Tilt in Place Microscopy: a Simple, Low-Cost Solution to Image Neural Responses to Body Rotations

Kyla R. Hamling, Yunlu Zhu, Franziska Auer, and David Schoppik

Departments of Otolaryngology and Neuroscience and Physiology, Neuroscience Institute, New York University Grossman School of Medicine, New York, New York 10016

Animals use information about gravity and other destabilizing forces to balance and navigate through their environment. Measuring how brains respond to these forces requires considerable technical knowledge and/or financial resources. We present a simple alternative—Tilt In Place Microscopy (TIPM), a low-cost and noninvasive way to measure neural activity following rapid changes in body orientation. Here, we used TIPM to study vestibulospinal neurons in larval zebrafish during and immediately after roll tilts. Vestibulospinal neurons responded with reliable increases in activity that varied as a function of ipsilateral tilt amplitude. TIPM differentiated tonic (i.e., sustained tilt) from phasic responses, revealing coarse topography of stimulus sensitivity in the lateral vestibular nucleus. Neuronal variability across repeated sessions was minor relative to trial-to-trial variability, allowing us to use TIPM for longitudinal studies of the same neurons across two developmental time points. There, we observed global increases in response strength and systematic changes in the neural representation of stimulus direction. Our data extend classical characterization of the body tilt representation by vestibulospinal neurons and establish the utility of TIPM to study the neural basis of balance, especially in developing animals.

Key words: balance; imaging; posture; vestibular; vestibulospinal; zebrafish

Significance Statement

Vestibular sensation influences everything from navigation to interoception. Here, we detail a straightforward, validated, and nearly universal approach to image how the nervous system senses and responds to body tilts. We use our new method to replicate and expand on past findings of tilt sensing by a conserved population of spinal-projecting vestibular neurons. The simplicity and broad compatibility of our approach will democratize the study of the response of the brain to destabilization, particularly across development.

Introduction

Animals must transform forces acting on the head/body into commands to stabilize gaze/posture and orient, navigate, and regulate physiology (Angelaki and Laurens, 2020; Chen et al., 2021; Daltorio and Fox, 2018; Yates et al., 2013; Yoder and Taube, 2014). Both sensory organs and bodies change as animals develop and age. Studying neuronal representations of forces, particularly over time, presents a profound challenge—to measure brain activity while applying destabilizing forces. For more than a century novel apparatuses have met this challenge, primarily in studies of the vertebrate vestibular system, from observational chambers (Lowenstein and Roberts, 1949; Mach, 1886; Straka et al., 2021), translating sleds (Fleisch, 1922), and later to electrophysiology-compatible rotating swings (Adrian, 1943), modern platforms with six degrees of freedom (Angelaki et al., 1999; Branoner and Straka, 2018), and wireless recording (Carriot et al., 2017). Forces are usually delivered sinuositydally, conferring mechanical advantages and facilitating linear systems analysis and modeling of neuronal/behavioral responses (Laurens et al., 2017). Although studies of vestibular processing have advanced nearly every area of systems neuroscience (Goldberg et al., 2012a), each apparatus and stimulus paradigm represents a set of necessary trade-offs. The specialized hardware used in most experiments is both a cost and knowledge barrier. Further, few existing approaches permit repeated measures of activity from the same neurons over days, key to
understanding changes that accompany development (Beraneck et al., 2014; Peusner, 2014) and learning (Goldberg et al., 2012b). Here, we detail and validate an approach for longitudinal measures of vestibular sensitivity.

Imaging neuronal activity by measuring changes in fluorescence of genetically encoded calcium indicators has transformed neuroscience. The vestibular field has been comparatively slow to adopt this technology, largely because imaging neurons while animals are challenged with vestibular stimuli is technically demanding. Recent efforts include microscopes that rotate (Migault et al., 2018), image a rotating sample (Hennesstad et al., 2021; Tanimoto et al., 2022), and optically trap and move part of the vestibular apparatus (Favre-Bulle et al., 2018). Together, these studies clearly illustrate the promise of using modern microscopy and genetics to understand the vestibular system. However, each requires specialized optical and/or engineering expertise to implement, limiting their impact. Motivated by these studies, we sought to develop a complementary low-cost, straightforward means to image neuronal activity following body tilts.

We focused our efforts on a model vertebrate with a rich vestibular repertoire—the larval zebrafish. At 4 days postfertilization (dpf) larval zebrafish swim freely and maintain posture and stabilize gaze (Bagnall and Schoppik, 2018). Both behaviors require central vestibular neuronal circuits (Schoppik et al., 2017), with considerable development between 4 and 7 dpf (Bianco et al., 2012; Ehrlich and Schoppik, 2017, 2019). Importantly, the larval zebrafish is both transparent and genetically accessible, facilitating measurements of calcium indicators in vestibular neurons and/or loss-of-function assays in mutants (Whitfield et al., 1996). Finally, like all vertebrates, the larval zebrafish has a small population of vestibulospinal neurons (~50 cells) with reliable and well-characterized responses to tilt stimulation (Hamling et al., 2021; Liu et al., 2020). Together, the zebrafish is a powerful model to develop new methods to investigate vestibular function.

Here, we present data from a new approach to imaging neuronal activity in response to body rotations that we call Tilt In Place Microscopy (TIPM), which allows extremely rapid (<6 ms) whole-body rotations toward and away from the horizon, allowing precise characterization of tilt sensitivity. We validated TIPM by characterizing the roll responses, topography, and development of larval zebrafish vestibulospinal neurons. We found that vestibulospinal neurons respond reliably to ipsilateral steps with parametrically increasing activity, consistent with prior electrophysiological measurements in fish and mammals (Peterson, 1970; Rovainen, 1979). We repeated TIPM sequentially on the same fish and found that trial-to-trial variation was likely intrinsic to vestibulospinal responses, not because of our approach/apparatus. Vestibulospinal neurons had a comparatively small response to phasic stimulation; neurons that sensed phasic components were preferentially located in the ventral lateral vestibular nucleus. The bulk of the vestibulospinal response was derived from utricular sensation. Finally, we measured responses from the same neurons at two behaviorally relevant time points, revealing increased response strength in older neurons and systematic changes in directional selectivity as neurons develop. We end with a discussion of the advantages of our method (low cost, broad compatibility, extensibility) and its limitations. Our method will facilitate investigation of neuronal responses to tilt stimulation, particularly in small model animals such as Drosophila, Caenorhabditis, Danio, Danionella, and Xenopus.

Materials and Methods

Fish care
All procedures involving zebrafish (Danio rerio) larvae were approved by the Institutional Animal Care and Use Committee of New York University Grossman School of Medicine. Fertilized eggs were collected and maintained at 28.5°C on a standard 14–10 h light/dark cycle. All experiments were performed on larvae between 4 and 7 dpf. During this time, zebrafish larvae have not yet differentiated their sex into male/female. Before 5 dpf, larvae were maintained at densities of 20–50 larvae per 10-cm diameter, filled with 25–40 ml E3 with 0.5 ppm methylene blue. After 5 dpf, larvae were maintained at densities under 20 larvae per Petri dish in E3 and were fed cultured rotifers (Reed Mariculture) daily.

Line fish
All experiments were done on the mitfa+/− background to remove pigment for imaging. All larvae were labeled with Tg(nefma:GAL4;UAS:GCaMP6s) (Liu et al., 2020; Thiele et al., 2014). For experiments testing utricular origin of responses, Tg(nefma:GAL4;UAS:GCaMP6s) fish with a homozygous recessive loss-of-function mutation of the inner ear-restricted gene otogelin (otog−/−), also called rock solo+/− (Whitfield et al., 1996), were visually identified by a bilateral lack of utricular otoliths.

Imaging setup and apparatus
Larval fish (4–7 dpf) were mounted in a small drop of 2% low-melting-point agarose (catalog #16520, Thermo Fisher Scientific) in the center of the uncoated side of a mirror galvanometer (catalog #GVS0111, Thorlabs). E3 was placed over the agarose, and the galvanometer mirror was placed under the microscope.

For routine imaging, a baseline voltage was applied to the galvanometer to set it at one end of its range, allowing up to 40° of rotation away from the horizontal plane in one direction. Stimuli were capped at ±30° to allow the experimenter to apply an additional small offset voltage to correct for slight deviations from horizontal incurred while mounting the fish, and because of steric limitations relative to the objective. All trials (three trials per step size, two stimuli repeats per trial) in one direction were run with that baseline voltage manually set at horizontal, then a new baseline voltage was applied, and the galvanometer was recentered at horizontal to continue performing trials in the opposite tilt direction. Experiments with rock solo fish were performed with a different stimulus paradigm; in these experiments no baseline voltage was applied to the galvanometer before it was positioned at horizontal, and the maximum voltage drive was then applied during the stimulus to rotate the sample to ±20°.

For experiments where responses at horizontal were compared with responses measured directly at the eccentric angle, fish were mounted and first imaged on return to horizontal. Then the microscope was manually focused to allow the fish to be in focus at an eccentric angle for subsequent trials. Fish were then anesthetized by applying 0.2 mg/ml ethyl-3-aminobenzoic acid ethyl ester (MESAB, catalog # E10521, Sigma-Aldrich) to the fish mounted in agarose. The fish was allowed to sit with the anesthetic for 10 min before imaging recommenced to ensure it had reached full effect; MESAB remained on the fish for the rest of the imaging session to keep the fish properly anesthetized. The imaging was then repeated in the anesthetized fish with the fish in focus on return to horizontal and subsequently in focus at the eccentric angle.

Galvanometer control was done in MATLAB 2019b software (MathWorks) using the Data Acquisition Toolbox to interface with a data acquisition card (PCIe-6363, National Instruments). Custom code was written to simultaneously (1) deliver an analog waveform to control the galvanometer, (2) measure the analog voltage that corresponded to the galvanometer position, and (3) deliver synchronizing digital pulses to begin imaging. For all step and impulse stimuli, the galvanometer was allowed to step to or away from the eccentric angle at the maximum angular velocity/acceleration achievable (Table 1). Impulse stimuli were delivered in both directions. A microscope (Thorlabs Bergamo) was used to measure fluorescence elicited by multiphoton excitation (920 nm) from a pulsed infrared laser (Mai Tai HP). Fast volumetric scanning was
achieved using a piezo actuator (catalog #PFM450E, Thorlabs) to move the objective. Each frame of the volume (416 × 64 pixels) was collected with a 1 μs pixel dwell time (18.6 frames/s). Volumes ranged from six to nine planes in depth (6 μm between planes) to cover the entire vestibulospinal nucleus, resulting in a mean effective volume rate of 2.2 volumes per second (range 1.9–2.7 volumes/s). Fish that were imaged multiple times were imaged using the same scan settings across both sessions. The rock solo mutant fish and their wild-type siblings were imaged before the addition of the piezo actuator; in the place of volumetric imaging, for these experiments single z-planes were imaged separately (two to three planes per fish) at a frame rate of 6.6 frames/s.

Data analysis and statistics

Calcium response extraction and analysis. Data analysis was performed using custom code in MATLAB 2017b software (MathWorks). All datasets and analysis code have been deposited at Open Science Framework and are publicly available at https://osf.io/8rftf/ (Schoppik, 2023). We preprocessed the imaging data with code adapted from the CalmAn package (Giovannucci et al., 2019) and then performed NoRMCorr rigid motion correction (Pnevmatikakis and Giovannucci, 2017) on our GCaMP6s signal across all concatenated trials of the same stimulus type for each fish. We then hand drew polygon ROIs in Fiji software (Schindelin et al., 2012) around each vestibulospinal cell on the maximum intensity projection of all the motion-corrected frames. We imported ROIs into MATLAB using ReadImageJROI (Muir and Kampa, 2014) and extracted the mean pixel value across each ROI for all time points of each trial and performed normalization of the raw fluorescence trace.

For experiments imaged at horizontal, we quantified the peak calcium response to each stimulus as the mean F/F in the first second after the sample returned to horizontal. For experiments imaged at the eccentric angle, responses calculated for comparison to those at horizon- tal were the mean AF/F in the last second before the sample returned to horizontal. A cell was determined to have a significant response to a stimulus if the peak calcium responses across all trials were significantly higher (one-tailed t test, p < 0.05) than the mean calcium responses during the first second of the baseline period of that same cell. For each cell, a directionality index was calculated by taking the difference between the peak calcium response to ipsilateral and contralateral 30° steps, normalized by their sum.

To calculate the sensitivity of peak calcium responses to step magnitude, we fit a line with two free parameters to the peak calcium responses from all trials of all step magnitudes in a single direction (ipsilateral or contralateral). During analyses of longitudinal calcium imaging, cells with a significant sensitivity increase/decrease between time points was defined as follows: To determine a cutoff for significantly increasing/decreasing slopes, for each fish we shuffled the peak calcium responses across all trials from both ages in the same direction and used the shuffled responses to calculate a best-fit line with a slope. We then took the difference of the shuffled slopes between 4 and 7 dpf for each cell for the ipsilateral and contralateral direction. The cutoff for significant sensitivity change was defined as the mean of the shuffled slopes ± 2 SDs. In longitudinal experiments, Early Contra Responders were defined as cells that had a contralateral slope at 4 dpf greater than the mean + 2 SDs of contralateral 4 dpf shuffled slopes.

To determine any field-of-view shifts after an eccentric step, we performed a two-dimensional normalized cross-correlation between the frame before the stimulus and the frame after the stimulus for each plane of the volume. These analyses were performed on frames from unprocessed volumes. Field-of-view shift in the x-axis and y-axis was determined by finding the position of highest correlation coefficient within the resulting matrix and corresponding that matrix with an x-axis and y-axis shift in pixels (reported in text after conversion to microns). Mean correlation coefficients for each fish were calculated from the center of the correlation coefficient matrix (i.e., the correlation between the two frames without any x-shift or y-shift).

Correlation was evaluated using Pearson’s correlation coefficient (r). We report slope fits and 95% confidence intervals (CI). Significance level was defined throughout at 0.05. Paired t-tests were used when assessing differences between calcium responses in repeated imaging experiments; for all other comparisons of means between two groups, unpaired t-tests were used. Three-way ANOVA with factors of genotype, stimulus direction, and stimulus type were used to test for effects of otoglobin mutation on calcium responses. A repeated-measures ANOVA with factors of age and stimulus direction were used to test for effects of age on response sensitivity. In both cases, significant interaction effects were followed with Tukey’s post hoc tests.

Normalization

When comparing activity in the same neuron measured at horizontal only (our standard imaging paradigm), we normalized the fluorescence against the mean fluorescence value in the last 5 s of the baseline period within each trial. When comparing activity in the same neuron measured at different angles, we used the fluorescence measured in an anesthetized condition at the angle at which the imaging was done. Our rationale is as follows: The intensity of a genetically encoded calcium indicator reflects a number of variables, necessitating normalization. The baseline level is usually derived during a neutral condition with respect to the stimulus, correcting for differences in expression levels and variable imaging conditions (e.g., infrared light penetration or scattering of emitted photons). Further, vestibular neurons might have different basal activity when held at eccentric positions. We assert that the fluorescent intensity measured in a given neuron in an anesthetized animal will only reflect basal expression levels and variability because of imaging conditions. Consequently, it is an easily accessible baseline that permits us to compare responses in the same neuron when held at different angles.

Vestibulospinal cell body position and roll angle analysis

For each fish, an additional two-photon volumetric stack was taken with scan settings optimized for a high-signal, low-speed image (2 μs scan speed, cumulative averaging across four frames, 1 μm between z-planes). This stack was used for localizing the vestibulospinal cell bodies in three dimensions, defined relative to the Mauthner cell lateral dendrite. To define these XYZ positions, we first placed reference point ROIs in Fiji software at the lateral-most tip of the Mauthner cell lateral dendrite in both brain hemispheres. We then dropped point ROIs on all vestibulospinal cells that were analyzed in our calcium imaging trials, placing the ROI at the center of the cell body at the z-plane where the cell was most in focus. Using the Fiji Measure tool, we measured the XYZ position of each ROI in microns and exported these data to Microsoft Excel. For each vestibulospinal cell position, we subtracted off the XYZ position of the Mauthner lateral dendrite in its corresponding brain hemisphere to convert absolute position to relative position and then imported the relative XYZ position data to MATLAB for plotting.

For calculating the roll tilt angle of each fish, we used the left and right Mauthner lateral dendrite reference ROIs to find the distance between the two hemispheres in depth (z-axis). We then calculated the average mediolateral (x-axis) distance between the Mauthner lateral...
nd the roll activity as a function of roll tilt angle, with a strong preference for ipsilateral roll. Vestibulospinal neurons respond reliably to ipsilateral step stimuli. We refer to this stimulus as an “impulse.” Together, step and impulse stimuli allow for characterization of both the tonic (i.e., steady-state body tilt) and phasic (i.e., rapidly changing) components of the response of a neuron. The key to our method is therefore delivery of rapid rotations to the preparation.

Here, we used a mirror galvanometer as a platform to rapidly rotate an immobilized larval zebrafish. Mirror galvanometers are the tool of choice to steer light to particular angles for their precision and rapid response. We mounted a larval zebrafish in a small drop of low melting agarose directly on the uncoated side of the mirror (Fig. 1A). We could rotate the platform through nearly the full range of the galvanometer both rapidly (5.3 ms for a 30° step; Table 1), and precisely (Fig. 1B–D).

To image fluorescence, the platform is mounted underneath a water-dipping objective on a two-photon microscope capable of rapid volumetric scanning. All experiments were conducted in complete darkness. A drop of water, held in place by surface tension, sits between the agarose and the objective. Before starting an experiment, the platform is adjusted to sit horizontally underneath the objective so that the neurons of interest are in focus. We measured fluorescence across the volume to define a baseline for the neurons. For the step stimuli, we rotated the platform to an eccentric angle (where the neurons of interest are no longer in focus), held the platform at that orientation for 15 s, and then returned the platform back to horizontal (Fig. 1C). There, we measured the changes in fluorescent intensity in response to our stimulus.

Vestibulospinal neurons respond reliably to ipsilateral step stimuli

We began by measuring responses to roll tilts at 4 dpf in vestibulospinal neurons (Fig. 1E) labeled in transgenic line, Tg(nefma: GAL4;14xUAS:GCaMP6s) (Liu et al., 2020). Previous work in larval zebrafish (Liu et al., 2020) and other animals (Fujita et al., 1968) established that vestibulospinal neurons increase their activity as a function of roll tilt angle, with a strong preference for tilts in the direction of the recorded neuron (i.e., a cell in the left hemisphere responds when the left ear is rolled down; hereafter called ipsilateral roll). Vestibulospinal neurons therefore provide an excellent test bed to evaluate new tilt paradigms, such as the step and impulse stimuli we use here.

Figure 1. TIPM produces reliable, directional, and magnitude-dependent responses following roll tilts. A, A 4 dpf larval zebrafish mounted in agarose for roll stimuli on a mirror galvanometer. B, Schematic of our experimental paradigm. Baseline fluorescence (used for normalization) is measured when the platform is horizontal. The galvanometer is then stepped and held at an eccentric angle (Stimulus) where fluorescence is not recorded, then quickly returned to horizontal, whereupon fluorescent fluorescence (used for normalization) is measured when the platform is horizontal. The galvanometer is then stepped and held in agarose for roll stimuli on a mirror galvanometer.

Results

Rationale, apparatus, and stimulus for tilt in place microscopy

We developed a simple method, TIPM, to permit imaging of neuronal activity following body tilts. The vestibular end organs in vertebrates detect either linear accelerations (such as orientation relative to gravity) or rotational accelerations. We reasoned that the most straightforward way to assay this activity would be to image the same volume before and after such stimulation to avoid image registration challenges and to maximize compatibility with different microscope architectures. The kinetics of fluorescent indicators of neuronal activity are slow to decay (Chen et al., 2013). Consequently, a sufficiently rapid step back to the horizon from an eccentric orientation would produce a response with two components. The first component would reflect the steady-state activity of the neurons encoding linear acceleration because of gravity (i.e., the tilt of the body) before the step. The second component would reflect the phasic response to the step itself, if any. We refer to the stimulus that elicits these combined responses as a “step.” Complementarily, a second stimulus composed of a rapid step to an eccentric angle and back would generate a response to an impulse of rotation, devoid of any steady-state component. We refer to this stimulus as an “impulse.” Together, step and impulse stimuli allow for characterization of both the tonic (i.e., steady-state body tilt) and phasic (i.e., rapidly changing) components of the response of a neuron. The key to our method is therefore delivery of rapid rotations to the preparation.

To image fluorescence, the platform is mounted underneath a water-dipping objective on a two-photon microscope capable of rapid volumetric scanning. All experiments were conducted in complete darkness. A drop of water, held in place by surface tension, sits between the agarose and the objective. Before starting an experiment, the platform is adjusted to sit horizontally underneath the objective so that the neurons of interest are in focus. We measured fluorescence across the volume to define a baseline for the neurons. For the step stimuli, we rotated the platform to an eccentric angle (where the neurons of interest are no longer in focus), held the platform at that orientation for 15 s, and then returned the platform back to horizontal (Fig. 1C). There, we measured the changes in fluorescent intensity in response to our stimulus.

Vestibulospinal neurons respond reliably to ipsilateral step stimuli

We began by measuring responses to roll tilts at 4 dpf in vestibulospinal neurons (Fig. 1E) labeled in transgenic line, Tg(nefma: GAL4;14xUAS:GCaMP6s) (Liu et al., 2020). Previous work in larval zebrafish (Liu et al., 2020) and other animals (Fujita et al., 1968) established that vestibulospinal neurons increase their activity as a function of roll tilt angle, with a strong preference for tilts in the direction of the recorded neuron (i.e., a cell in the left hemisphere responds when the left ear is rolled down; hereafter called ipsilateral roll). Vestibulospinal neurons therefore provide an excellent test bed to evaluate new tilt paradigms, such as the step and impulse stimuli we use here.
We mounted the fish parallel to the axis of rotation of the platform to provide both ipsilateral and contralateral roll tilts of 10, 20, and 30° (Fig. 1C). We detected significant changes in fluorescence relative to the baseline of each cell (hereafter called responsive cells; see above, Materials and Methods) in 94% of neurons (67/71 neurons from 10 fish; Fig. 1F, example responsive trace). Important responses were reliable across repeated trials, with a median coefficient of variation across trials of 0.19 (Fig. 1F,G). Consistent with prior reports, responses were direction dependent. The majority of neurons had a larger response to ipsilateral roll compared with contralateral (Directionality Index = 0.46 ± 0.43; Fig. 1H,I). Additionally, we found that the strength of responses to roll stimuli increased with the size of the step. For steps in both the ipsilateral and contralateral direction, we observed that the peak response, defined as the mean response within the first second after the end of the stimulus, scaled linearly with the magnitude of the roll step (Fig. 1H,F; mean slope of responsive neurons = 0.07 ± 0.06 ΔF/F° ipsilateral, 0.02 ± 0.02 ΔF/F° contralateral). We conclude that our apparatus can elicit reliable, parametric, and directionally sensitive responses following roll tilts of different amplitude in vestibulospinal neurons.

TIPM is robust to extrinsic sources of variability

There are several potential sources of variability that could compromise detection of reliable fluorescent changes following stimulation. First we measured response variation from the following sources: (1) field-of-view movement during imaging and (2) mounting variability. We then measured intrinsic trial-to-trial variability that presumably reflects biological sources such as changes in intraneuronal calcium, spike rate fluctuations, or state of the animal (Schoppik et al., 2008). If the variability observed from trial to trial is greater than extrinsic variability, we would conclude that our approach is sufficiently robust.

The dynamic nature of TIPM introduces the potential for the field of view of our sample to move during the course of imaging. Sample movement has the potential to cause variability in fluorescence intensity that does not reflect an underlying calcium fluctuation. Qualitatively, we did not observe field-of-view shifts acutely between the baseline recording period and after an eccentric step. We quantified such changes by performing a cross-correlation of each frame before and after the eccentric step. To eliminate signal changes from neuronal fluctuations, we performed this analysis on unprocessed volumes measured in anesthetized fish. The frames before and after the eccentric step were most highly correlated with each other when they were not shifted relative to each other (mean shift = 0.03 μm in x-axis, −0.02 μm in y-axis; mean correlation without shift = 0.5). Additionally the mean peak fluorescence change after the eccentric step in anesthetized fish was very low (0.05 ± 0.08 ΔF/F anesthetized vs 2.2 ± 1.8 ΔF/F unanesthetized, n = 26 neurons), indicating there is very little variation in the fluorescence signal that results from acute shifts during imaging. We conclude that TIPM as implemented here introduces tolerable levels of sample movement.

Notably, as with any long-term imaging experiment, we did observe that some samples slowly drift in X/Y/Z between trials. We estimate this drift at ~1 μm/min in all axes. These slow shifts can be easily corrected either manually between trials or by post hoc motion correction and so do not introduce appreciable variability into measured responses.

We next addressed the variability because of mounting. For each imaging experiment, larval fish are manually mounted on the galvanometer in agarose in a dorsal-up position. Every attempt is made to minimize roll, pitch, and yaw relative to the axis of rotation, but manual mounting is subject to small variations. These variations would impose linear accelerations that scale with the distance from the center of the axis of rotation. Such shifts would be challenging to quantify and, if large, might compromise longitudinal experiments.

To estimate how much variation in response originated from variation in mounting, we performed a repeated imaging experiment. We mounted and imaged a fish, then removed the fish from agarose and remounted the same fish and repeated our imaging protocol (Fig. 2A). We were able to reliably identify...
neurons between the first and second mounts (Fig. 2B). To estimate the roll tilt, we calculated the bilateral difference in z-position of the tips of the left and right Mauthner lateral dendrites and used this to calculate a roll angle of the head. We observed only minor rotation of the baseline position of the fish in the roll axis between the first and second mounts (2.4 ± 1.0°, N = 5 fish).

We saw a strong correlation (ρ = 0.83) in the response of individual neurons between the first and second mount (Fig. 2C, D). We did not observe a significant shift in responses between the first and second mounts (paired t test, p = 0.64; n = 34 neurons, N = 5 fish), and the responses of the neurons fell nearly along the unity line (slope = 0.75 ± 0.18 CI).

To contextualize the magnitude of the mount-to-mount variability we observed, we compared it to trial-to-trial variability. Response correlation between TIPM sessions is comparable to the response correlation between two subsequent trials (Fig. 2E) within a single imaging session (ρ = 0.79, slope = 0.79 ± 0.09 CI, n = 34 cells, N = 5 fish). These data suggest that most of the variability in response magnitude we see mount to mount reflects inherent variability.

Together these experiments establish best practices to estimate variability when using TIPM to measure neural responses. We conclude that variability because of our apparatus or mounting are relatively minor concerns for estimating neural response magnitude in our preparation.

Neural activity imaged after a step reflects the encoding of body tilt before the step

Our interpretation of the response rests on the assumption that the activity observed after the platform returns to the horizon primarily reflects the activity of the neuron at the eccentric position. To test this assumption, we compared the response of neurons at eccentric angles to that after a step returning the fish to horizontal. In this experiment, we presented the same 30° roll stimulus to fish while measuring activity first on return to the horizontal plane as previously described (Fig. 3A, black), then on subsequent trials measuring activity directly at the eccentric 30° angle (Fig. 3A, magenta). Because the light path to the neuron changes as a function of eccentricity, we normalized fluorescence to a baseline stack taken at either the horizontal or the eccentric angle while the fish was anesthetized. We compared fluorescence in vestibulospinal neurons in the first second on return to horizontal to the responses of the same neurons in the last second of the eccentric step. Neural responses at eccentric angles were closely correlated (ρ = 0.94) with the responses measured at horizontal (Fig. 3B, C). The strong similarity in response amplitude supports two conclusions: First, the response of the neuron on return to the horizontal is indeed a reasonable proxy for the activity of a neuron at an eccentric angle in the moment just before the return step. Second, by inference, larval zebrafish vestibulospinal neurons should have comparatively small responses to phasic stimulation.

Notably, while measuring fluorescent intensity at the eccentric angle, we observed quite different dynamics among vestibulospinal neurons. The most striking variation was in the decay rate of the fluorescent intensity (Fig. 3D). Some neurons had a distinct peak followed by a fast decay (Fig. 3B, left), whereas others had plateau-like responses that had little to no decay over the 15 s hold (Fig. 3B, right). In all neurons measured, fluorescent intensity reached its peak within 10 s of being at the eccentric angle (median 5.2 s; Fig. 3E). We conclude that for vestibulospinal neurons, a 10 s step would be sufficient to ensure accurate detection of the peak response on return to horizontal (correlation between eccentric calcium response at its peak and at 10 s, ρ = 0.91). As this value will vary between neuronal populations, preliminary experiments to set the optimal window should be done for each new population of interest. By adjusting the length of the TIPM eccentric step to one’s own experimental goals and observed calcium dynamics, the experimenter can use the return to horizontal response as a proxy to measure the magnitude of either the peak or steady-state calcium responses. Together, measuring fluorescence on return to horizontal can be used to accurately extrapolate information about the response of the neuron at the eccentric angle. Further, although imaging at horizontal alone cannot provide information...
about temporal dynamics, we demonstrate here how TIPM can be modified to allow for imaging at an eccentric angle to study variations in response dynamics within a population of neurons.

**Vestibulospinal neurons respond weakly to impulses of angular acceleration**

To measure the impulse response of vestibulospinal neurons, we delivered rapid roll steps to 10, 15, or 30° and then back to horizontal in ∼13 ms (Fig. 4). We observed significant changes in fluorescence to the impulse stimulus in a moderate fraction (35.4%) of neurons (n = 22/62 neurons from N = 6 fish). The average peak fluorescence observed to the impulse stimulus was small (0.53 ± 0.39 ΔF/F for an ipsilateral 30° stimulus) compared with the response to the tilt stimulus. Impulse responses are more variable across trials than responses to the step stimuli (median coefficient of variation = 0.44 vs 0.19; Fig. 4D). Unlike responses to steps of different amplitudes, peak fluorescent intensity did not vary systematically with the magnitude of the impulse (slope of peak fluorescence = 0.002 ± 0.01 ΔF/F° ipsilateral, −0.002 ± 0.02 ΔF/F° contralateral; Fig. 4F). Additionally, impulse responses did not show a consistent directional preference. Most neurons responded equally strongly to ipsilateral and contralateral steps (Directionality Index = 0.10 ± 0.37; Fig. 4E,G).

We asked whether there was topographical organization to these responsive neurons within the lateral vestibular nucleus. Although nonresponsive neurons are found distributed evenly throughout the lateral vestibular nucleus, neurons with a significant response to the impulse stimulus are located more ventrolaterally (mean dorsoventral position relative to Mauthner lateral dendrite = 3.6 ± 7.0 μm responsive neurons vs 2.2 ± 11.0 μm nonresponsive neurons; unpaired t test, p = 0.03; Fig. 4H,I). There may therefore be topographic differences in innervation by VIIIth nerve afferents that relay phasic vestibular inputs relative to tonic inputs such that impulse-responsive afferents target only a subset of ventrolateral vestibulospinal neurons.

Together, our data argue that vestibulospinal neurons are more sensitive to the tonic component of the step stimulus than to an impulse stimulus. We infer that the response of vestibulospinal neurons predominantly reflects static encoding of body tilt.

The utricle is indispensable for the bulk of vestibulospinal neuron responses

Loss-of-function experiments assaying both behavior (Bianco et al., 2012; Ehrlich and Schoppik, 2019; Mo et al., 2010) and neuronal responses (Liu et al., 2020) support the proposal that in larval zebrafish, the bulk of the vestibular response is derived...
from a single vestibular end organ, the utricle. However, TIPM is inherently multimodal and might activate other systems in addition to the utricle. Angular accelerations can be transduced by the semicircular canals. Although the semicircular canals are too small to be activated under natural conditions (Beck et al., 2004; Lambert et al., 2008), they can be activated by sufficiently strong stimuli in comparably small vertebrates (Branoner and Straka, 2004). Translational forces along the body might be encoded by the trigeminal system (Ribera and Nüsslein-Volhard, 1998). As vestibulospinal neurons are known in other animals to receive a wide variety of multimodal inputs (Sarkisian, 2000), we sought to clarify the role of utricular sensation.

We adopted a genetic loss-of-function approach to assay the contribution of the utricle to vestibulospinal responses. Mutants in *otogelin*, also known as *rock solo* fish (Whitfield et al., 1996), do not form a utricular otolith in the first 10 dpf (Roberts et al., 2017). *Ototelin* is selectively expressed in the inner ear (Stooke-Vaughan et al., 2015), avoiding off-target confounds. We tested the responses of *rock solo* mutants to both a 20° step and impulse stimulus. We provided both ipsilateral and contralateral impulses; as we did not previously observe systematic differences, we aggregated the data across both directions to assay impulse responses.

We observed that vestibulospinal responses to ipsilateral roll steps in *rock solo* fish were severely compromised. The *rock solo* mutants were less likely to show significant changes in fluorescent intensity following a 20° ipsilateral step compared with their wild-type siblings (96% responsive WT, n = 24/25 neurons from N = 3 fish; 42% responsive mutants, n = 13/31 neurons from N = 3 fish). When there were suprathreshold responses, the magnitude of peak fluorescence in mutants was strongly attenuated (2.7 ± 2.3 ΔF/F WT vs 0.21 ± 0.24 ΔF/F mutants; three-way ANOVA, interaction effect, p = 1.1 × 10⁻⁵; Tukey’s post hoc test, p = 5.9 × 10⁻⁵; Fig. 5A,B).

In contrast, responses were not significantly different between wild-type siblings and *rock solo* mutants following contralateral steps (0.38 ± 0.41 ΔF/F WT vs 0.26 ± 0.30 ΔF/F mutant; Tukey’s post hoc test, p = 0.99), nor were responses significantly different to impulse steps (0.42 ± 0.48 ΔF/F WT vs 0.32 ± 0.39 ΔF/F mutant; Tukey’s post hoc test, p = 0.99; Fig. 5A,B). We conclude that contralateral eccentric and impulse responses are predominantly driven by extraurticular sources. Following both contralateral steps and impulse stimuli, we observed a decrease in the fraction of neurons that responded to the stimulus in *rock solo* fish (Contralateral step = 72% responsive WT vs 39% responsive mutant; Impulse = 72% responsive WT vs 44% responsive mutant). Changes to the fraction of neurons that have suprathreshold responses reflect an increase in variability of baseline calcium fluctuations in *rock solo* mutants (baseline SEM, 0.019 WT vs 0.032 *rock solo*), consistent with electrophysiological observations (Hamling et al., 2021; Liu et al., 2020).

We conclude that the changes in fluorescence we observe in vestibulospinal neurons following ipsilateral body tilts predominantly reflects utricular transduction.

**Vestibulospinal neuron responses develop systematically**

A distinct advantage of TIPM is its minimally invasive nature. As such, it is well suited for experiments that require monitoring the same neurons across multiple time points. We asked whether TIPM could detect developmental changes in individual vestibulospinal neurons on two different days. Prior behavioral work established that larval zebrafish use vestibular information to balance and locomote in different ways at 4 and 7 dpf (Ehrlich and Schoppik, 2017, 2019). We therefore picked 4 and 7 dpf to assay differences in body tilt-evoked responses.

We imaged fluorescence after return to horizontal from 10, 20, and 30° step stimuli in the same fish at two ages, 4 and 7 dpf. We were able to reliably identify the same neurons across imaging sessions (Fig. 6A). Peak calcium responses within the same neuron were correlated (ρ = 0.51) between 4 and 7 dpf (n = 71 cells, N = 10 fish; Fig. 6B) but were more variable than neurons in repeated imaging sessions performed on the same day (Fig. 6B, gray bar) suggesting developmental changes in neuronal encoding. We observed that responses were more variable in our second imaging session than the first (median coefficient of variation = 0.29 at 7 dpf vs 0.19 at 4 dpf); we therefore chose to compare the slope of peak fluorescence responses (Roll Sensitivity, a common metric of sensory encoding capacity; Lannou et al., 1979) of neurons across development, instead of a metric-like mutual information that takes response variability into account (Quiroga and Panzeri, 2009). Across all neurons that responded to the stimulus at either age, calcium response sensitivity to ipsilateral eccentric rolls strengthened between 4 and 7 dpf (mean slope = 0.07 vs 0.10 ΔF/F°; repeated-measures ANOVA, interaction effect, p = 0.001; Tukey’s post hoc test, p = 0.002); n = 70 cells), and sensitivity to contralateral roll did not decrease significantly (mean slope = 0.02 vs 0.01 ΔF/F°; Tukey’s post hoc test, p = 0.09). Our data suggest that the population of vestibulospinal neurons improves its ability to encode eccentric roll tilts during this developmental window.
Figure 6. Longitudinal imaging suggests systematic changes in the complement of presynaptic inputs in developing vestibulospinal neurons. A, Two-photon volumes of vestibulospinal neurons in a Tg(nefma:GAL4);Tg(UAS:GCaMP6s) larva during longitudinal imaging experiments at 4 and 7 dpf. Colored overlays indicate the same neurons located in the volume across the two time points. B, Mean peak calcium response of all neurons at 4 dpf are correlated with their mean peak response at 7 dpf (r = 0.51) but is more variable than expected by remounting alone (fit line ± SD of residuals from repeated mounting experiment in Fig. 2D). C, Mean peak ΔF/F responses across all responsive neurons for ipsilateral and contralateral rolls of 10, 20, and 30° magnitudes at 4 dpf (black) and 7 dpf (magenta). Error bars indicate ± SEM. D, Probability distributions of longitudinal changes in ipsilateral (top) and contralateral (bottom) calcium response sensitivity (n = 70 cells; see above, Materials and Methods). Dashed vertical lines represent cutoffs for significant sensitivity change. E, Longitudinal sensitivity changes in individual neurons across ipsilateral and contralateral roll tilts. F, Longitudinal sensitivity changes for ipsilateral (top) and contralateral (bottom) roll tilts split by neurons that have significant magnitude-dependent responses to roll steps in that direction at 4 dpf (Early-Tuned, green) versus nontuned at 4 dpf (Non-Tuned, black).

Additionally, beyond effects on the population, our longitudinal imaging paradigm allowed us to ask (1) how sensitivity of an individual neuron to ipsilateral or contralateral eccentric roll angle changed across development and (2) whether these changes are systematically patterned. First, we investigated whether developmental changes within the population of vestibulospinal cells were homogeneous. To do so, we examined the distribution of developmental sensitivity changes in individual cells. We then identified cells with a significant change in roll sensitivity by comparing the observed change in the sensitivity of a cell to a cutoff generated from sensitivity changes in age-shuffled data (see above, Materials and Methods). We found that individual cells had heterogeneous and asymmetric sensitivity changes to ipsilateral rolls (Fig. 6D, top). In response to ipsilateral rolls, individual vestibulospinal cells experienced either no change in sensitivity (53/70 cells) or a significant increase in sensitivity (14/70 cells) but very rarely experienced a significant decrease in ipsilateral sensitivity between 4 and 7 dpf (3/70 cells). This asymmetry explains the overall increase in ipsilateral responses observed across the whole population at 7 dpf. In comparison, the distribution of contralateral sensitivity changes was heterogeneous and approximately symmetric (Fig. 6D, bottom), with most cells experiencing no significant change (51/70 cells) and comparable numbers experiencing a significant sensitivity increase (8/70 cells) or decrease (11/70 cells). Together, these findings allow us to conclude that the vestibulospinal population is not homogeneous in how tilt responses develop. Specifically, a majority of cells do not change between 4 and 7 dpf, whereas small subpopulations either increase or decrease their sensitivity in a directional-dependent manner.

We then asked whether the developmental changes an individual cell experiences in the ipsilateral and contralateral directions are correlated; we found that there was no significant correlation between sensitivity changes across these two directions (Fig. 6E; r = −0.14, p = 0.25). Together with previous findings, these data support the existence of different patterns of functional development occurring within the vestibulospinal nucleus that are not coordinated between response directions. Although ipsilateral responses strengthen and rarely weaken, the same pattern does not apply to contralateral responses. Additionally, developmental change in one direction does not predict change in the opposing direction. The lack of correlation observed between ipsilateral and contralateral developmental changes suggests that the refinement of these two directions is driven by separate mechanisms.

To explore potential mechanisms for systematic changes in response properties across development, we attempted to predict how ipsilateral and contralateral roll sensitivity within a neuron would change based on the responses we observed at 4 dpf. We asked whether neurons with a significant, magnitude-dependent response to roll stimuli early in development (Early-Tuned) would selectively strengthen or weaken their responses as they develop. For ipsilateral stimuli, there was no significant difference between sensitivity change distributions when split between early-tuned (42/70 neurons) and nontuned cells (Fig. 6F, top; two-sample Kolmogorov–Smirnov test, p = 0.95). In contrast, we found that neurons that were early tuned for contralateral stimuli (24/70 cells) had a significantly different distribution of developmental sensitivity changes compared with nontuned neurons (Fig. 6F, bottom; two-sample Kolmogorov–Smirnov test, p = 2.46 × 10⁻⁷). Specifically, early-tuned contralateral neurons were only observed to decrease (11/24 neurons), or have no change (13/24 neurons) to their contralateral sensitivity between 4 and 7 dpf, and never strengthened their contralateral responses.
Neurons that did not have significant contralateral responses at 4 dpf either strengthened their contralateral sensitivity between 4 and 7 dpf (8/46 neurons) or experienced no change (38/46 neurons). We can therefore conclude that contralateral, but not ipsilateral, sensitivity changes are patterned by the responses of a neuron early in development. Such inferences are new to the vestibular field and are made possible by our ability to follow the same neurons over multiple days. We propose that our novel approach is therefore well suited for discovering biologically relevant changes responsible for improvements to neuronal control of posture as animals develop.

Discussion
Here, we report a new method, Tilt In Place Microscopy, to measure neuronal responses following vestibular stimulation. To test TIPM, we mounted a larval zebrafish on a rotating platform (mirror galvanometer) and measured fluorescence as the fish returned from an eccentric tilt. Consistent with prior work (Peterson, 1970; Rovainen, 1979), we observed reliable responses that vary with tilt magnitude. We tested the reproducibility of our method by mounting the same fish repeatedly, finding that TIPM produced little change in the strength and reliability of neuronal responses. By imaging the same neurons both at an eccentric angle and at the horizon, we confirmed that the response reflected the steady-state activity while tilted. We next delivered impulse steps, and discovered topographically organized responses. Consistent with other work, vestibulospinal neurons in mutant zebrafish without utricles responded minimally following tilts. Finally, we measured activity from a set of vestibular neurons at both 4 and 7 dpf and reveal systematic changes in sensitivity and selectivity across time. Below we compare TIPM to other approaches/apparatus, discuss limitations and potential extensions, and contextualize our findings.

Comparison to other apparatus/approaches
Other groups have tackled the challenge of imaging neural activity while measuring vestibular responses; each approach requires considerable technical expertise and financial resources. One approach involved adapting a powerful technique, the optical trap, to directly displace the utricle (Favre-Bulle et al., 2018). Another approach cleverly miniaturized the hardware to permit an entire light sheet microscope to rotate stably (Migault et al., 2018). Together, these apparatuses allowed these investigators to characterize vestibular responses across an entire vertebrate brain—a considerable advance. Recently, another group developed a rotating stage that allowed them to examine the vestibular periphery (Tanimoto et al., 2022). Finally, a complementary approach used for in vivo electrophysiology may be compatible with imaging, provided that the microscope was sufficiently small—mounting the preparation on an air-bearing sled (Liu et al., 2020). All four solutions require a familiarity with optical physics and/or engineering to implement and calibrate. All four require specialized and expensive hardware.

In contrast, TIPM offers a number of advantages. It is comparatively low cost and compatible with any microscope (multiphoton or otherwise) with a long working distance objective that can accommodate a simple rotating platform. Importantly, the only requirement to control the stimulus is the ability to deliver an analog voltage to control the galvanometer and a digital pulse for calibration. We propose that the simplicity, flexibility, and low cost of TIPM facilitate the study of the neuronal encoding of vestibular responses.

Limitations of TIPM
The design choices we made facilitate certain experiments but are not without their own trade-offs. TIPM was designed to image the response to tilts at one particular orientation, to facilitate comparison to baseline measurements so crucial for imaging fluorescence from calcium indicators. Responses must be therefore measured after stimulation is complete. Further, we chose to move the preparation relative to a stationary microscope objective, the light path to the same neuron will change with each change in orientation. Consequently, comparisons across orientations (Fig. 3) requires normalization to a baseline that accounts for different scattering such as an anesthetized baseline (see above, Materials and Methods). The inability to measure during stimulation and the challenge of comparing across orientations means that our method is likely incompatible with a sinusoidal rotation.

The vestibular periphery has been been modeled as an linear, time-invariant system (Laurens et al., 2017). By and large, the measurements underlying this powerful framework are derived from sinusoidal stimulation at different frequencies while recording neuronal responses across vestibular areas. However, sinusoidal stimulation is not the only way to measure the response of a linear, time-invariant system. Impulse stimuli (Fig. 4A) contain power at a wide range of frequencies. Such click stimuli are common in characterizing auditory responses, and the head impulse test is commonplace during clinical evaluation of semicircular canal function (Halmagyi et al., 2017). Similarly, step stimuli such as we have used here allow evaluation of the DC component (i.e., the steady-state response to gravity at a particular orientation). Although TIPM as presented here is incompatible with sinusoidal rotation, we propose that evaluating the responses to impulses (Fig. 4) and steps (Fig. 1) as we have here will serve comparably for linear systems analysis of the vestibular system.

Unlike other systems that can rotate a full circle, TIPM is constrained to a smaller range of angles because of three factors. First, the galvanometer itself can only rotate 40°. Second, our preparation relies on surface tension to keep the water between the sample and the objective. In practice rotations >40° relative to the horizon risk spilling the water. Finally, steric considerations limit the achievable rotation. To rotate 90°, the platform would have to be sufficiently narrow to fit entirely within the working distance of the objective (2 mm) Such a narrow platform would be unwieldy to mount and hold too little water. We therefore do not believe our apparatus will be able to tilt much beyond what we report here. Experiments that require a wider range of angles are better performed on apparatus that can rotate more.

Ways to extend TIPM
For imaging experiments, the choice of indicator and field of view set fundamental limitations in time and space. Here, we used a slow calcium indicator (GCaMP6s) to measure neuronal activity. All our estimates of vestibular response are convolved with the spike-to-calcium kernel (Chen et al., 2013). This low-pass filter constrains our ability to measure vestibular responses regardless of whether stimuli are sinusoidal, impulses, or steps. Recent advances in genetically encoded voltage indicators suggest that fluorescent imaging of membrane potential is on the horizon (Böhm et al., 2022), or perhaps here (Liu et al., 2022). As TIPM delivers rapid changes to tilt, and is straightforward to
integrate with advanced microscopes, we anticipate that it will be ideal for voltage imaging experiments. Similarly, TIPM translates readily to microscopes with wider fields of view, facilitating whole-brain approaches.

TIPM can accommodate a wide variety of existing hardware to accommodate in vivo imaging in different preparations. Although we used a mirror galvanometer, any device that can rapidly and precisely rotate away from and back to a given angle will work. Small direct-drive rotation mounts such as Thorlabs DDR25, or larger options such as the Newport RGV100 series offer rapid and precise rotation and allow for larger loads than the mirror galvanometer. A low-cost option is similarly available by substituting a DC stepper motor and a driver with microstepping capability to permit smooth acceleration. Both options would also permit compatibility with current platforms for in vivo imaging in Drosophila (Aragon et al., 2022) and Caenorhabditis (Smith et al., 2022). Naturally, our approach is compatible with head-mounted microscopes (Aharoni and Hoogland, 2019; Zong et al., 2022) and stably mounted high-density probes of electrical activity (Steinmetz et al., 2021) in rodents. We anticipate that laboratory staff looking to adopt TIPM will select the device that best rotates their existing preparation.

TIPM can be easily extended to permit measuring tail, fin, and/or eye movements in zebrafish. Similar adaptations allow for measurement of leg/wing movements in other animals. First, it is necessary to replace the mirror galvanometer with a transparent platform. A camera mounted below the apparatus can measure the tail bends and eye movements in the horizontal plane with freely available software such as Stytta (Stih et al., 2019). To measure eye movements in the torsional plane, a glass coverslip can be glued perpendicular to the plane of the slide. A camera can then measure torsional eye movements that follow pitch tilts, as done in Bianco et al. (2012). As tilt stimuli reliably elicit compensatory postural and ocular behaviors, such apparatus would provide valuable context to the measures of neuronal activity we report here.

Insights into encoding of roll tilt by developing vestibulospinal neurons

The responses to roll tilts in vestibulospinal neurons reported here largely agree with and extend prior reports, bolstering confidence in TIPM, and go on to describe novel development findings that have not previously been observed in vestibular nuclei in any species. We see much stronger responses to ipsilateral than contralateral steps, replicating findings from larval nuclei in any species. We see much stronger responses to ipsilateral roll reported for vestibulospinal neurons (Peterson, 1970; Rovainen, 1979). By following the same cells over time, TIPM longitudinal imaging also allows us to make new observations about patterns of functional vestibular development. We found that response sensitivity changes do not appear at random but instead follow structured patterns, which, for some response types, are related to response properties early in development. Our analyses point the way forward to use early response properties to predict how neurons will change across development.

Conclusion

From birth to death, the sense of gravity of an animal and other accelerations profoundly shapes its physiology (Yates et al., 2013) and journey through the world (Angelaki and Laurens, 2020). Perhaps, unsurprisingly, studies of the vestibular system have informed nearly every aspect of modern systems-level neuroscience (Goldberg et al., 2012a). Advances in imaging neuronal activity have similarly shaped modern neuroscience. Others have brought imaging and vestibular stimulation together with custom microscopes (Favre-Bulle et al., 2018; Migault et al., 2018; Tanimoto et al., 2022), but adoption requires considerable expertise and financial resources. Here, we describe and validate a novel apparatus/analysis approach we call TIPM to image neuronal responses to body tilts. TIPM is comparatively easy to implement, compatible with a large set of existing microscope designs, low cost, noninvasive, extensible to a wide variety of preparations, and compatible with longitudinal measurements. We support this claim by confirming and extending our understanding of tilt representation by developing vestibulospinal neurons in the larval zebrafish. Specifically, we observed preferential sensitivity to tonic stimulation, rough topographic organization, tractable levels of extrinsic variability, and systematic changes across early development. Although not without trade-offs, we hope that the simplicity and broad compatibility of TIPM will democratize the study of the response of the brain to destabilization, particularly across development.

References

Adrian ED (1943) Discharges from vestibular receptors in the cat. J Physiol 101:389–407.
Aharoni D, Hoogland TM (2019) Circuit investigations with open-source miniaturized microscopes: past, present and future. Front Cell Neurosci 13:141.
Angelaki DE, Laurens J (2020) The head direction cell network: attractor dynamics, integration within the navigation system, and three-dimensional properties. Curr Opin Neurobiol 66:136–144.
Angelaki DE, McHenry MQ, Dickman JD, Newlands SD, Hess BJM (1999) Computation of inertial motion: neural strategies to resolve ambiguous otolith information. J Neurosci 19:316–327.
Aragon MJ, Mok AT, Shea J, Wang M, Kim H, Barkdull N, Xu C, Yapien N (2022) Multiphoton imaging of neural structure and activity in Drosophila through the intact cuticle. Elife 11:e69094.
Bagnall MW, Schopik D (2018) Development of vestibular behaviors in zebrafish. Curr Opin Neurobiol 53:83–89.
Beck JC, Gilland E, Tank DW, Baker R (2004) Quantifying the ontogeny of optokinetic and vestibulococular behaviors in zebrafish, medaka, and goldfish. J Neurophysiol 92:3546–3561.
Beranek M, Lambert FM, Sadeghi SG (2014) Functional development of the vestibular system. In: Development of auditory and vestibular systems (Romand R, Varela-Nieto I, eds), pp 449–487. Amsterdam: Elsevier.
Bianco IH, Ma LH, Schoppik D, Robson DN, Orger MB, Beck JC, Li JM, Schier AF, Engert F, Baker R (2012) The tangential nucleus controls a gravito-inertial vestibulo-ocular reflex. Curr Biol 22: 1285–1295.

Böhm UL, Kimura Y, Kawashima T, Ahrens MB, Higashijima SI, Engert F, Cohen AE (2022) Voltage imaging identifies spinal circuits that modulate locomotor adaptation in zebrafish. Neuron 118:1211–1222.e4.

Brannon F, Straka H (2015) Semicircular canal-dependent developmental tuning of translational vestibulo-ocular reflexes in Xenopus laevis. Dev Neurobiol 75:1051–1067.

Brannon F, Straka H (2018) Semicircular canal influences on the developmental tuning of the translational vestibulo-ocular reflex. Front Neural 9:404.

Carriot J, Jamali M, Chacron MJ, Cullen KE (2017) The statistics of the vestibular input experienced during natural self-motion differ between rodents and primates. J Physiol 595:2751–2766.

Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499:395–399.

Chen WL, Ko H, Chuang HS, Raizen DM, Bau HH (2021) Caenorhabditis elegans exhibits positive gravitaxis. BMC Biol 19:186.

Daltorio K, Fox J (2018) Haltere removal alters responses to gravity in stand-ard larvae. J Neurosci 38:51–105.

Dijkgraaf S (1963) The functioning and significance of the lateral-line organs. Gustav Fischer.

Ehrlich DE, Schoppik D (2017) Control of movement initiation underlies the development of balance. Curr Biol 27:334–344.

Ehrlich DE, Schoppik D (2019) A primal role for the vestibular sense in the development of coordinated locomotion. Elife 8:e45839.

Favre-Bulle IA, Vanwalleghem G, Taylor MA, Rubinstein-Dunlop H, Scott EK (2018) Cellular-resolution imaging of vestibular processing across the larval zebrafish brain. Curr Biol 28:3711–3722.e3.

Fleisch A (1922) Das labyrinth als beschleunigungsempfindendes organ. J Physiol 110:947–964.

Fujita Y, Rosenberg J, Segundo JP (1968) Activity of cells in the lateral vestibular nucleus as a function of head position. J Physiol 196:1–18.

Giovannucci A, Friedman J, Gunn P, Kalfon J, Brown BL, Koay SA, Taxidis J, Najafi F, Gauthier LS, Zhou P, Khakh BS, Tank DW, Chklovskii DB, Pnevmatikakis EA (2019) CaImAn an open source tool for scalable calcium imaging data analysis. Elife 8:e28173.

Goldberg JM, Wilson VJ, Cullen KE, Angelaki DE, Broussard DM, Buttner-Ennever J, Fukushima K, Minor LB (2012a) The vestibular system: a sixth sense. New York: Oxford UP.

Goldberg JM, Wilson VJ, Cullen KE, Angelaki DE, Broussard DM, Buttner-Ennever JA, Fukushima K, Minor LB (2012b) Learning and compensation in the vestibular system. In: The vestibular system: a sixth sense (Goldberg JM, Wilson VJ, Cullen KE, Angelaki DE, Broussard DM, Buttner-Ennever J, Fukushima K, Minor LB, eds), pp 408–442. New York: Oxford UP.

Halmagyi GM, Chen L, MacDougall HG, Weber KP, Garvin LAI, Curthoys IS (2017) The video head impulse test. Front Neurol 8:258.

Hamling KR, Harmon K, Greaney MR, Dobler Z, Kimura Y, Higashijima S, Goldberg JM, Wilson VJ, Cullen KE, Angelaki DE, Broussard DM, Büttner-Ennever J, Fukushima K, Minor LB (2012a) The vestibular system: a sixth sense. New York: Oxford UP.

Hamling KR, Harmon K, Greaney MR, Dobler Z, Kimura Y, Higashijima S, Pnevmatikakis EA (2019) Otolith tethering in the zebrafish otic vesicle requires Otoegin and alpha-Tectorin. Development 142:1137–1145.

Hennestad E, Witoelar A, Chambers AR, Vervaeke K (2021) Mapping vestibular and visual contributions to angular head velocity tuning in the cortex. Cell Rep 37:110134.

Hershey K, Beck JC, Baker R, Straka H (2008) Semicircular canal size determines the developmental onset of angular vestibulococular reflexes in larval Xenopus. J Neurosci 28:8086–8095.

Lannou J, Precht W, Cazin L (1979) The postnatal development of functional properties of central vestibular neurons in the rat. Brain Res 175:219–232.

Laurens J, Liu S, Yu XJ, Chan R, Dickman D, DeAngelis GC, Angelaki DE (2017) Transformation of spatiotemporal dynamics in the macaque vestibular system from otolith afferents to cortex. Elife 6: e20787.

Liu Z, Kimura Y, ichi Higashijima S, Häderbrand DG, Morgan JL, Bagann MW (2020) Central vestibular tuning arises from patterned convergence of otolith afferents. Neuron 108:748–762.e4.

Liu Z, et al. (2022) Sustained deep-tissue voltage recording using a fast indicator evolved for two-photon microscopy. Cell 185:3408–3425.e29.

Lowneisen O, Roberts TDM (1949) The equilibrium function of the otolith organs of the thornback ray Raja clavata. J Physiol 110:392–415.

March E (1886) Beiträge zur Analyse der Empfindungen. Jena, Germany: Gustav Fischer.

Migault G, van der Plas TL, Trentesaux H, Panier T, Cancelleri R, Provirel V, Englitz B, Debrégeas G, Bornuth V (2018) Whole-brain calcium imaging during physiological vestibular stimulation in larval zebrafish. Curr Biol 28:3723–3735.e6.

Mo W, Chen F, Nechiporuk A, Nicolson T (2010) Quantification of vestibular-induced eye movements in zebrafish larvae. BMC Neurosci 11:110.

Muir DR, Kampa BM (2014) FocusStack and StimServer: a new open-source MATLAB toolchain for visual stimulation and analysis of two-photon calcium neuronal imaging data. Front Neuroinform 8:e10.

Petersen BW (1970) Distribution of neural responses to tilting within vestibular nuclei of the cat. J Neurophysiol 33:750–767.

Peusner KD (2014) Development of the central vestibular system. In: Development of auditory and vestibular systems (Romand R, Varela-Nieto I, eds), pp 413–447. Amsterdam: Elsevier.

Pnevmatikakis EA, Giovannucci A (2017) NoRMCorr: an online algorithm for piecewise rigid motion correction of calcium imaging data. J Neurosci Methods 291:1–17.

Quiroga RQ, Panzeri S (2009) Extracting information from neuronal populations: information theory and decoding approaches. Nat Rev Neurosci 10:173–185.

Ribera AB, Nüsslein-Volhard C (1998) Zebrafish touch-insensitive mutants reveal an essential role for the developmental regulation of sodium current. J Neurosci 18:9181–9191.

Roberts R, Elsner J, Bagann MW (2017) Delayed otolith development does not impair vestibular circuit formation in zebrafish. JARO 18:415–425.

Rovainen CM (1979) Electrophysiology of vestibulospinal and vestibuloreticulospinal systems in lampreys. J Neurophysiol 42:745–766.

Sarkissian VH (2000) Input-output relations of Deiters’ lateral vestibulospinal neurons with different structures of the brain. Arch Ital Biol 138:295–353.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomanak C, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682.

Schoppik D, Nagel KI, Lüscher SG (2008) Cortical mechanisms of smooth eye movements revealed by dynamic covariations of neural and behavioral responses. Neuron 58:248–260.

Schoppik D, Bianco IH, Prober DA, Douglass AD, Robson DN, Li JM, Greenwood JS, Soucy E, Engert F, Schier AF (2017) Gaze-stabilizing central vestibular neurons project asymmetrically to extraocular motoneuron pools. J Neurosci 37:11353–11365.

Schoppik D (2023) Data Associated with ‘Tilt In Place Microscopy: A Simple, Low-Cost Solution to Image Neural Responses to Body Rotations. OSF. Available at https://osf.io/brtbf/.

Smith JJ, Kenny IW, Wolff C, Ray K, Kumar A, Sherwood DR, Matus DQ (2022) A light sheet fluorescence microscopy protocol for Caenorhabditis elegans larvae and adults. Front Cell Dev Biol 10:1201820.

Steinmetz NA, et al. (2021) Neuropixels 2.0: a miniaturized high-density probe for stable, long-term brain recordings. Science 372:697–707.

Stith V, Petrucco L, Kast AM, Portugues R (2019) Stytra: an open-source, integrated system for stimulation, tracking and closed-loop behavioral experiments. PLOS Comput Biol 15:e1006699.

Stooke-Vaughan GA, Obholzer ND, Baxendale S, Msegson GW, Whitfield TT (2015) Otolith tethering in the zebrafish otic vesicle requires Otoegin and alpha-Tectorin. Development 142:1137–1145.
Straka H, Paulin MG, Hoffman LF (2021) Translations of Steinhausen’s publications provide insight into their contributions to peripheral vestibular neuroscience. Front Neurol 12:676723.

Tanimoto M, Watakabe I, Higashijima S (2022) Tilttable objective microscope visualizes discrimination of static and dynamic head movement originates at hair cells. Research Square. Advance online publication. Retrieved December 20, 2022. https://doi.org/10.21203/rs.3.rs-1845426/v1.

Thiele TR, Donovan JC, Baier H (2014) Descending control of swim posture by a midbrain nucleus in zebrafish. Neuron 83:679–691.

Whitfield T, Granato M, van Eeden F, Schach U, Brand M, Furutani-Seiki M, Haftler P, Hammerschmidt M, Heisenberg C, Jiang Y, Kane D, Kelsh R, Mullins M, Odenthal J, Nüsslein-Volhard C (1996) Mutations affecting development of the zebrafish inner ear and lateral line. Development 123:241–254.

Yates BJ, Kerman IA, Jian BJ, Wilson TD (2013) The vestibulo-autonomic system. In: Oxford textbook of vertigo and imbalance (Bronstein AM, ed), pp 49–62. Oxford: Oxford UP.

Yoder RM, Taube JS (2014) The vestibular contribution to the head direction signal and navigation. Front Integr Neurosci 8:32.

Zong W, Obenhaus HA, Skyytoen ER, Eneqvist H, de Jong NL, Vale R, Jorge MR, Moser MB, Moser EI (2022) Large-scale two-photon calcium imaging in freely moving mice. Cell 185:1240–1256.e30.