Characterizing trajectories of innate immune cells in larval zebrafish

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Abstract

It is well established from in vitro studies of immune cells that stimulation by a wide range of potential signals leads to motility and morphology changes. How these physical behaviors manifest inside a living animal remains unclear due to limitations of conventional imaging and analysis approaches. Here, we establish a quantitative framework for imaging and tracking neutrophil and macrophage dynamics in larval zebrafish, spanning a large fraction of the animal for multi-hour timescales with few-minute temporal resolution. We focus especially on the gut, examining innate immune responses to different preparations of the intestinal microbiome. Using light sheet fluorescence microscopy and trajectory analysis of hundreds of individual cells, we characterize speeds, directional persistence measures, and cellular morphology to reveal distinct population behaviors. Individual immune cells exhibit stable motility phenotypes, favoring predominantly motile or non-motile states rather than frequent transitions between them. Gut architecture constrains migration patterns as demonstrated by preferential anterior-posterior movement and a high probability of cells remaining in the vicinity of the gut throughout the imaging duration. Macrophages display significantly reduced sphericity during motile periods compared to non-motile periods, providing a morphological signature that may enable inference of dynamic behavior from static snapshots. Surprisingly, migration patterns remain consistent across diverse microbial conditions – germ-free, conventionally reared, and colonized by two strains of a zebrafish-native *Vibrio* species – indicating that tissue structure exerts a stronger influence than bacterial stimuli on immune surveillance dynamics. Previously observed tissue damage by the wild-type *Vibrio* strain, and the resulting recruitment of immune cells towards the damage site, provided the only microbe-specific cellular behavior. These findings reveal innate immune surveillance as a stereotyped process whose characteristics reflect both cellular decision-making and larger-scale anatomical structure.

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I. INTRODUCTION

The innate immune system serves as the first line of defense against pathogens and supports tissue homeostasis throughout the body [1, 2]. Both functions are especially important around the gastrointestinal tract, which serves as an interface between interior and exterior spaces and between the cells of the host and vast numbers of microbes that include commensal as well as pathogenic organisms. Neutrophils and macrophages, the primary effector cells of innate immunity, communicate with each other and with other cell types through complex, multi-layered chemical networks involving numerous signaling molecules and regulatory pathways [3–6]. Immune cells are also highly motile and physically dynamic, and their strategies for navigating complex, three-dimensional, heterogeneous environments are an integral component of their overall efficacy. In vitro systems, for example collagen gels, have enabled insights into chemotaxis, cell polarity, activation, and force generation by neutrophils and macrophages [7–9]; while they afford experimental control and clarity, their geometry and homogeneity differ considerably from that encountered in live animals. Our understanding of how innate immune cells move in vivo, especially in and around gut-associated tissues, remains minimal. Questions including whether cell shape correlates with motility, and whether the presence of gut microbes alters immune cell surveillance behavior, remain unanswered.

Imaging immune cells in live animals is technically challenging but has progressed considerably in recent years. Studies using intravital multi-photon microscopy have revealed the dynamics of immune cells in mammals, but require extensive surgical preparation that can compromise physiological relevance [10–14]. Larval zebrafish (*Danio rerio*), due to their transparency and genetic tractability, provide an especially useful vertebrate model system for studies of the innate immune system [15, 16]. Studies using zebrafish have shown, for example, fewer numbers of gut-associated neutrophils in germ-free animals than in animals colonized by microbes[17, 18], different speeds and modes of migration (mesenchymal versus amoeboid) for neutrophils and macrophages [19], tissue-dependent T-cell motility modes likely reflecting mechanical constraints [20], and a two step search and run response of neutrophils to in-vivo chemical gradients [21]. Furthermore, cutting the caudal fin has become a well-established wound and regeneration assay, which has been used for example to

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show that neutrophils perform both forward and reverse migration in response to localized damage [22–26], and to test therapeutics for inflammation [27].

Despite the increasing number of investigations, there remains a lack of imaging-based approaches of innate immune cells in volumes large enough to span the entire larval zebrafish gut and over durations large enough to characterize motion types. We therefore used light sheet fluorescence microscopy to image fluorescent neutrophils (transgenic line *Tg(mpx:eGFP)*) and macrophages (*Tg(mpeg1:mCherry)*) [28, 29] in live fish over length scales of millimeters and time scales of a few hours. Light sheet fluorescence microscopy provides rapid, high-volume, three-dimensional imaging with minimal phototoxicity [30–32], and its utility for imaging zebrafish intestinal bacteria and immune cells has been repeatedly demonstrated [33–37]. The resulting image datasets are large, roughly 24 GB per time-point and 1.92 TB per fish for typical dataset, necessitating efficient segmentation and tracking methods to yield immune cell trajectories.

We first validate this imaging and analysis approach with caudal fin cut experiments, providing a test of our analysis methods under conditions of known, directed immune cell migration before applying them to the more complex gut-associated microenvironment. Then we describe neutrophil and macrophage trajectories in larval fish under various gut microbial preparations: germ-free, colonized by ambient microbes ("conventional"), or colonized solely by one of two variants of a zebrafish-isolate *Vibrio* strain, ZWU0020. In past work, we have shown that this strain induces inflammation and other physiological responses in the host animal, responses that require the presence of the actin crosslinking domain (ACD) of the bacterial Type VI Secretion System (T6SS). *Vibrio* with a complete T6SS causes tissue damage, induces macrophage redistribution towards the damage site, and leads to a roughly 100% increase in the amplitude of zebrafish intestinal contractions compared to *Vibrio* lacking the ACD or to non-*Vibrio* bacteria [37]. As described below, we find that under all bacterial conditions, both neutrophils and macrophages show stable motility states, i.e. remaining either motile or non-motile over few-hour durations, maintain a stable proximity to the gut, and migrate preferentially along the intestinal anterior-posterior axis, suggesting that gut architecture is the primary determinant of immune cell movement patterns. Notably, macrophages but not neutrophils show significantly higher sphericity during non-motile periods, providing a morphological indicator of motility state that reflects the greater pseudopodial extension of migrating macrophages.

II. RESULTS

A. Validation of 3D immune cell tracking using the caudal fin cut wound model

We performed transverse amputations of the caudal fin in 5 days post-fertilization (dpf) zebrafish larvae, cutting midway between the posterior end of the notochord and the distal tip of the fin (Figure 1A). Using the dual transgenic line *Tg(mpx:GFP; mpeg1:mCherry)*, we simultaneously imaged neutrophils (cells expressing the myeloperoxidase, *mpx*) and macrophages (cells expressing macrophage-expressed gene-1, *mpeg1*) during the wound response [28, 29] (Figure 1B and Supplemental Videos 1, 2). Time-lapse imaging was performed on a custom-built light sheet fluorescence microscope from 1 to 5 hours post-amputation, capturing a tissue volume of approximately $4.8 \times 10^7 \mu m^3$ (400μm along the dorsal-ventral, 800μm along the anterior-posterior, and 150μm along the left-right axes), with images acquired every 3 minutes.

Following image pre-processing and drift correction, individual immune cells were segmented and tracked across time points to generate single cell trajectories (see Methods). Simple cell counts showed more number of neutrophils in the field of view of the fin cut assay than the control; this difference was not seen with the macrophages (Supplemental Figure 1A). The resulting trajectory data clearly demonstrated directed migration of both neutrophils and macrophages toward the amputation site (Figure 1C, D), consistent with the known recruitment of immune cells to sites of tissue damage [22–26]. All cell trajectories are provided as Supplementary Data.

B. Establishment of motion classification criteria

To extract meaningful physical parameters from cellular trajectories, we developed a computational framework that accounts for the intermittent nature of immune cell migration. Individual cells exhibit alternating periods of active migration and relative quiescence, a characteristic feature of immune cell behavior in tissue environments [38]. To capture this temporal heterogeneity, we subdivided each complete trajectory into "subtracks" based on frame-to-frame displacement. Specifically, a new subtrack was initiated whenever the displacement transitioned above or below a threshold of half the mean cell diameter per 3 minute interval. Subtrack classification, however, does not characterize the form of the

motion, for example directed versus random, calling for additional measures. We computed the spatial extent of each subtrack as the diameter of the smallest circumscribing sphere. We calculated the velocity cosine similarity of each subtrack, a measure of directional persistence defined as the cosine of the angle between consecutive velocity vectors, averaged over the subtrack. Evaluating immune cells in the caudal fin area, pooling both cut and control fish, plots of spatial extent versus velocity cosine similarity for each subtrack show a positive correlation for both neutrophils and macrophages (Figure 2A, B), indicating that more spatially extended tracks are also more directionally persistent. (Such a correlation would not exist, for example, for random walks.) To classify subtracks as motile or non-motile, we define a simple threshold of spatial extent being at least twice the mean cell diameter. To justify this beyond its intuitive validity, we consider for each cell type a linear fit of spatial extent versus cosine similarity; the spatial extent corresponding to zero cosine similarity 47.2μm and 42.6μm for neutrophils and macrophages, respectively, is close to twice the mean cell diameter, 20.4μm and 19.5μm for neutrophils and macrophages, respectively (Figure 2A, B), implying that our motility criterion also distinguishes persistent migration from random, small-scale motion likely due to cell shape changes or tracking uncertainties.

Applying this motility classification scheme to the fin cut assay demonstrates its effectiveness in capturing differences in immune cell behavior. Motile subtracks from fin-cut fish exhibited significantly higher velocity cosine similarity compared to motile subtracks from the control, uninjured group (Figure 2C), confirming that our analysis pipeline successfully identified the expected increase in directional migration of both neutrophils and macrophages following tissue damage. In control fish, motile subtracks show a velocity cosine similarity near zero, indicating largely random motion, while in injured fish the innate immune cells show persistent motion, visually identified as being towards the wound site.

Having validated our motion analysis pipeline, we turn to investigations of immune cell behavior in response to different gut microbiota inoculations.

C. Three-dimensional immune cell tracking reveals gut-associated motility patterns

To investigate immune cell dynamics around the larval zebrafish gut in response to varied commensal bacterial groups, we assessed germ-free fish, conventionally reared fish with

normal parental tank microbiota, and initially germ-free fish inoculated at 5 or 6 days post-fertilization (dpf) with one of two variants of the zebrafish-native *Vibrio* strain ZWU0020. As noted above, we showed in earlier work that colonization with wild-type *Vibrio* ZWU0020 (which we denote $Vibrio^{WT}$) causes tissue damage near the zebrafish vent (the posterior opening of the gut), which recruits macrophages to the vent from their normal midgut positions during a period of roughly 14 to 20 hours post inoculation (hpi) [37]. Vent damage and immune activation are minimal, with levels similar to germ-free fish, in larvae colonized with a *Vibrio* mutant lacking the actin crosslinking domain (ACD) of the Type VI Secretion System (T6SS), (denoted $Vibrio^{\Delta ACD}$) [37]. In prior work [37], macrophages positions were assessed at 20 minute intervals and neutrophils were not tracked.

In the studies described here, we began imaging at 9 hpi and tracked neutrophil and macrophage cells at 3-minute intervals through 13 hpi. Germ-free, conventionally reared, or *Vibrio*-inoculated zebrafish were imaged with light sheet fluorescence microscopy at 3-minute intervals at 5 or 6 dpf from 9 to 13 hpi for inoculated fish or the same age for other non-inoculated fish. The imaging volume covered the entire gut: approximately 1600μm along the anterior-posterior (AP) axis, 100μm left-right (LR), and 400μm dorsal-ventral (DV) (Figure 3A). Using the dual transgenic line *Tg(mpx:GFP; mpeg1:mCherry)*, we imaged and tracked individual neutrophils and macrophages throughout the 4-hour observation period (Figure 3B, C and Supplemental Video 3, 4). All cell trajectories are provided as Supplementary Data. The mean and median track lengths were 23.7 and 15 frames for neutrophils and 31.8 and 24 frames for macrophages respectively, pooled across all groups. These cells showed a similar relationship between spatial extent and velocity cosine similarity as immune cells in the fin cut assay (Supplemental Figure 2). Vent width measurements confirmed the expected T6SS-dependent tissue damage at the end of the imaging window, with $Vibrio^{WT}$-associated fish showing the largest increase compared to other groups (Figure 3D, Supplemental Figure 3).

D. Immune cells maintain distinct motility states with robust motility patterns across microbial conditions

Using our framework for classifying the motility of subtracks, we found that both neutrophils and macrophages were predominantly either motile or non-motile throughout the

four-hour imaging duration, rather than showing frequent transitions between motility states. Histograms of the fraction of each track showing motile behavior, grouped across all bacterial conditions, show for both neutrophils and macrophages over an order of magnitude greater probability near 0 and 1 than at intermediate fractions (Figure 4A; bin width $0.0\overline{6}$). Analysis included only tracks with minimum track lengths of 5 frames to ensure robust motility classification. This bimodal distribution was also consistent across all four experimental groups and in the fin cut assay (Supplemental Figure 4, 5). Over four hours, therefore, innate immune cells generally adopt a consistent motile or non-motile phenotype.

To more finely assess motility behaviors and possible dependence on microbial stimulation, we examined the number and fraction of motile and non-motile cells (subtracks) at each timepoint from 9 to 13 hpi. The numbers of motile neutrophils and macrophages remained fairly constant and similar across all four conditions (germ-free, conventional, $Vibrio^{WT}$, and $Vibrio^{\Delta ACD}$) (Figure 4B, C). Non-motile neutrophils increased in number over time in $Vibrio^{WT}$-inoculated fish (Figure 4B), which was mainly driven by the increase in the number of non-motile neutrophils near the vent region (Supplemental Figure 6). Macrophages showed no significant temporal changes in non-motile subtrack numbers across any group (Figure 4C).

E. Macrophage morphology correlates with motility state

To investigate whether immune cell motility correlates with detectable morphological changes, we quantified cellular sphericity, defined as the ratio of the surface area of a sphere with the same volume as the measured cell to the measured surface area of the cell. Perfect spheres have a sphericity value of 1.0, whereas shape distortions reduce this value.

Neutrophils exhibited no significant differences in sphericity between motile and non-motile states across all experimental groups (Figure 5A). In striking contrast, macrophages demonstrated significantly higher sphericity during non-motile periods compared to motile periods, indicating increased cellular rounding when stationary. This morphological distinction suggests that motile macrophages extend pseudopodia more extensively than their non-motile counterparts, providing a snapshot-based indicator of motility state that does not require temporal tracking to assess. This behavior is also observed in the response of these cells in the fin cut assay (Supplemental Figure 1B). We examined numerous addi-

tional cellular parameters, including volume and normalized anterior-position position, but sphericity was one of the few metrics that distinguished motile from non-motile behavior across all groups (Supplemental Figure 7A, B and 1C).

F. Gut architecture constrains immune cell migration patterns

We manually segmented images to identify gut-associated regions, including the intestinal lumen and surrounding tissue (Figure 3B and Supplemental Video 5). We classify as "gut-associated" immune cells that are located within these boundaries at any point during imaging. To assess the persistence of gut association over extended periods, we considered subtracks with a duration of at least five frames and examined the fraction of time spent in the gut volume. Non-motile subtracks spent more than 80% of their time within the gut, considering averages across all experimental groups(Figure 5B). Surprisingly, motile subtracks also demonstrated high gut residence probability ($> 70\%$ across all groups). Simulating random walks with the same average frame-to-frame displacement as immune cells and assessing their residence in a volume like that of the larval gut gives a residence probability of $30-40\%$ for walkers with the same median track lengths of the observed neutrophils and macrophages, indicating that gut-associated immune cells migrate predominantly along the boundary of the gut.

Analysis of motile subtrack characteristics revealed that most kinetic parameters, including mean speed and velocity components, showed no significant differences between experimental groups (Figure 5C and Supplemental Figure 8). We also provide these parameters for the fin cut assay (Supplemental Figure 9). However, we found that immune cell migration shows a strong directional bias. We calculated all cells' velocity anisotropy, the ratio of the anterior-posterior velocity component to the perpendicular component, scaled to the range 0-1, i.e.:

$$\text{Velocity anisotropy} := \sqrt{\frac{2 \cdot V_{\text{AP}}^2}{V_{\text{DV}}^2 + V_{\text{LR}}^2}},$$

where V_{AP}, V_{DV}, and V_{LR} represent velocity components along the anterior-posterior, dorsal-ventral, and left-right axes, respectively. This velocity anisotropy consistently exceeded 1.0 across all experimental conditions (Figure 5D). indicating preferential movement along the anterior-posterior axis compared to dorsal-ventral or left-right directions. Com-

bined with the high gut residence probability of motile cells, this result demonstrates that gut-associated immune cells migrate primarily along the intestinal length (along anterior-posterior axes) rather than in left-right or dorsal-ventral directions, suggesting that gut architecture fundamentally constrains immune cell movement patterns regardless of microbial stimulus.

III. DISCUSSION

To date, there have been few quantitative analyses of immune cell dynamics in living organisms over volumes that span a large fraction of the animal, and to our knowledge, this study is the first that characterizes innate immune dynamics around the gastrointestinal tract. Given strong, multifaceted interactions between the intestinal microbiota and the host immune system [39–42], it is natural to expect that different microbial stimuli should lead to different immune cell motility patterns. Surprisingly, we find little difference in motility characteristics in germ-free larval zebrafish, conventionally reared fish, and fish inoculated with a pathobiont *Vibrio* species, other than spatial redistribution to the site of *Vibrio*-induced tissue damage [37]. Immune cell motion is, however, strongly influenced by the anatomical context of the host. Neutrophils and macrophages that encounter the gut tend to spend a high fraction of their time near the gut, and migrate largely along the anterior-posterior axis. This strong axial preference was consistent across all microbial conditions, indicating that gut structure, not microbial signals, primarily dictates migration patterns.

On the timescale of a few hours, we found stable motility states (either motile or non-motile) for both neutrophils and macrophages, rather than frequent transitions in behavior. While our study made use of live imaging over several hours, it is interesting to ask whether motility states can be gleaned from single snapshots, potentially even from non-living animals or histological samples. We found no notable morphological differences between motile and non-motile neutrophils. Macrophages, in contrast, were much less spherical when motile. While the protrusions of macrophages and the relatively more spherical shapes of neutrophils in general are well known, distinction of macrophage sphericity between motile and non-motile states are not, and this feature may allow the inference of dynamic behavior from static snapshots.

The remarkable consistency of immune cell behavior across microbial conditions—germ-free, conventional, *Vibrio*^{WT}, and *Vibrio*$^{\Delta ACD}$—demonstrates robust migration patterns independent of bacterial stimuli. The bimodal distribution of motility states, constant numbers of motile cells, and invariant kinetic parameters across all conditions suggest that gut architecture exerts stronger influence than microbial signals during the 9-13 hpi window. Only the localized accumulation of non-motile neutrophils near *Vibrio*-induced tissue damage deviated from this pattern, indicating that while bacterial activity can create specific retention sites, it does not fundamentally alter migration dynamics. These findings suggest that therapeutic strategies targeting immune motion should consider the dominant role of tissue architecture over microbial influences. The quantitative framework established here to track and characterize hundreds of cells in-vivo in 3D across multi-hour timescales should translate to other model systems and additional cell types (such as T-cells) enabling systematic comparison of immune surveillance mechanisms across diverse immunogenic conditions. Our computational pipeline is made available as open-source code (see the Image Analysis section of Methods). The combination of stable motility states, architectural constraints, and cell-type-specific morphological signatures reveals immune surveillance as a stereotyped process whose characteristics reflect both cellular decision-making and larger-scale structure.

IV. METHODS AND MATERIALS

A. Zebrafish husbandry and transgenic lines

All experiments with zebrafish followed standard procedures [43] and were performed in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee (protocol numbers AUP-22-02 and AUP-20-16). Fish from which the larvae used in these studies were derived were maintained in the University of Oregon Zebrafish Facility at 28°C with a 14-h/10-h light/dark cycle.

The transgenic line Tg(mpeg1:mCherry), allele number: gl23, is described in reference [28]. The transgenic line Tg(tnfα:GFP);Tg(mpeg1:mCherry), allele number pd1028Tg, is described in reference [44].

B. Gnotobiotic derivation and bacterial colonization

Embryos were derived germ-free following established protocols [45]. Fertilized eggs were incubated for 6 hours in sterile embryo medium (EM) containing antibiotics (100 μg/mL ampicillin, 10 μg/mL gentamycin, 1 μg/mL tetracycline, 1 μg/mL chloramphenicol, 250 ng/mL amphotericin B). After surface sterilization with 0.003% sodium hypochlorite and 0.1% polyvinylpyrrolidone-iodine, embryos were distributed into T25 flasks at one embryo per mL in 15 mL sterile EM.

Vibrio ZWU0020 (PRJNA205585) was previously isolated from the zebrafish intestinal tract. From freezer stocks kept at -80°C, cultures were grown overnight in Luria Broth at 30°C with agitation. Then, bacterial cultures were pelleted through centrifugation for 3 min at 7,000 × g, washed once in sterile EM, and inoculated at 10^6 CFU/mL directly into flask water. Larvae were colonized at 5 or 6 dpf, with time-lapse imaging from 9 to 13 hpi.

C. Construction of *Vibrio* ZWU0020 T6SS mutants

The actin crosslinking domain deletion mutant (Vibrio$^{\Delta ACD}$) was constructed using markerless allelic exchange with the pAX2 vector as previously described [37, 46]. In brief, splice-by-overlap extension generated deletion cassettes with the following primers: 5' HR: 5'-CCCAATGATAGCCACGGTTG-3' + 5'-CCATTCCATTTTCCACTAGGCTAAAGGACACACCTT-3'; 3' HR: 5'-AAGGTGTGTCCTTTAGCCTAGTGGAAAATGGAATGG-3' + 5'-GGCGCAAGATTTTCAATCA-3'. After conjugation into *Vibrio* using *E. coli* SM10, merodiploids were selected on TSA-gentamicin at 37°C. Deletion was confirmed by PCR using flanking primers: 5'-CGGAGCTTTGGTCAATCTCA-3' + 5'-AGGTCTCTCCGTGGAAAACA-3'.

D. Caudal fin amputation

For wound response assays, 5 dpf larvae were anesthetized in 120 μg/mL MS-222 before mounting on glass slides using 4% methyl-cellulose. The caudal fin was transected midway between the notochord end and fin tip using a sterile scalpel. Larvae recovered in fresh EM at 28°C before imaging.

E. Light sheet fluorescence microscopy

Imaging was performed using a custom-built light sheet fluorescence microscope described in detail elsewhere [47, 48]. A galvanometer-scanned light sheet of excitation illumination from 448 or 561 nm lasers (Coherent Sapphire, operating at 5 mW) was oriented perpendicular to the optical axis of a 40× 1.0 NA detection objective lens. Images were captured by a 5.5 MP sCMOS camera (Excelitas pco.edge). Three-dimensional volumes were acquired by scanning the sample along the detection axis with 33 ms exposures per plane. All hardware components were controlled using μManager software [49].

For gut imaging, four overlapping sub-regions covering the entire intestine (∼1,200 × 300 × 150 μm^3) were sequentially acquired and computationally registered. Time series were collected at 3-minute intervals for 4 hours (9-13 hpi). For fin wound assays, single volumes were acquired at 3-minute intervals for 4 hours (1-5 hours post-amputation). Original voxel dimensions were 1 μm (z-axis) × 0.1625 μm (y-axis) × 0.1625 μm (x-axis).

F. Sample mounting

Larvae were anesthetized in 120 μg/mL MS-222 and embedded in 0.7-1.0% low-melt agarose within glass capillaries. Specimens were partially extruded to avoid optical interfaces and oriented with ventral illumination. Up to six samples were mounted simultaneously in sterile EM at 28°C.

G. Gut Segmentation

Binary masks that identified the larval zebrafish gut region in images were manually segmented using a Wacom Cintiq® graphics tablet on brightfield images acquired at the median sagittal plane. To optimize efficiency while maintaining accuracy, masks were initially drawn at every 10th timepoint and subsequently interpolated across all 80 imaging timepoints (Supplemental Video 5). The resulting 2D masks were extruded along the left-right axis to encompass the complete gut volume within the z-stack imaging boundaries.

H. Image analysis

All image processing and data analysis were performed with code written in Python using the NumPy [50], SciPy [51], scikit-image [52], and pandas [53] libraries. Code is available at `https://github.com/rplab/Immune_Cell_Motion_Analysis_2025`.

1. Preprocessing

Each 2D slice of a 3D image volume (z-stack) was downsampled by a factor of 4 in both x and y directions to reduce computation time. Raw image stacks were then drift-corrected using phase correlation between bright field images from consecutive time points. For gut imaging, 4-5 overlapping fields of view along the anterior-posterior axis were acquired. Stage positions saved in the acquisition metadata were extracted from configuration files and represent global coordinates of the images which were then transformed to local pixel coordinates which was used to stitch different fields of view to generate complete gut volumes.

2. Cell segmentation

Immune cells were segmented using a two-stage approach combining iterative thresholding with morphological Active Contour Without Edges (ACWE) [54, 55].

In brief, we applied iterative simple thresholding to get loose boundaries around objects. Initial thresholding used median + 2σ, where σ is the standard deviation of pixel intensities. Objects exceeding 10^5 μm^3, considerably larger than the possible volume of a single cell, were iteratively re-thresholded at their local median + nσ, incrementing n until all objects fell below the volume limit or n reached 7. Objects smaller than a sub-cellular threshold of 10^3 μm^3 were removed.

For each remaining object, we tightened/refined boundaries around the object using morphological ACWE with the thresholded region as the initial level set. The algorithm ran for 10 iterations with parameters: smoothing=1, λ_1=1, λ_2=4, with early stopping after 3 iterations of no change.

3. *Cell property analysis*

Cell properties including volume, centroid, and mean intensity were extracted for each segmented object.

Surface area was calculated via a marching cubes algorithm on Gaussian-smoothed segmented objects with 5-pixel padding to prevent edge artifacts Supplemental Video 7 shows the generated surfaces on the segmented macrophages. Sphericity quantified morphological complexity and was computed as:

$$\text{Sphericity} = \frac{(36\pi V^2)^{1/3}}{A} \tag{1}$$

where V is cell volume and A is surface area. This metric equals 1 for perfect spheres and decreases with surface complexity, providing a quantitative measure of pseudopodial extension. As this metric is meaningful only between 0 and 1, the resulting values were clipped to this range.

Gut association was determined by spatial overlap with manually defined gut boundaries.

4. *Tracking and trajectory analysis*

Segmented cells were tracked across time points using a bidirectional nearest-neighbor algorithm. For each consecutive time point pair, we computed Euclidean distances between all cell centroids in 3D space. A cell at time t was linked to its nearest neighbor at time $t + 1$ only if the reverse mapping (from $t + 1$ to t) also identified the original cell as the nearest neighbor. This bidirectional validation prevented spurious connections. Trajectories were subdivided into "subtracks" at transitions above/below a displacement threshold of 0.5× mean cell diameter, separating motile from non-motile phases. A patience parameter (n=3 timepoints) was applied to ensure subdivision only occurred after cells maintained the new motility state for 3 consecutive timepoints to prevent oversegmentation from transient behavior.

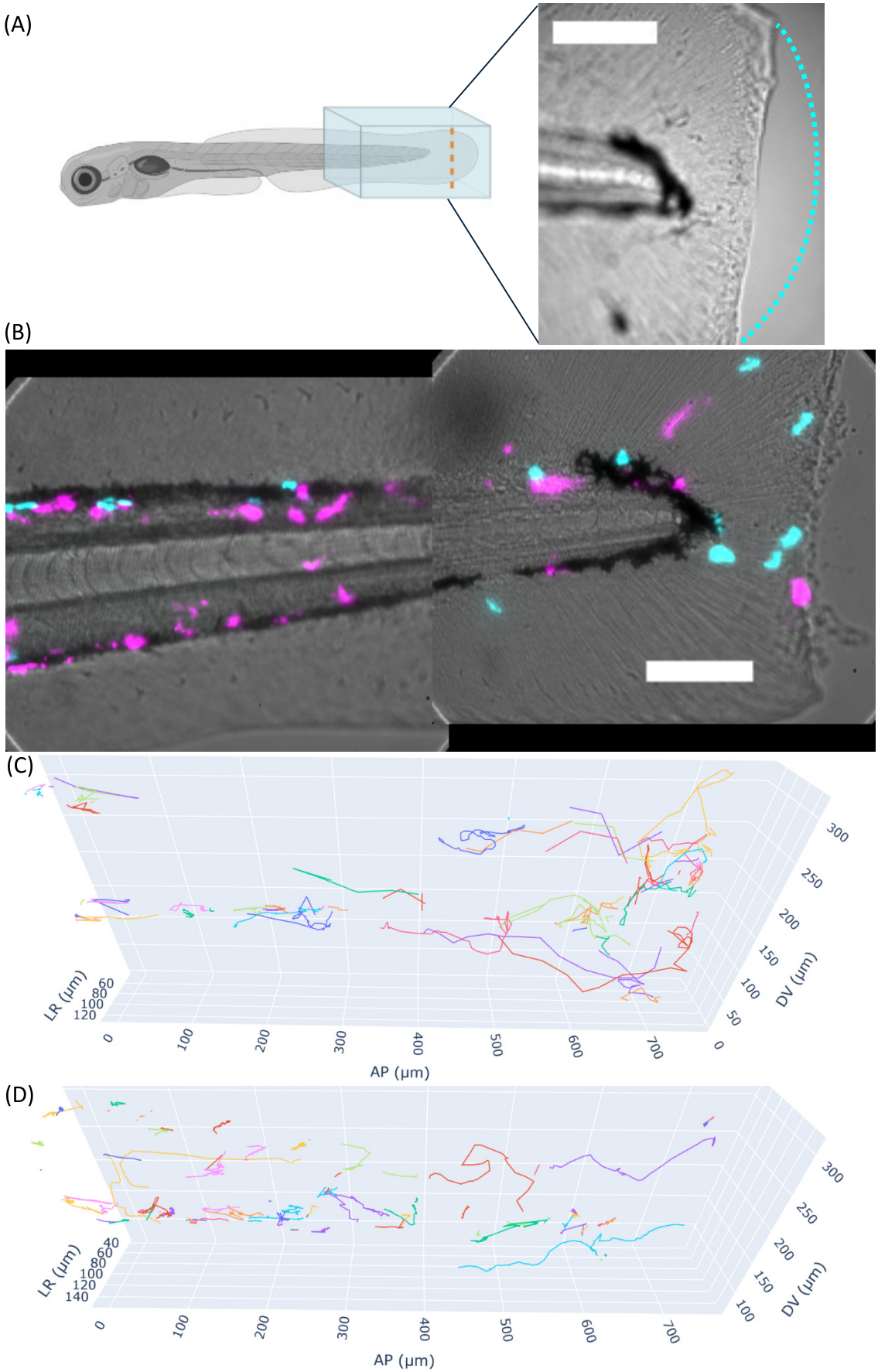
For each subtrack, we calculated:

- **Velocity components**: Frame-to-frame velocity components V_{AP}, V_{DV}, and V_{LR} along anterior-posterior (AP), dorsal-ventral (DV), and left-right (LR) axes

- **Velocity cosine similarity**: $\cos(\theta)$ between consecutive velocity vectors
- **Spatial extent**: Diameter of smallest circumscribing sphere of the subtrack
- **Velocity anisotropy**: $\sqrt{2V_{\mathrm{AP}}^2/(V_{\mathrm{DV}}^2 + V_{\mathrm{LR}}^2)}$

I. Statistical analysis

Custom code written in Python was used for data analysis and plotting. All p values reported are from Mann–Whitney U tests, which are nonparametric and so do not assume normally distributed data. Unless otherwise noted, uncertainties are the 16th and 84th percentile of the distribution calculated from bootstrap subsampling.


(A)
(B)
(C)
LR (μm)
60
80
100
120
0
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300
400
500
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700
AP (μm)
0
50
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DV (μm)
(D)
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40
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600
700
AP (μm)
100
150
200
250
300
DV (μm)

FIG. 1: (A) Left: illustration of a larval zebrafish. The blue box shows the imaging volume for fin cut assays, roughly 800μm in length along the anterior-posterior axis, and the orange dashed line indicates the location of the caudal fin transection. Right: a representative brightfield image showing the fin clip, with the original fin shape approximated by the dotted cyan curve. (B) A representative image around 1 hour post fin clip showing the caudal fin cut in brightfield, with fluorescence images of macrophages (magenta) and neutrophils (cyan) overlaid. All scale bars: 100μm. (C) Cumulative tracks of macrophages and (D) neutrophils over the 1–5 hours post-fin cut imaging window. Different colors represent individual cell tracks, illustrating the dynamic migration of both cell types toward the fin cut site, which is to the right of the figure. AP: anterior-posterior; DV: dorsal-ventral; LR: left-right.

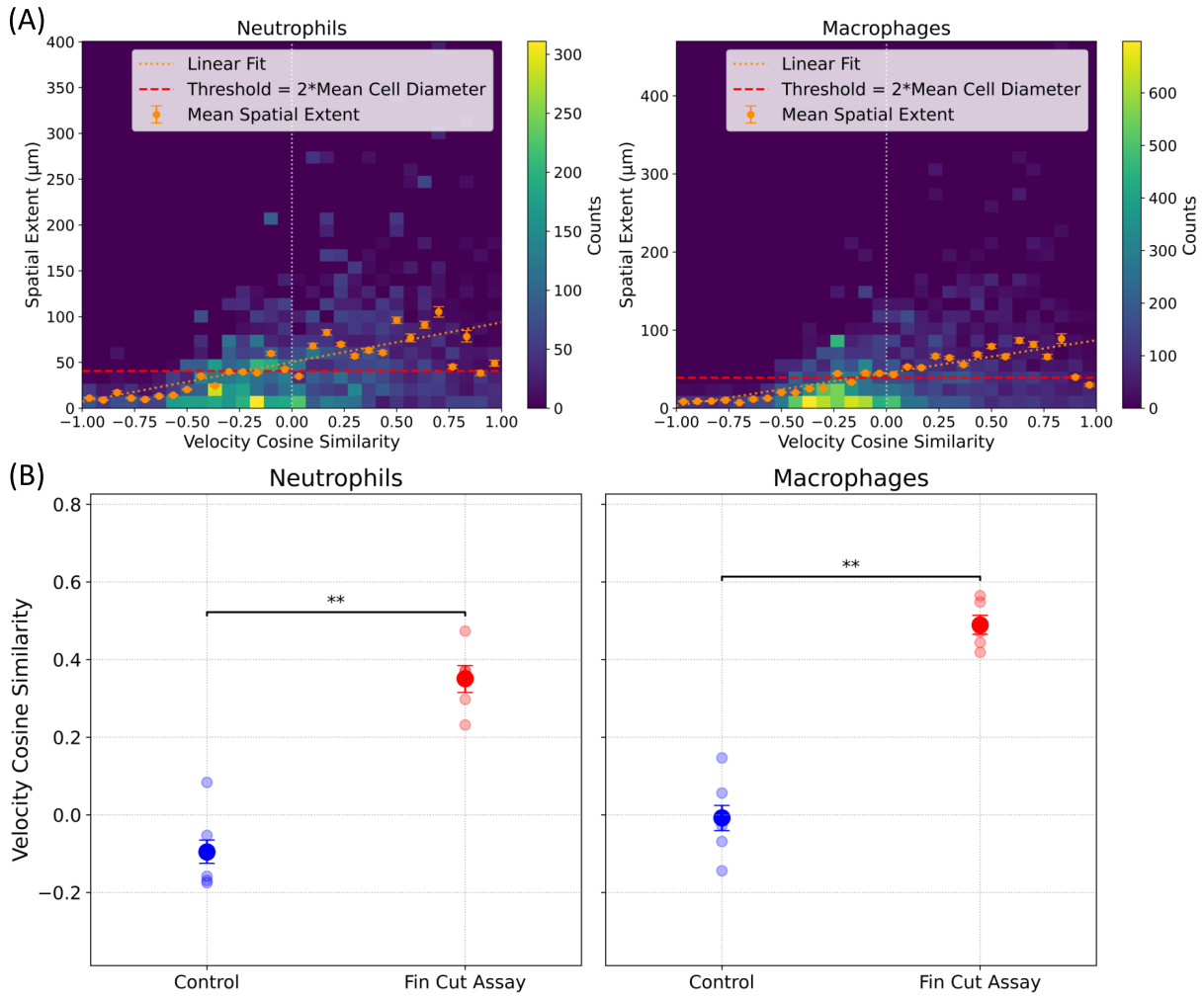

FIG. 2: 2D Histograms of Spatial extent vs mean velocity cosine similarity for neutrophils (left) and macrophages (right), from each subtrack of cells in the caudal fin area, pooling control and fin-cut fish. The horizontal red line shows the motility classification threshold of twice the mean cell diameter. Orange points are binned by velocity cosine similarity, with a linear regression plotted as an orange dashed line. (B) Velocity cosine similarity for motile subtracks in fin cut and control fish . Neutrophils (left) and macrophages (right) show significantly higher velocity cosine similarity for the fin cut assay compared to the control, indicating more consistent directional movement. $N = 6$ fish per group. $* : p < 0.05$, $** : p < 0.01$.

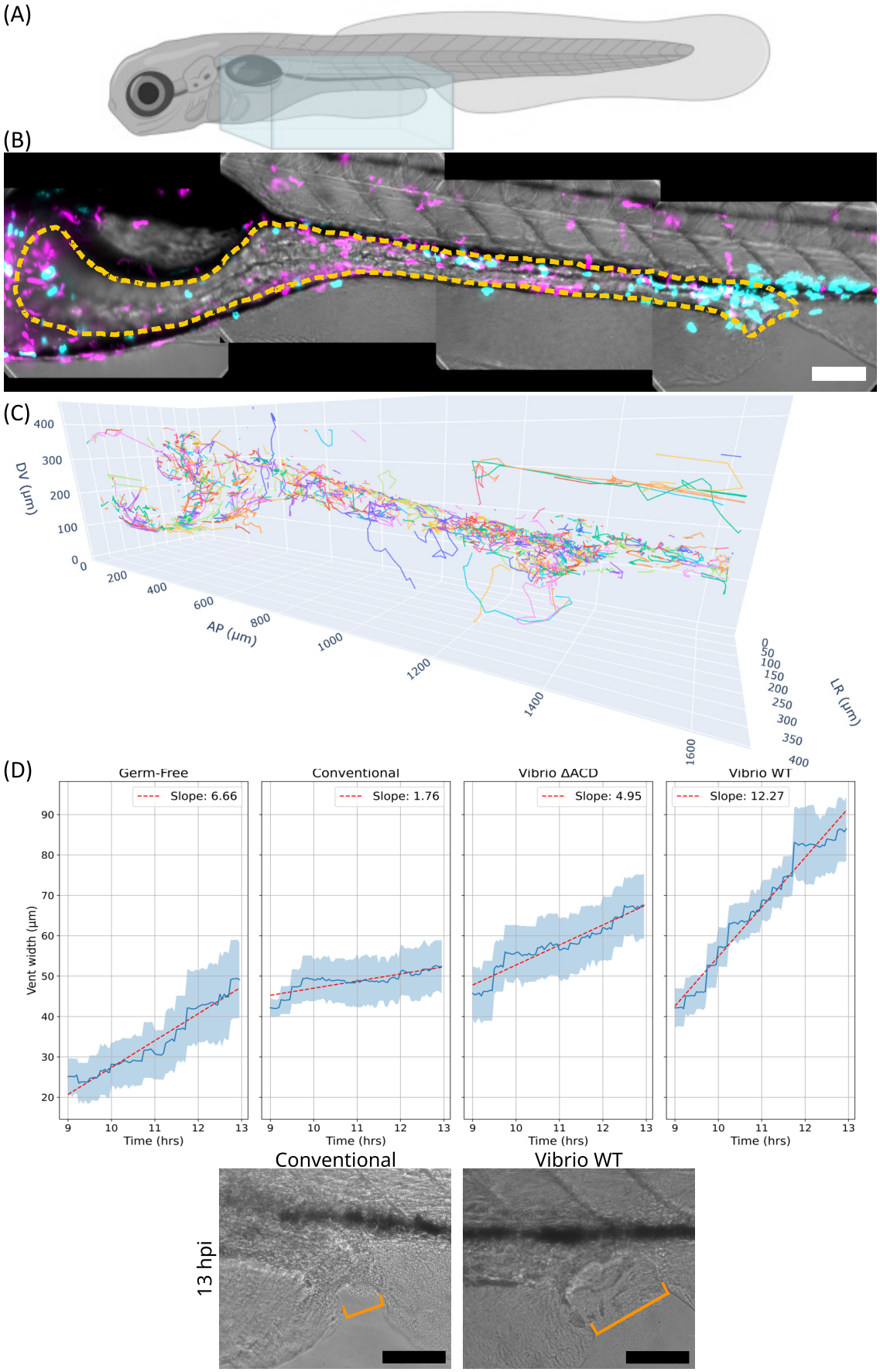
(A)
(B)
(C)
DV (µm)
AP (µm)
LR (µm)
(D)
Germ-Free
Conventional
Vibrio ΔACD
Vibrio WT
Slope: 6.66
Slope: 1.76
Slope: 4.95
Slope: 12.27
Vent width (µm)
Time (hrs)
Conventional
Vibrio WT
13 hpi

FIG. 3: (A) An illustration of a larval zebrafish showing the imaging volume spanning the gut region as a blue box. (B) Composite maximum intensity projection image at a single time point (11 hpi) showing brightfield (gray), macrophages (magenta), and neutrophils (cyan). The orange dashed line outlines the gut. (C) Cumulative tracks of neutrophils over the 9 to 13 hpi imaging window. Different colors represent individual cell tracks, illustrating the dynamic migration of neutrophils in the gut. (D) Upper: The width of the zebrafish posterior vent over the course of imaging for the different experimental groups: Germ-free, conventionally reared (Conventional), $Vibrio^{\Delta ACD}$-inoculated, and $Vibrio^{WT}$-inoculated fish. Increased vent width is an indicator of tissue damage. Lower: Brightfield images showing vent widths for conventionally raised and $Vibrio^{WT}$-inoculated fish at the end of the imaging window. Orange lines indicate the vent width. All scale bars: 100μm.

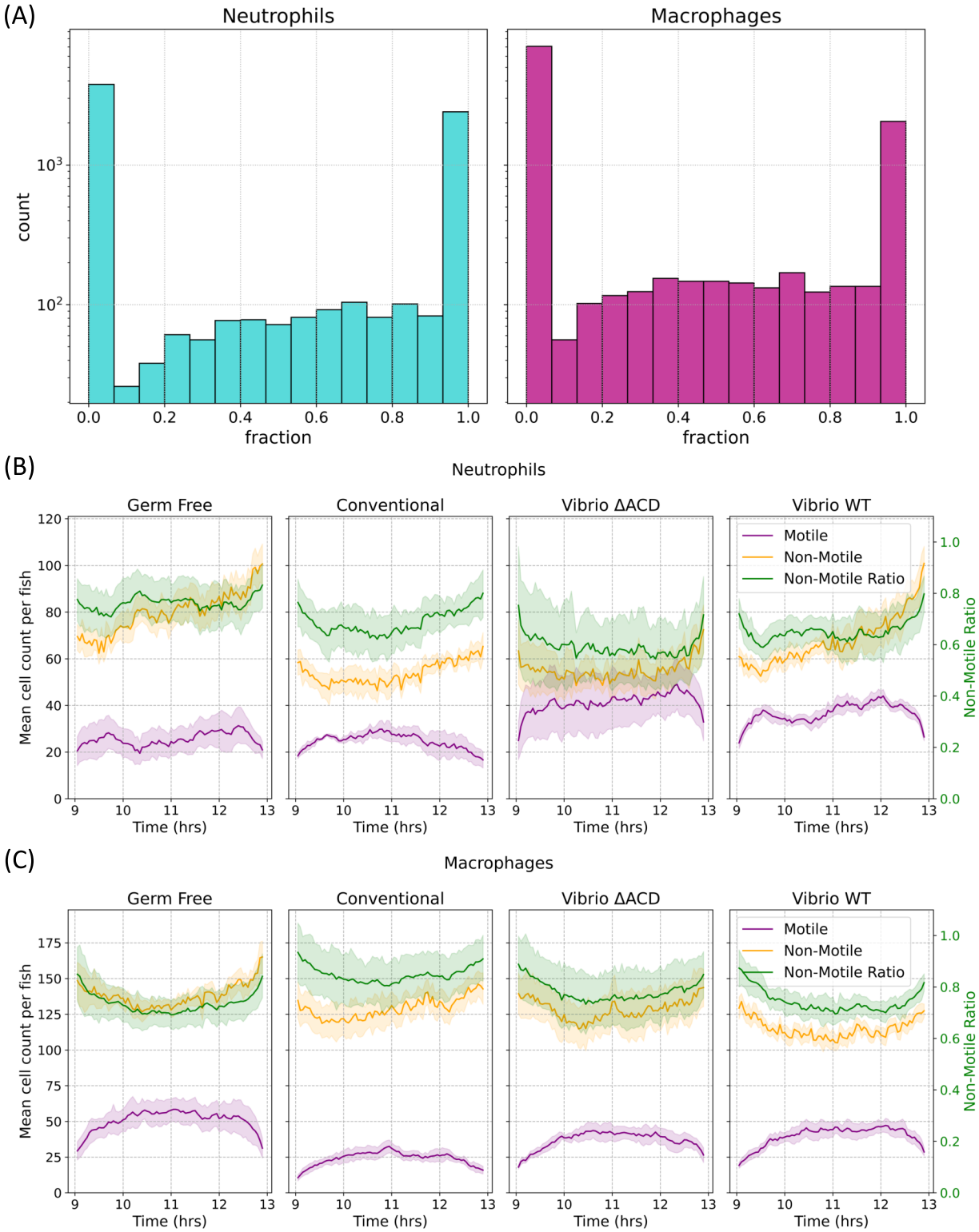
(A)
Neutrophils
Macrophages
count
fraction
(B)
Neutrophils
Germ Free
Conventional
Vibrio ΔACD
Vibrio WT
Motile
Non-Motile
Non-Motile Ratio
Mean cell count per fish
Time (hrs)
(C)
Macrophages

FIG. 4: (A) Histograms showing the fraction of each complete cell track exhibiting motile behavior for neutrophils (left) and macrophages (right), pooled across all experimental groups. The sharp peaks at 0 and 1 demonstrate that individual immune cells maintain predominantly motile or non-motile phenotypes throughout the 4-hour imaging period, with minimal intermediate behavior. Bin width $= 0.0\overline{6}$. (B, C) Counts of motile and non-motile subtracks, and the ratio of non-motile to total subtracks from 9 to 13 hpi for (B) neutrophils and (C) macrophages across experimental groups: Germ-free (GF), conventionally reared (Conventional), $\mathit{Vibrio}^{\Delta ACD}$-inoculated, and Vibrio^{WT}-inoculated fish. Motile cell numbers remain relatively constant across conditions, while non-motile neutrophil numbers increase over time in germ-free and Vibrio^{WT} groups. Error bars represent uncertainties from bootstrapping. $N = 5$–6 fish per group.

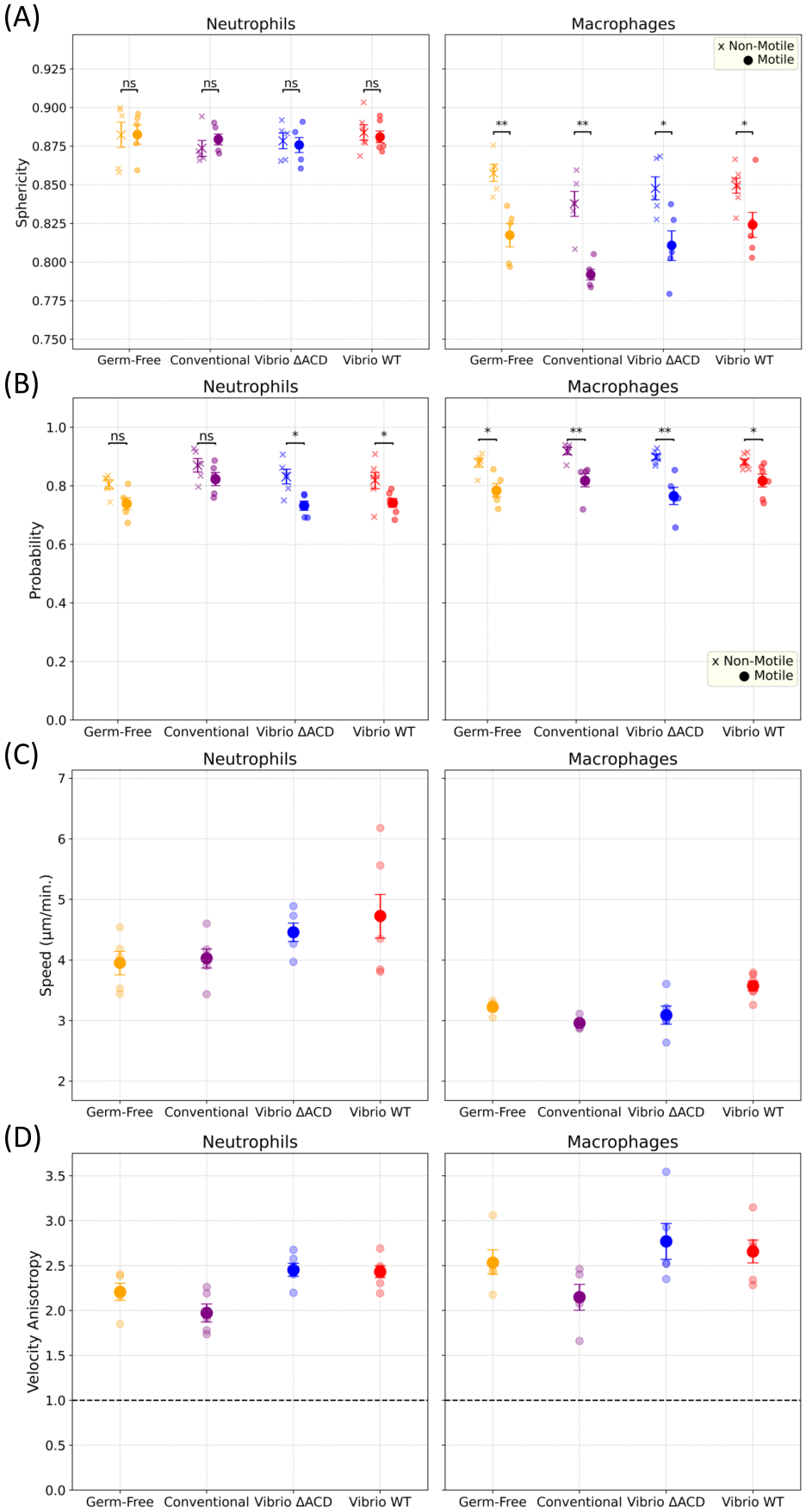
(A)
Neutrophils
Macrophages
Sphericity
0.925
0.900
0.875
0.850
0.825
0.800
0.775
0.750
ns
**
*
x Non-Motile
● Motile
Germ-Free
Conventional
Vibrio ΔACD
Vibrio WT
(B)
Probability
1.0
0.8
0.6
0.4
0.2
0.0
(C)
Speed (μm/min.)
7
6
5
4
3
2
(D)
Velocity Anisotropy
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

FIG. 5: (A) Sphericity comparison between motile and non-motile subtracks for neutrophils (left) and macrophages (right) across all experimental groups. Macrophages exhibit significantly higher sphericity during non-motile periods, indicating increased cellular rounding when stationary, while neutrophils show no morphological distinctions between motility states. (B) Gut residence probability, defined as the fraction of time a cell that is ever in the vicinity of the gut remains in the vicinity of the gut, for motile and non-motile subtracks, demonstrating that both populations spend the majority of their time within gut-associated regions across all experimental conditions. (C) The average speed of motile subtracks, calculated from frame-to-frame displacements, shows no significant differences between experimental groups for either cell type. (D) Velocity anisotropy quantifies preferential movement along the anterior-posterior axis relative to perpendicular directions. Values consistently exceed 1.0 across all conditions, indicating that immune cells migrate primarily along the intestinal length rather than in dorsal-ventral or left-right directions. Error bars represent uncertainties from bootstrapping. $* : p < 0.05$, $** : p < 0.01$, $*** : p < 0.001$. $N = 5$–6 fish per group.

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