whether any calcium transients in the cell are related to the stimulus.

5. Microscopy procedures for recording calcium responses

Identify the cell to be recorded using anatomical landmarks and morphological features of individual labeled cells. Most neurons that descend from the brain to the mid spinal cord in zebrafish larvae can be unambiguously identified using published criteria (Metcalfe et al 1986). The inner ear, which can be seen under light microscopy without labeling, is a useful landmark because it is centered at hindbrain rhombomere 4, adjacent to the Mauthner neuron. The clearest way to indentify a specific RS neuron is by examining a 3D confocal reconstruction. First, take a series of 2D images through the depth plane of a neuron or group of neurons at about 4µm intervals. Examine the series for diagnostic features such as the size, shape, and position of the soma and the projection of dendrites and axons. Once the targeted cell or cells is identified, the confocal microscope should be operated in line scan mode for calcium imaging, which maximizes imaging speed, (Figure 4). The line to be scanned should be drawn through the brightest point in the cell so any movement of the cell will cause fluorescence to decrease, not increase, thus minimizing the potential for mistaking movement artifacts for actual calcium-activated fluorescence responses. A line may be drawn through multiple cells if they occur in the same depth plane in the image, as is often the case with bilaterally paired neurons. Recording from multiple neurons simultaneously is a particular advantage when the goal is to assess network dynamics. Line scans should be scanned at a rate of at least 250 Hz, which is fast enough to detect a single action potential in the Mauthner cell (Figure 4).

**Representative Results:**

The larva should show normal swimming behavior by 24 hours after dye injection into the ventral spinal cord at the level of the 20th myotome. Injections into more anterior spinal cord may result in abnormal swimming, however, because anterior injections can disconnect RS axons from more of their postsynaptic target neurons posterior to the injection. Successful injections should label up to 100 RS neurons in the hindbrain, though there is considerable variability in the number of labeled cells. There should be sufficient anatomical detail of cell body morphology and dendritic and axonal processes of most cells to allow for identification based on previously published characteristics (Metcalfe et al., 1986).

Baseline fluorescence intensity during line scan imaging should show no more than 2% variability during a 1 second line scan when no stimulus is given. Laser exposure may result in some dye bleaching during a scan and cause fluorescence intensity to gradually decreases, but that decrease should be small relative to the fluorescence increase associated with action potential firing. A cell that responds to the stimulus should show an increase in fluorescence intensity over baseline of ≥10% within 250ms after the stimulus is delivered (Gahtan and O’Malley, 2002; Figure 4). The latency of fluorescence increase after action potential firing in a neuron is much shorter, on the order of milliseconds, as intracellular free calcium spikes rapidly during neural activity. But since RS neurons generate motor commands, they become active only after sensory processing of an effective stimulus. Intracellular calcium-activated fluorescence dynamics associated with an action potential typically show a rapid (<10ms) rise to peak and a much slower (>500ms) decay back to baseline. This is the expected pattern for the Mauthner neuron, which is known to fire a single action potential after an effective stimulus (Figure 4), but firing of multiple action potentials in other neurons may result in a slower decay in fluorescence intensity.

**Discussion:**

This protocol represents one application of in vivo optical physiological recording from neurons in a popular model organism, zebrafish larvae. Advantages of optical calcium imaging in zebrafish larvae include the ability to record populations of identified neurons noninvasively in living, intact animals. This method is well suited to studying neural network dynamics.

The scientific goal of this application is to investigate topographical mapping in hindbrain reticulospinal sensorimotor nuclei. The basic methods, however, can be varied in several ways to address other scientific questions. Intracellular calcium imaging can be done in any neuron in which an optical calcium indicator can be introduced. In this protocol, a fluorescent calcium indicator is injected into targeted neurons, but recent advances in transgenics has allowed fluorescent calcium indicators to be genetically encoded and expressed in specific subsets of neurons (Dombeck et al., 2010).

Recorded neurons may be stimulated in numerous ways. For example, calcium imaging from RS neurons in zebrafish larvae has been done using visual (Orger et al., 2008) and tactile (O’Malley et al., 1996; Gahtan et al., 2002) stimulation. As the sensory stimulus and scientific goals are varied, the criteria for cell responsiveness should also be reconsidered. For example, calcium imaging can be used to record subcellular calcium dynamics (Dombeck et al., 2011) or subthreshold neural activity as opposed to action potential firing (Li et al., 2010), in which cases much smaller changes in fluorescence intensity may be of interest. One important limitation to all of these approaches is that the preparation must remain immobilized under a microscope. A related limitation is the difficulty of using optical physiology to record spontaneous activity of neurons since prolonged exposure to excitation laser light can be toxic to neurons.

A confocal microscope is the most specialized and expensive piece of equipment needed for the protocol described here. But in some cases fluorescent calcium imaging can be performed using less expensive CCD cameras that can be operated in line scan mode (Li et al., 2010). Multiphoton microscopy is also well suited for optical calcium imaging. While multiphoton imaging systems are usually larger and more expensive than confocals, they offer several important advantages, including the ability to focus deeper into tissues, to optically penetrate opaque tissue such as mammalian cortex, and to do so with less phototoxicity than traditional lasers (Dombeck et al., 2011). Calcium imaging has quickly become a mainstream tool in behavioral neuroscience. The application of calcium imaging in zebrafish larvae, a versatile and widely used model organism promises continued discoveries about neural circuit mechanisms of adaptive behavior.

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**Disclosures:** I have nothing to disclose.

**Table of specific reagents and equipment:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Name of the reagent** | **Company** | **Catalogue number** | **Comments (optional)** |
| Alexa Fluor 568 | Invitrogen | D22912 |  |
| Oregon green 488 BAPTA-1 | Invitrogen | O6798 |  |
| Sulfonic Acid salt, 98% (MS-222) | Acros Organics | 118000500 |  |
| Zebrafish egg water |  |  | Recipe from the Zebrafish Book at http://zfin.org |
| Agarose, low melting point | Sigma | A9414 |  |
| Tubocurarine hydrochloride | Sigma | T2379 |  |
| 1.5mm OD capillary glass | AM Systems | 603000 |  |
| Pipette puller | Sutter | P-97 |  |
| Pipette beveller | Sutter | BV-10 |  |
| Low melting temperature agar |  |  |  |
| MicroFil pipette loader | World Precision Instruments | # MF34G-5 |  |
| Confocal imaging system | Olympus | FV1000 |  |
| Dissecting microscope | Nikon | SZM1500 |  |
| Picospritzer | Parker |  |  |

**Figures legends:**

Figure 1. The setup for dye injections into the spinal cord of a 5 day old zebrafish larva to label hindbrain reticulospinal neurons. The glass microinjection needle is poised above the target site, at about the 25th myotome. The correct orientation of the needle, pointing toward the anterior-ventral surface of the larva, is shown, and the swim bladder and eyes are labeled as anatomical landmarks to aid in orienting the preparation. Sufficient dye is shown loaded into the needle to complete approximately 75 spinal injections.

Figure 2. A confocal projection of RS neurons labeled by spinal injection in a living 6 day old zebrafish larva. The injection was done approximately 20 hour prior to imaging. The image is a maximum projection of depth series of 30 confocal images taken at 2µm intervals through the larval hindbrain. Several identifiable RS neurons are labeled, following established nomenclature. The box within the larval silhouette at top shows the approximate location of the imaged area, and the arrow head the approximate site of the spinal injection.

Figure 3. The setup for targeted stimulation of lateral line sensory neuromasts with water current during calcium imaging.

Figure 4. Calcium imaging line scan example. A. The Mauthner cell (M) was located in the array of backfilled RS neurons (anterior is to the left in the image). B. A scan line was set through the brightest point in the middle of the cell (vertical white line). C. The line scan image. Lines were scanned repeatedly at 2ms intervals. A water current stimulus was applied to a localized area of the skin using a vibrating glass probe after recording 150ms of baseline fluorescence intensity. A robust calcium response of 65% above baseline occurred within 4ms after the stimulus, visible in the line scan image (C) and in a corresponding chart that quantifies calcium fluorescence intensity (D).

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