

Characterizing Trajectories of Innate Immune Cells in Larval Zebrafish

Piyush Amitabh^{*} and Raghuvveer Parthasarathy[†]

Department of Physics, *University of Oregon*, Eugene, Oregon, USA



(Received 13 August 2025; accepted 9 February 2026; published 20 March 2026)

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 multihour 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 nonmotile 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 nonmotile 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 toward the damage site, provide 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.

DOI: [10.1103/wx8f-b1p9](https://doi.org/10.1103/wx8f-b1p9)

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, multilayered 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, leaving open questions about what cellular features might characterize distinct motility states and whether gut microbes influence surveillance behavior.

Imaging immune cells in live animals is technically challenging but has progressed considerably in recent years. Studies using intravital multiphoton 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 show that neutrophils perform both forward and reverse migration in response

^{*}Contact author: pamitabh@uoregon.edu

[†]Contact author: raghu@uoregon.edu

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 examinations of innate immune cells in volumes large enough to span the entire larval zebrafish gut and over durations long enough to characterize motion types. We therefore use 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 timescales 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 a 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 toward 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 nonmotile over roughly hour-long durations, maintain 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 nonmotile periods, providing a morphological indicator of motility state that reflects the greater pseudopodial extension of migrating macrophages.

II. RESULTS

A. Validation of three-dimensional immune cell tracking using the caudal fin cut wound model

We perform transverse amputations of the caudal fin in 5 days postfertilization (dpf) zebrafish larvae, cutting midway between the posterior end of the notochord and the distal tip of the fin [Fig. 1(a)]. Using the dual transgenic line Tg(*mpx*:GFP; *mpeg1*:mCherry), we simultaneously image neutrophils (cells expressing the myeloperoxidase, *mpx*) and macrophages (cells expressing macrophage-expressed gene-1, *mpeg1*) during the

wound response [28,29] (Fig. 1(b) and Supplemental Videos 1 and 2 [38]). Time-lapse imaging is performed on a custom-built light sheet fluorescence microscope from 1 to 5 h postamputation, capturing a tissue volume of approximately $4.8 \times 10^7 \mu\text{m}^3$ (400 μm along the dorsal-ventral, 800 μm along the anterior-posterior, and 150 μm along the left-right axes), with images acquired every 3 min.

Following image preprocessing and drift correction, individual immune cells are segmented and tracked across time points to generate single cell trajectories (see Sec. IV). Simple cell counts showed a greater number of both cell types in the field of view of the fin cut assay than the control (Supplemental Fig. 1(a) [38]). The resulting trajectory data clearly demonstrate directed migration of both neutrophils and macrophages toward the amputation site (Fig. 1(c), D; Supplemental 3D Figs. 1–4 [38]), consistent with the known recruitment of immune cells to sites of tissue damage [22–26]. All cell trajectories are provided as data in the Supplemental Material [38].

B. Establishment of motion classification criteria

To extract meaningful physical parameters from cellular trajectories, we develop 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 [39]. To capture this temporal heterogeneity, we subdivide each complete trajectory into “subtracks” based on frame-to-frame displacement. Specifically, a new subtrack is initiated whenever the displacement transitions above or below a threshold of half the mean cell diameter per 3 min interval. We compute the spatial extent of each subtrack as the diameter of the smallest circumscribing sphere. We calculate 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 [Fig. 2(a)], indicating that more spatially extended tracks are also more directionally persistent.

To compare this correlation with what would arise from undirected random motion with the same track features and sampling as in the data, we generate simulated tracks by randomizing step directions while preserving each track’s step-size distribution and track length, with detector noise matching experimental imaging conditions (see Sec. IV). Simulated tracks show no such correlation, with velocity cosine similarity clustered near zero regardless of spatial extent (Supplemental Fig. 11(a) [38]), and both velocity cosine similarity and velocity anisotropy being significantly lower than experimental values (Supplemental Figs. 11(b) and 11(c) [38]). These results confirm that the observed directional signatures reflect intrinsic directional bias rather than kinematic artifacts. To classify subtracks as motile or nonmotile, 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

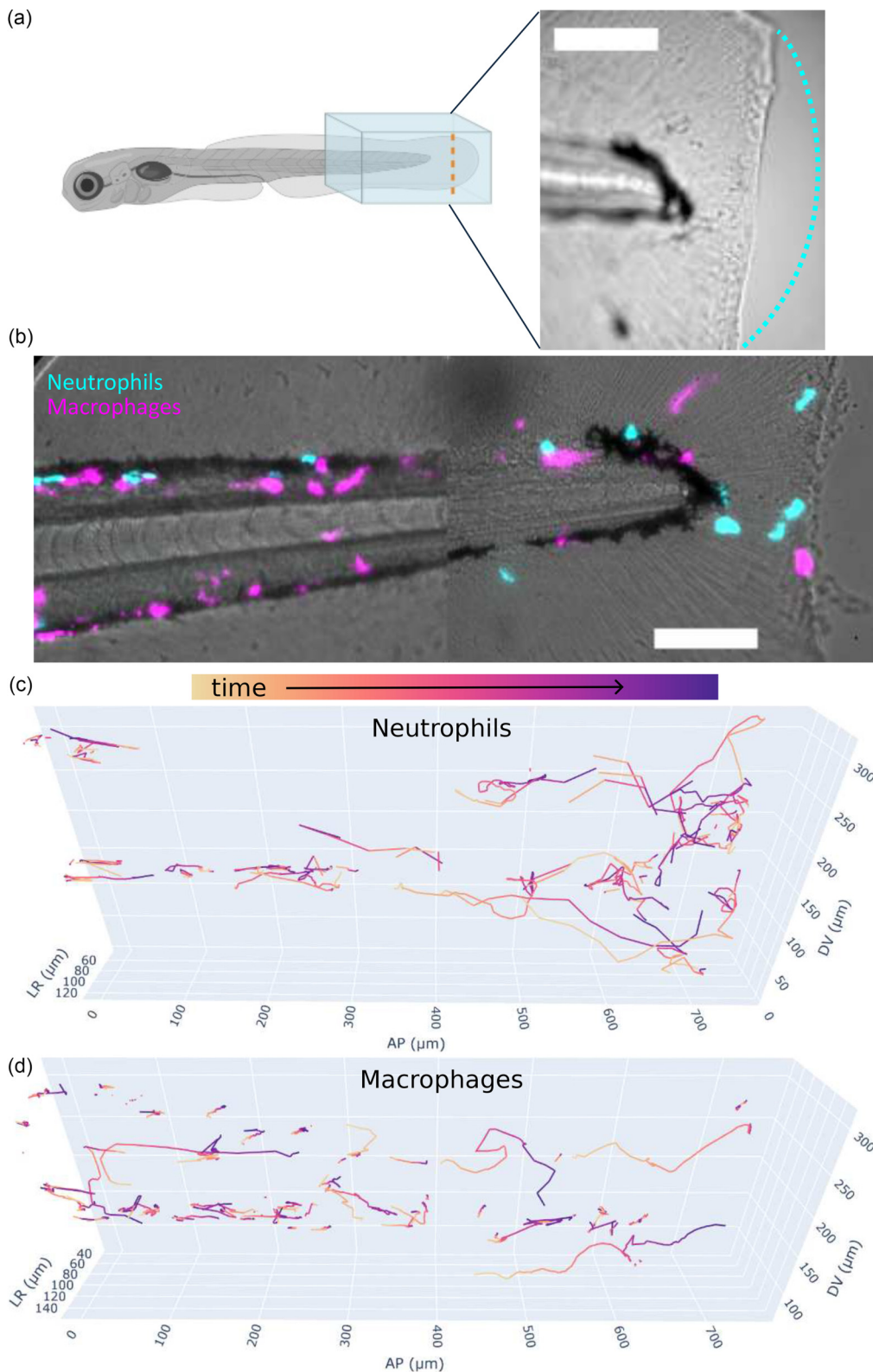


FIG. 1. (a) Left: illustration of a larval zebrafish. Blue box indicates the imaging volume ($\approx 800 \mu\text{m}$ along the anterior-posterior axis), with its posterior boundary aligned to the caudal fin tip; dashed orange line indicates the location of the caudal fin transection. Right: representative bright-field image showing the fin clip, with the original fin shape approximated by the dotted cyan curve. (b) Representative composite image at 1 h post fin cut showing bright field, macrophages (magenta), and neutrophils (cyan). [(c), (d)] Cumulative tracks of (c) neutrophils and (d) macrophages over 1–5 h post fin cut from a representative specimen. Colors represent normalized time from track initiation (yellow) to termination (purple). Both cell types show migration toward the fin cut site (right). See Supplemental Material Figs. 1–4 for interactive three-dimensional (3D) trajectories [38]. AP, anterior-posterior; DV, dorsal-ventral; LR, left-right. All scale bars: $100 \mu\text{m}$.

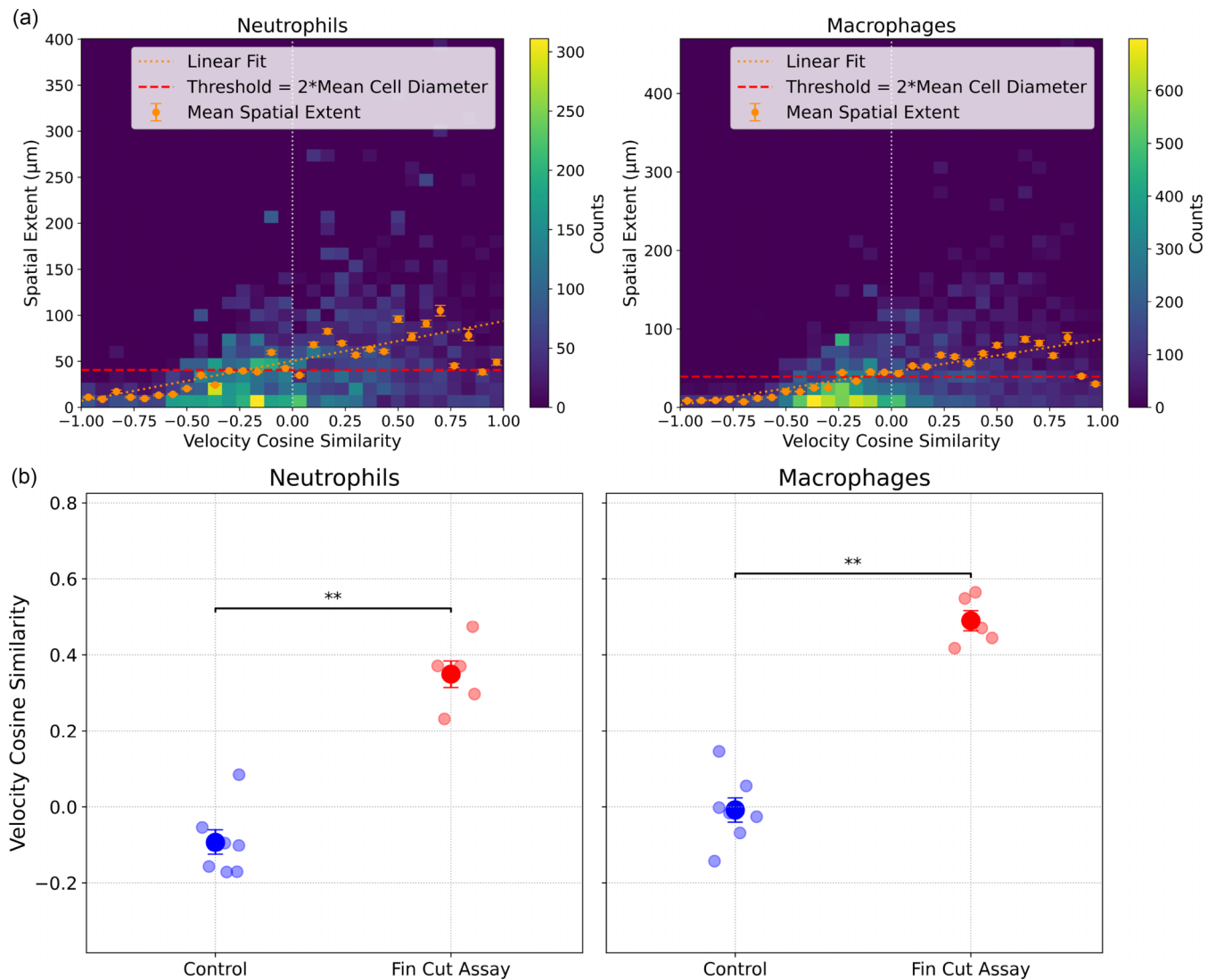


FIG. 2. Characterization of immune cell trajectory properties in the caudal fin region for neutrophils (left) and macrophages (right). (a) Two-dimensional (2D) histograms of spatial extent versus mean velocity cosine similarity for subtracks in the caudal fin region, pooling control and fin-cut fish. The horizontal red line shows the motility classification threshold of twice the mean cell diameter. Orange points show mean spatial extent binned by velocity cosine similarity; the dashed orange line is a linear regression fit to all data points (see main text). (b) Velocity cosine similarity for motile subtracks in fin-cut and control fish. Both cell types show significantly higher velocity cosine similarity for the fin-cut assay compared to the control, indicating more consistent directional movement. $n_{\text{control}} = 5$, $n_{\text{fin-cut assay}} = 7$; total $N = 12$. * $p < 0.05$, ** $p < 0.01$.

velocity cosine similarity; the spatial extent corresponding to zero velocity cosine similarity is $47.2 \mu\text{m}$ ($R^2 = 0.109$, $n = 13\,882$ data points) for neutrophils and $42.6 \mu\text{m}$ ($R^2 = 0.115$, $n = 23\,765$) for macrophages, close to twice the mean cell diameter of 20.4 and $19.5 \mu\text{m}$ for neutrophils and macrophages, respectively [Fig. 2(a)]. Since zero velocity cosine similarity corresponds to random or repetitive motion, including physiological noise from blood flow and small displacements from cell shape changes, this agreement implies that our motility threshold effectively separates persistent migration from nondirectional motion.

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

exhibit significantly higher velocity cosine similarity compared to motile subtracks from the uninjured control group [Fig. 2(b)], confirming that our analysis pipeline successfully identifies the expected increase in directional migration of both neutrophils and macrophages following tissue damage. In control fish, motile subtracks show velocity cosine similarity near zero, indicating largely random motion; small deviations from zero reflect residual noise not fully eliminated by the analysis pipeline. In injured fish, the innate immune cells show persistent motion, visually apparent as directed toward 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 assess germ-free fish, conventionally reared fish with normal parental tank microbiota, and initially germ-free fish inoculated at 5 or 6 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 14–20 hours postinoculation (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 ACD of the T6SS (denoted *Vibrio*^{ΔACD}) [37]. In prior work [37], macrophages' positions were assessed at 20 min intervals and neutrophils were not tracked.

In the studies described here, we begin imaging at 9 hpi and track neutrophil and macrophage cells at 3 min intervals through 13 hpi. This time window is chosen to investigate and capture immune cell behavior prior to the major macrophage redistribution to the vent previously observed at 14–20 hpi [37]. Gut dissection and plating at the end of the imaging period (13 hpi) confirms successful *Vibrio*^{WT} colonization at 10³ to 10⁴ colony-forming units (CFU) per gut (see Sec. IV). Imaging of *Vibrio*^{WT}-dTomato fluorescence at representative timepoints (10, 11, and 13 hpi) shows bacterial localization primarily at the gut bulb and the vent (see Sec. IV; see Supplemental Fig. 2 and Supplemental Video 3 [38]). Germ-free, conventionally reared, or *Vibrio*-inoculated zebrafish are imaged with light sheet fluorescence microscopy at 3 min intervals at 5 or 6 dpf from 9 to 13 hpi for inoculated fish or the same age for other noninoculated fish. The imaging volume covers the entire gut: approximately 1600 μm along the anterior-posterior (AP) axis, 100 μm along the left-right (LR) axis, and 400 μm along the dorsal-ventral (DV) axis [Fig. 3(a)]. Using the dual transgenic line Tg(*mpx*:GFP; *mpeg1*:mCherry), we image and track individual neutrophils and macrophages throughout the 4 h observation period (Figs. 3(b) and 3(c) and Supplemental Videos 4 and 5 [38]). All cell trajectories are provided as Supplemental Material Data [38]. The mean track lengths are 24 and 32 frames (69 and 93 min) for neutrophils and macrophages, respectively, pooled across all groups. These cells show a similar relationship between spatial extent and velocity cosine similarity as immune cells in the fin-cut assay (Supplemental Fig. 3 [38]). As with the fin-cut data, simulated random walks show no such correlation, with velocity cosine similarity clustering around zero, while experimental data show significantly higher directional persistence across nearly all conditions (Supplemental Figs. 12(a) and 12(b) [38]). Vent width measurements confirm 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 (Fig. 3(d), Supplemental Fig. 4 [38]).

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

Using our framework for classifying the motility of subtracks, we find that both neutrophils and macrophages show a tendency to remain either motile or nonmotile for extended periods of time comparable to the mean track duration of over 1 hour rather than showing frequent transitions between motility states. Histograms of the motile subtrack fraction per track, grouped across all bacterial conditions, reveal bimodal distributions with peaks at 0 and 1 for both neutrophils and macrophages [Fig. 4(a); bin width 0.06]. This bimodal pattern contrasts with the uniform distribution expected if there were frequent switching between motility states. While a subset of cells exhibits intermediate fractions of motile behavior, the observed distribution indicates that innate immune cells preferentially adopt and maintain a consistent motile or nonmotile phenotype over the 4 hour observation period. Analysis includes only tracks with minimum track lengths of 5 frames to ensure robust motility classification. This distribution pattern is consistent across all four experimental groups and in the fin-cut assay (Supplemental Figs. 5 and 6 [38]).

To more finely assess motility behaviors and possible dependence on microbial stimulation, we examine the number and fraction of motile and nonmotile cells (subtracks) at each time point from 9 to 13 hpi. The numbers of motile neutrophils and macrophages remain fairly constant and similar across all four conditions (germ-free, conventional, *Vibrio*^{WT}, and *Vibrio*^{ΔACD}) [Figs. 4(b) and 4(c)]. Nonmotile neutrophils increase in number over time in *Vibrio*^{WT}-inoculated fish [Fig. 4(b)], which is mainly driven by the increase in the number of nonmotile neutrophils near the vent region (Supplemental Fig. 7 [38]). Macrophages show no significant temporal changes in nonmotile subtrack numbers across any group [Fig. 4(c)]. We note that for some experimental conditions there is an initial increase and a late decrease in motile cells [Figs. 4(b) and 4(c)]. This is a likely consequence of the requirement of a minimum track length of 5 frames (15 min), as motile cells have an appreciable likelihood to enter or leave the field of view during the peripheral 15 min, and so are not counted.

E. Macrophage morphology correlates with motility state

To investigate whether immune cell motility correlates with detectable morphological changes, we quantify 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 elongation or any other shape distortions reduce this value.

Neutrophils exhibit no significant differences in sphericity between motile and nonmotile states across all experimental groups [Fig. 5(a)]. In striking contrast, macrophages demonstrate significantly higher sphericity during nonmotile periods compared to motile periods, indicating increased cellular rounding when stationary. Representative microscopy images reveal that motile macrophages display prominent pseudopodial protrusions, whereas nonmotile macrophages adopt a more rounded morphology (Fig. 5(b) and Supplemental Videos 6–9 [38]). This morphological distinction suggests that

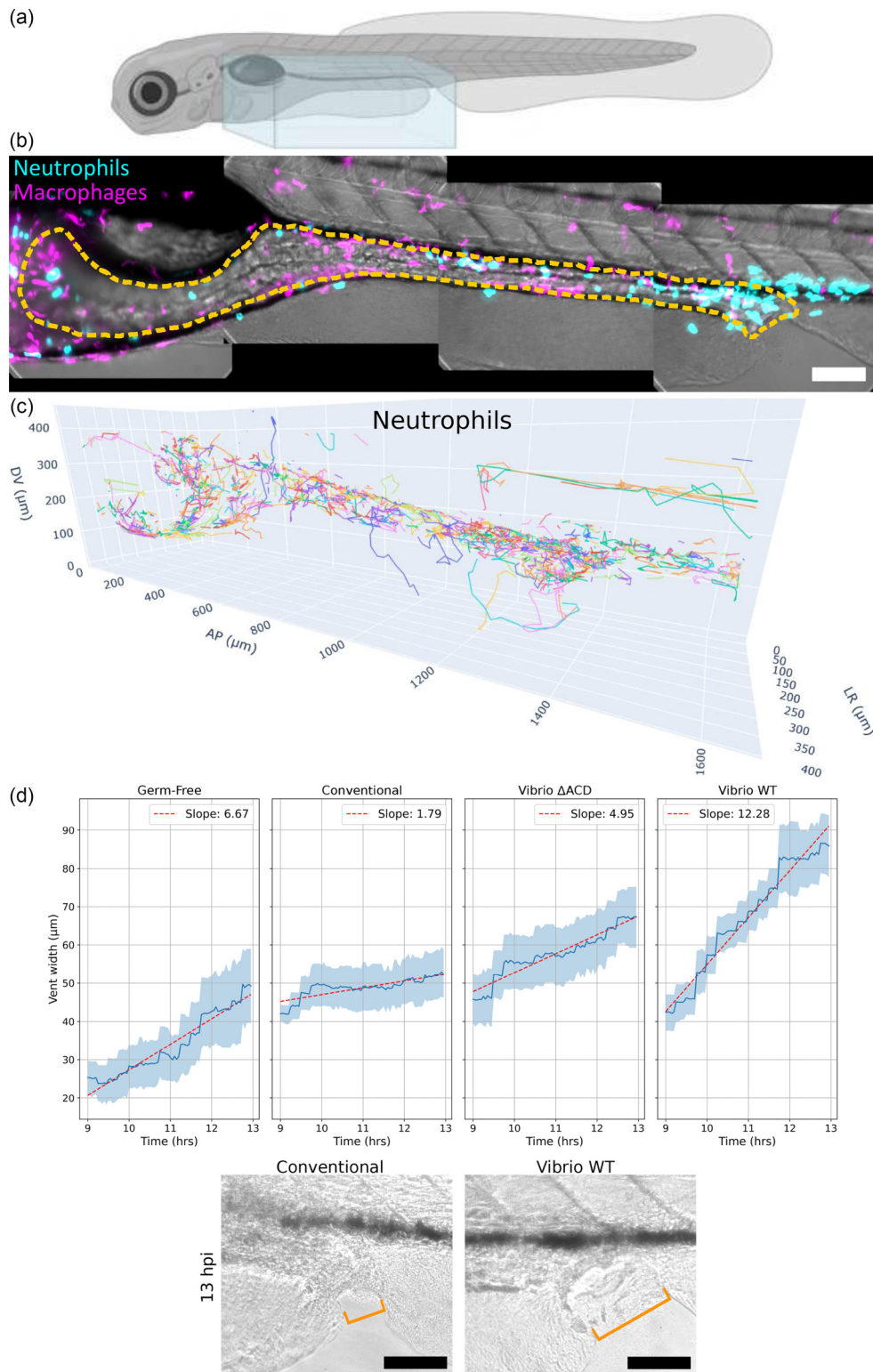


FIG. 3. (a) An illustration of a larval zebrafish showing the imaging volume spanning the gut region as a blue box. (b) Composite maximum intensity projection image at a single time point (11 hpi, midpoint of imaging window) showing bright field (gray), macrophages (magenta), and neutrophils (cyan). The dashed orange 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 movement of neutrophils. (d) Top: Zebrafish vent width over the course of imaging for the different experimental groups: Germ-free, conventionally reared (Conventional), *Vibrio* Δ ACD-inoculated, and *Vibrio* ^{WT} -inoculated fish. Increased vent width is an indicator of tissue damage. $n_{\text{germ-free}} = 5$, $n_{\text{conventional}} = 5$, $n_{\text{Vibrio}\Delta\text{ACD}} = 5$, $n_{\text{Vibrio}^{WT}} = 6$; total $N = 21$. Bottom: Bright-field 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: 100 μm .

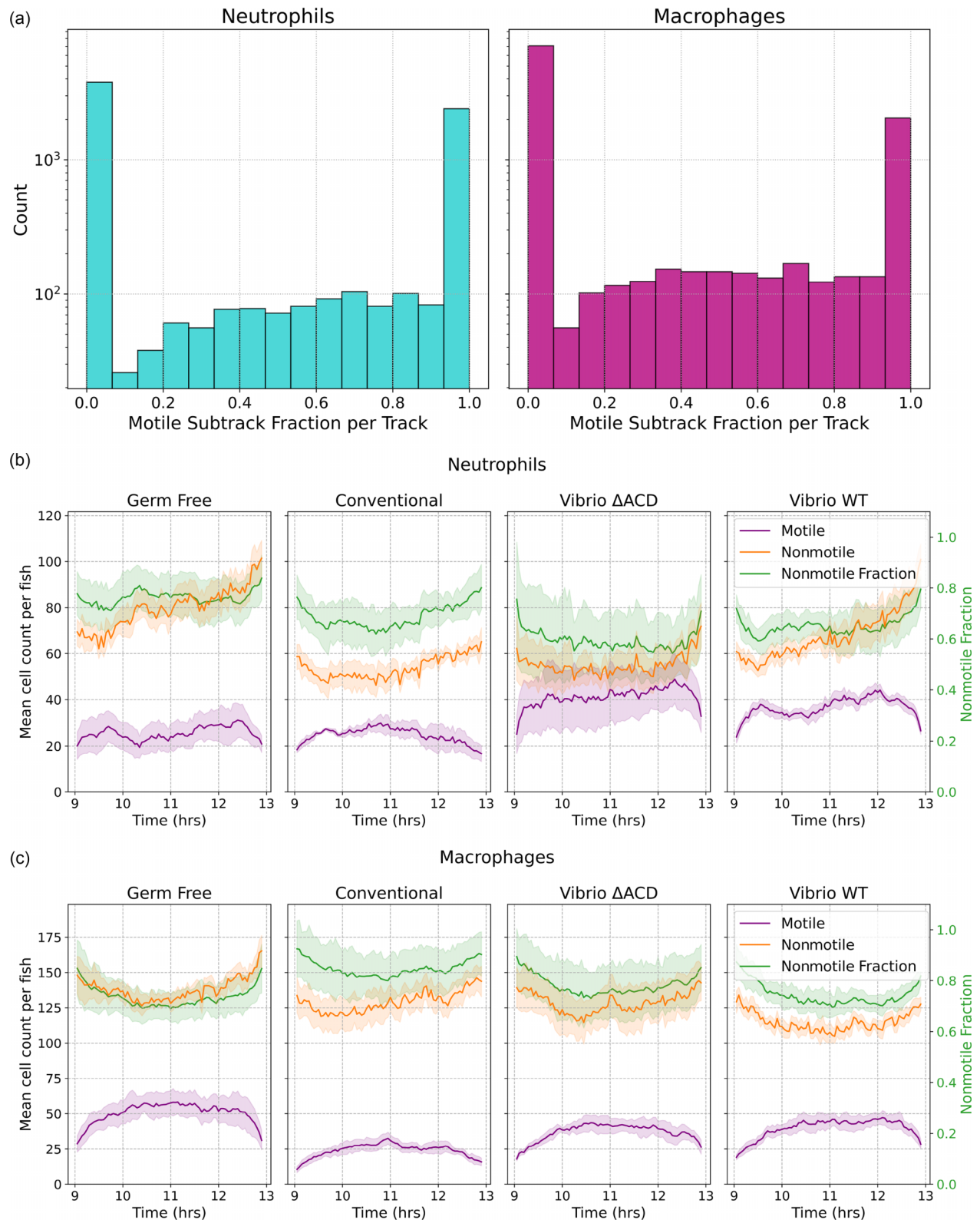


FIG. 4. (a) Histograms showing the motile subtrack fraction per track for neutrophils (left) and macrophages (right), pooled across all experimental groups. The distribution shows peaks at 0 and 1, indicating that immune cells generally adopt a consistent motile or nonmotile phenotype over the 4 hour observation period, though a subset of cells exhibits intermediate behavior. Bin width 0.06. [(b), (c)] Counts of motile and nonmotile subtracks, and the ratio of nonmotile to total subtracks from 9 to 13 hpi for (b) neutrophils and (c) macrophages across experimental groups: Germ-free, conventionally reared (Conventional), *Vibrio*^{ΔACD}-inoculated, and *Vibrio*^{WT}-inoculated fish. Motile cell numbers remain relatively constant across conditions. Error bars represent uncertainties from bootstrapping. $n_{\text{germ-free}} = 5$, $n_{\text{conventional}} = 5$, $n_{\text{Vibrio}\Delta\text{ACD}} = 5$, $n_{\text{VibrioWT}} = 6$; total $N = 21$.

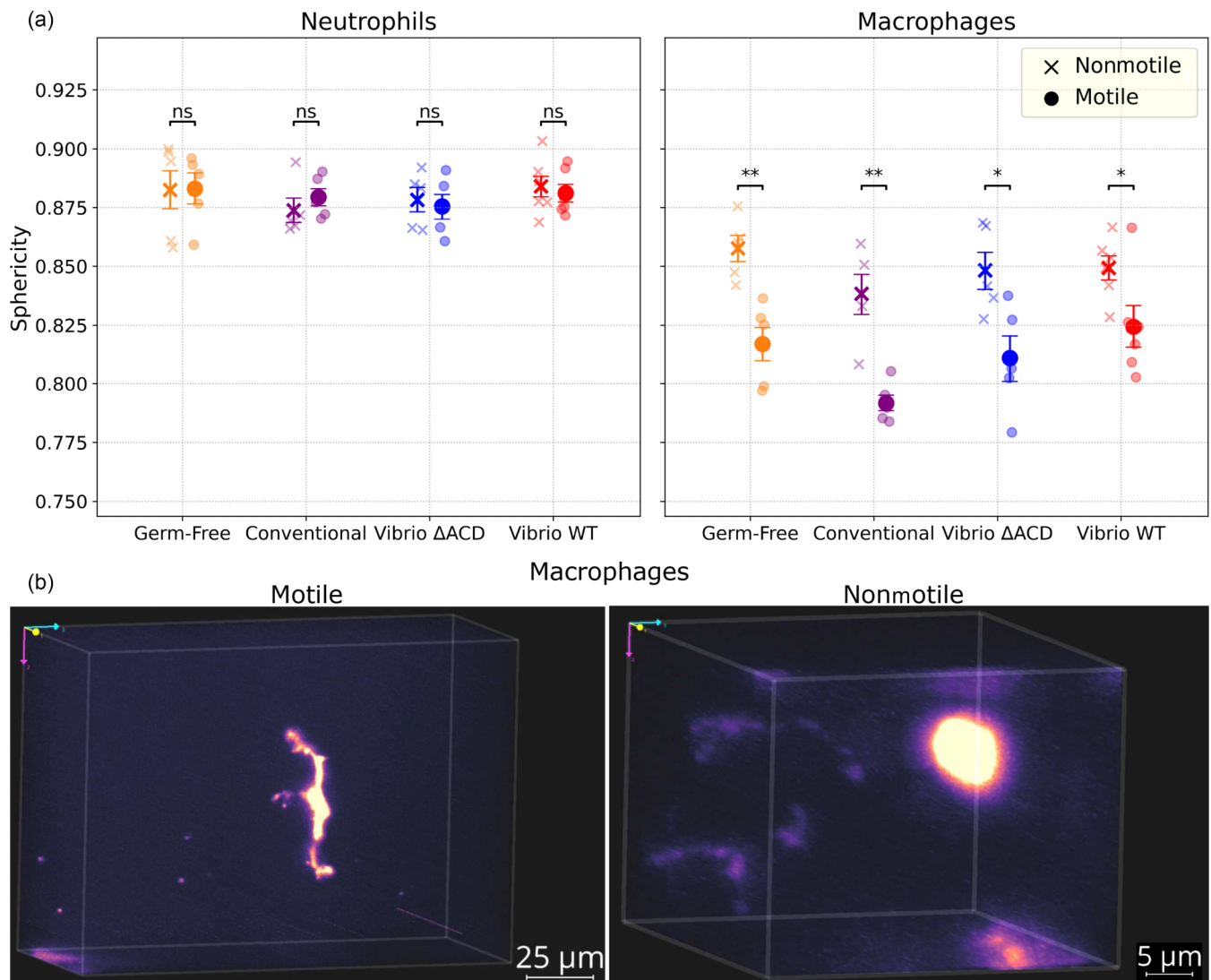


FIG. 5. Macrophages exhibit reduced sphericity associated with pseudopodial extensions during motile phases. (a) Sphericity comparison between motile and nonmotile subtracks for neutrophils (left) and macrophages (right) across all experimental groups. Macrophages exhibit significantly lower sphericity during motile periods compared to nonmotile periods, while neutrophils show no significant difference between motility states. (b) Representative images of macrophages during motile (left) and nonmotile (right) phases. Motile macrophages display prominent pseudopodial protrusions, whereas nonmotile macrophages adopt a more rounded morphology (Supplemental Videos 6–9 [38]). Error bars represent uncertainties from bootstrapping. $n_{\text{germ-free}} = 5$, $n_{\text{conventional}} = 5$, $n_{\text{Vibrio}\Delta\text{ACD}} = 5$, $n_{\text{VibrioWT}} = 6$; total $N = 21$. ns, not significant, $*p < 0.05$, $**p < 0.01$.

motile macrophages extend pseudopodia more extensively than their nonmotile 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 Fig. 1(b) [38]). We examine numerous additional cellular parameters, including volume and normalized anterior-posterior position, but sphericity is the only metric that distinguishes motile from nonmotile behavior across all macrophage groups (Supplemental Figs. 8(a), 8(b), and 1(c) [38]).

F. Gut architecture constrains immune cell migration patterns

We manually segment images to identify gut-associated regions, including the intestinal lumen and surrounding tissue

(Fig. 3(b) and Supplemental Video 3 [38]). 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 consider subtracks with a duration of at least 5 frames and examine the fraction of time spent in the gut volume. Nonmotile subtracks spend more than 80% of their time within the gut, considering averages across all experimental groups [Fig. 6(a)]. Surprisingly, motile subtracks also demonstrate 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

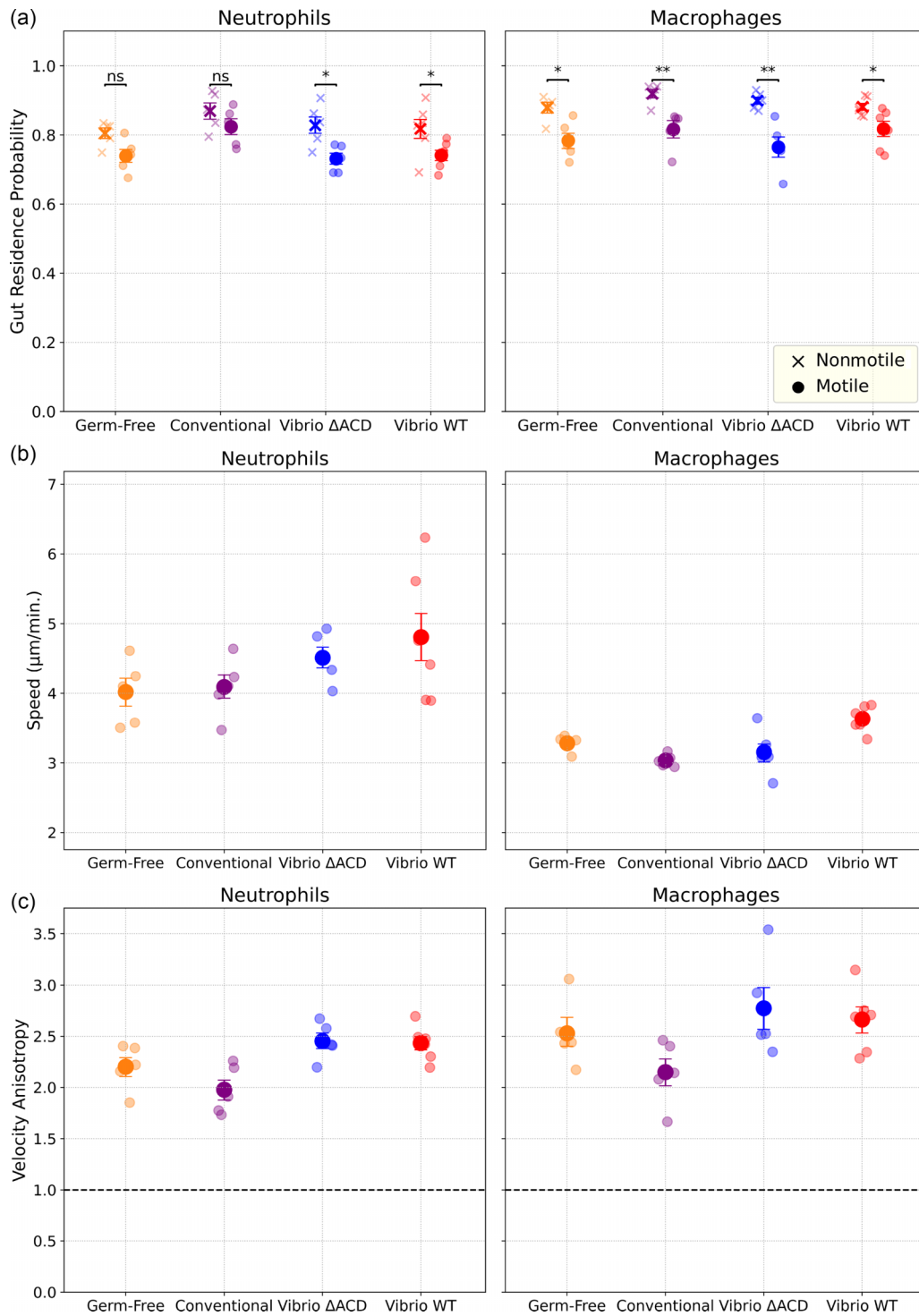


FIG. 6. Characterizations of immune cell residence and migration dynamics in gut-associated regions for neutrophils (left) and macrophages (right). (a) Gut residence probability, defined as the fraction of time a cell that is ever in the vicinity of the gut remains in the vicinity of the gut, for motile and nonmotile subtracks, demonstrating that both populations spend the majority of their time within gut-associated regions across all experimental conditions. (b) Average speed of motile subtracks, calculated from frame-to-frame displacements, shows no significant differences between experimental groups for either cell type. (c) Velocity anisotropy quantifies preferential movement along the anterior-posterior axis relative to perpendicular directions. Values consistently exceed 1.0 across all conditions, indicating migration primarily along the intestine rather than in dorsal-ventral or left-right directions. Error bars represent uncertainties from bootstrapping. $n_{\text{germ-free}} = 5$, $n_{\text{conventional}} = 5$, $n_{\text{Vibrio}\Delta\text{ACD}} = 5$, $n_{\text{VibrioWT}} = 6$; total $N = 21$. ns, not significant, * $p < 0.05$, ** $p < 0.01$.

gut-associated immune cells migrate predominantly along the boundary of the gut.

Analysis of motile subtrack characteristics reveals that most kinetic parameters, including mean speed and velocity components, show no significant differences between experimental groups (Fig. 6(b) and Supplemental Fig. 9 [38]). We also provide these parameters for the fin-cut assay (Supplemental Fig. 10 [38]). However, we find that immune cell migration shows a strong directional bias. We calculate all cells' velocity anisotropy, given by

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

where V_{AP} , V_{DV} , and V_{LR} represent velocity components along the anterior-posterior, dorsal-ventral, and left-right axes, respectively. Velocity anisotropy consistently exceeds 1.0, the value for isotropic motion, across all experimental conditions [Fig. 6(c)], and is significantly higher than the anisotropy of simulated random walks drawn from the experimental distribution of step sizes (see Sec. IV; Supplemental Fig. 12(c) [38]), indicating preferential movement along the anterior-posterior axis compared to dorsal-ventral or left-right directions.

Combined 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 [40–43], 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 among 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.

The remarkable consistency of immune cell behavior across microbial conditions—germ-free, conventional, *Vibrio*^{WT}, and *Vibrio*^{ΔACD}—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 nonmotile neutrophils near *Vibrio*-induced tissue damage deviate 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.

On the timescale of a few hours, we find stable motility states (either motile or nonmotile) for both neutrophils and macrophages, rather than frequent transitions in behavior. While our study makes use of live imaging over several hours, it is interesting to ask whether motility states can be gleaned from single snapshots, potentially even from nonliving animals or histological samples. We find no notable morphological differences between motile and nonmotile neutrophils. Macrophages, in contrast, are much less spherical when motile. This feature may allow the inference of macrophage dynamic behavior from static snapshots alone. Due to the lack of cell membrane and actin markers, we cannot definitively classify the cellular protrusions observed in the motile and less spherical subset of macrophages, though visual inspection suggests these protrusions are consistent with the general form of pseudopodia (see Supplemental Videos 6–9 [38]). Cellular protrusions can be classified into several types based on their morphology and cytoskeletal architecture, like lamellipodia (broad, sheetlike projections), filopodia (thin, fingerlike extensions), and blebs (pressure-driven spherical extensions) [44]. Characterizing the protrusion types present in migrating gut macrophages, along with their mechanical characteristics and modes of migration (amoeboid versus mesenchymal), represents an interesting avenue for future investigation.

A key open question concerns the mechanistic basis of immune cell guidance *in vivo*. Neutrophils and macrophages are well known to migrate along chemokine gradients toward sites of infection or tissue damage [5], yet the striking anatomical constraint we observe, cells remaining in the vicinity of the gut and migrating along its axis, suggests that physical cues also play a role. The gut is a tubular structure, presenting curved surfaces that could engage curvotaxis, whereby cells preferentially migrate along regions of negative curvature through nucleus-mediated mechanosensing [45,46]. Cell migration can also be directed by durotaxis (stiffness gradients) and haptotaxis (substrate-bound signals) [47,48]. The extracellular matrix (ECM) in zebrafish undergoes substantial remodeling during development, with region-specific changes in composition and mechanical properties [49,50]. Given that different anatomical regions exhibit distinct ECM characteristics at different developmental stages, the relative contributions of chemotaxis, curvotaxis, and durotaxis to immune cell navigation likely vary across tissue contexts. Future work employing membrane and cytoskeletal markers, combined with ECM characterization, would help dissect these contributions.

Our study focuses on neutrophils and macrophages in larval zebrafish at 5–6 days postfertilization, a stage when innate immunity predominates and adaptive immune cells have not yet matured [51]. Notably, heterogeneous motility phenotypes within immune cell populations have been

observed in other contexts. In lymph nodes, T-cell populations contain both highly motile naive cells undergoing random walks and arrested cells forming stable conjugates with dendritic cells during antigen recognition, with cells maintaining their respective motility states for periods of 8–12 h [13,14,52]. Similarly, heterogeneity in migration modes—mesenchymal versus amoeboid—has been described for neutrophils and macrophages in zebrafish tissues [19]. Whether the stable motile and nonmotile phenotypes we observe in gut-associated immune cells correspond to distinct functional subtypes, analogous to the naive versus antigen-engaged distinction in T cells, or represent different stages of a behavioral program remains an open question. More generally, motility patterns across the range of immune cell types remain underexplored. The immune systems of zebrafish and mammals are highly conserved but differ in key aspects, including, in zebrafish, the absence of lymph nodes and reliance on the kidney as the primary hematopoietic organ [53,54]. Extending our framework to mammals with more complex organ structure and tissue compartmentalization, to later developmental stages when adaptive immunity is established, and to other immune cell types would be important next steps and would reveal whether the behaviors uncovered in innate immune cells in larval zebrafish are reflected in other organisms and other stages of development. The ability to track hundreds of cells in three dimensions across multihour timescales, combined with emerging single-cell transcriptomic atlases [55], could ultimately link motility phenotypes to transcriptional states.

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. The quantitative framework established here and the open-source computational pipeline we provide should allow translation to other model systems and cell types, identifying conserved versus context-specific features of immune surveillance mechanisms during development and disease.

IV. MATERIALS AND METHODS

A. Zebrafish husbandry and transgenic lines

All experiments with zebrafish follow standard procedures [56] and were performed in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee (protocol nos. AUP-22-02 and AUP-20-16). Fish from which the larvae used in these studies are 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 Ref. [28]. The transgenic line Tg(*tnfr*:GFP);Tg(*mpeg1*:mCherry), allele number pd1028Tg, is described in Ref. [57].

B. Gnotobiotic derivation and bacterial colonization

Embryos are derived germ-free following established protocols [58]. Fertilized eggs are incubated for 6 h 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 are 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 are grown overnight in Luria Broth at 30 °C with agitation. Then, bacterial cultures are pelleted through centrifugation for 3 min at 7000g, washed once in sterile EM, and inoculated at 10⁶ CFU/mL directly into flask water. Larvae are 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*^{ΔACD}) is constructed using markerless allelic exchange with the pAX2 vector as previously described [37,59]. In brief, splice-by-overlap extension generates deletion cassettes with the following primers: 5' HR: 5'-CCCAATGATAGCCACGGTTG-3' + 5'-CCATTCCATTTTCCACTAGGCTAAAGGACACACCTT-3'; 3' HR: 5'-AAGGTGTGTCTTTAGCCTAGTGGAAAATGGAATGG-3' + 5'-GGCGCAAGATTTTCAATCA-3'. After conjugation into *Vibrio* using *Escherichia coli* SM10, merodiploids are selected on TSA-gentamicin at 37 °C. Deletion is confirmed by polymerase chain reaction (PCR) using flanking primers: 5'-CGGAGCTTTGGTCAATCTCA-3' + 5'-AGGTCTCTCCGTGGAAACA-3'.

D. Caudal fin amputation

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

E. Quantification of intestinal bacterial abundance

To quantify intestinal bacterial abundance, larval zebrafish are euthanized by hypothermic shock at the indicated time points [hours postinoculation (hpi)]. Intestines are removed under a dissection microscope, placed in 1 mL sterile embryo medium, and homogenized with zirconium oxide beads using a Bullet Blender® tissue homogenizer (Next Advance, Troy, NY). Homogenates are serially diluted 10^{−1} and 10^{−2}, and 100 µL volumes are spread onto LB-agar plates. Plates are incubated at 30 °C overnight, and colony-forming units (CFU) are enumerated to calculate bacterial load per intestine.

F. Light sheet fluorescence microscopy

Imaging is performed using a custom-built light sheet fluorescence microscope described in detail elsewhere [60,61]. A galvanometer-scanned light sheet of excitation illumination from 448- or 561-nm lasers (Coherent Sapphire, operating at 5 mW) is oriented perpendicular to the optical axis of a 40×, 1.0 numerical aperture (NA) water-immersion detection objective. Images are captured by a 5.5 MP sCMOS

camera (Excelitas pco.edge). Three-dimensional volumes are acquired by scanning the sample along the detection axis in 1 μm steps with 33 ms exposures per plane. The resulting image resolution is approximately 0.4 μm in the dimensions parallel to the sheet and 5 μm perpendicular to the sheet. All hardware components are controlled using $\mu\text{Manager}$ software [62].

For gut imaging, four overlapping subregions covering the entire intestine ($\approx 1200 \times 300 \times 150 \mu\text{m}^3$) are sequentially acquired and computationally registered. Time series are collected at 3 min intervals for 4 h (9–13 hpi). For fin wound assays, single volumes are acquired at 3 min intervals for 4 h (1–5 hours postamputation). Original voxel dimensions are 1 μm (z axis) $\times$ 0.1625 μm (y axis) $\times$ 0.1625 μm (x axis).

G. Sample mounting

Larvae are anesthetized in 120 $\mu\text{g/mL}$ MS-222 and embedded in 0.7–1.0 % low-melt agarose within glass capillaries. Specimens are partially extruded to avoid optical interfaces and oriented with ventral illumination. Up to four samples are mounted simultaneously in sterile EM at 28 $^{\circ}\text{C}$.

H. Bacterial distribution analysis

To quantify bacterial distribution along the gut during the imaging period, we analyze *Vibrio*^{WT}-dTomato fluorescence. To reduce host cell and background autofluorescence interference, we create 3D gut lumen masks to restrict analysis to regions where bacteria are present in the zebrafish gut. Using bright-field and dTomato fluorescence images, 2D gut lumen boundaries are manually drawn at every tenth z plane for each field of view. These 2D masks are extruded across the intermediate planes to generate complete 3D volumes, then stitched across all imaging positions to cover the entire gut (Supplemental Video 3 [38]). The gut is masked using this 3D mask and the resulting image is median background subtracted. Pixel intensities are binned along the anterior-posterior axis to generate line profiles of bacterial distribution. Anterior-posterior position is normalized such that 0 and 1 represent the start and end of the gut, respectively.

I. Gut segmentation

Binary masks that identify the larval zebrafish gut region in images are manually segmented using a Wacom Cintiq[®] graphics tablet on bright-field images acquired at the median sagittal plane. To optimize efficiency while maintaining accuracy, masks are initially drawn at every tenth time point and subsequently interpolated across all 80 imaging time points (Supplemental Video 10 [38]). The resulting 2D masks are extruded along the left-right axis to encompass the complete gut volume within the z -stack imaging boundaries.

J. Image analysis

All image processing and data analysis are performed with code written in Python using the NumPy [63], SciPy [64], scikit-image [65], and pandas [66] libraries. All code is available at [67]. Figures 1(a) and 3(a) are created in Inkscape using icons from BioRender.

1. Preprocessing

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

2. Cell segmentation

Immune cells are segmented using a two-stage approach combining iterative thresholding with morphological Active Contours Without Edges (ACWE) [68,69].

In brief, we apply iterative simple thresholding to get loose boundaries around objects. Initial thresholding uses median $+2\sigma$, where σ is the standard deviation of pixel intensities. Objects exceeding $10^5 \mu\text{m}^3$, considerably larger than the possible volume of a single cell, are iteratively rethresholded at their local median $+n\sigma$, incrementing n until all objects fall below the volume limit or n reaches 7. Objects smaller than a subcellular threshold of $10^3 \mu\text{m}^3$ are removed.

For each remaining object, we tighten and refine boundaries around the object using morphological ACWE with the thresholded region as the initial level set. The algorithm is run for ten iterations with the following parameters: smoothing = 1, $\lambda_1 = 1$, $\lambda_2 = 4$, with early stopping after three iterations of no change.

3. Cell property analysis

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

Surface area is calculated via a marching cubes algorithm on Gaussian-smoothed segmented objects with 5-pixel padding to prevent edge artifacts. Supplemental Video 11 shows the generated surfaces on the segmented macrophages [38]. Sphericity quantified morphological complexity and is computed as

$$\text{sphericity} = \frac{(36\pi V^2)^{1/3}}{A}, \quad (1)$$

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 are clipped to this range.

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

4. Tracking and trajectory analysis

Segmented cells are tracked across time points using a bidirectional nearest-neighbor algorithm. For each consecutive time-point pair, we compute Euclidean distances between all cell centroids in 3D space. A cell at time t is linked to its nearest neighbor at time $t + 1$ only if the reverse mapping

(from $t + 1$ to t) also identifies the original cell as the nearest neighbor. This bidirectional validation is a stringent criterion, such that tracks terminate when cells exit the imaging volume or when local crowding prevents unambiguous assignment; however, it also prevents spurious connections. The total number of tracked cells at each time point shows gradual changes without large fluctuations (Supplemental Figs. 13 and 14 [38]), consistent with stable tracking over time. Trajectories are subdivided into “subtracks” at transitions above or below a displacement threshold of 0.5 times the mean cell diameter, separating motile from nonmotile phases. A patience parameter ($n = 3$ time points) is applied to ensure subdivision only occurs after cells maintain the new motility state for three consecutive time points to prevent oversegmentation from transient behavior.

For each subtrack, we calculate 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, using $\cos(\theta)$ between consecutive velocity vectors; spatial extent, via diameter of smallest circumscribing sphere of the subtrack; and velocity anisotropy, $\sqrt{2V_{AP}^2/(V_{DV}^2 + V_{LR}^2)}$.

K. Simulated random walk

To compare the observed spatial extent and velocity cosine similarity relations with what would arise from randomly directed motion, we generate simulated trajectories for each experimental track. For each track, we generate random walks with the same starting position, number of time points, and set of frame-to-frame step magnitudes, drawing step sizes at random and assigning directions drawn uniformly from the unit sphere. Positions are computed by cumulative summation from the original starting position. To replicate imaging, we apply simulated detection effects: Gaussian localization noise

($\sigma_{xy} = 0.21 \mu\text{m}$, $\sigma_z = 0.43 \mu\text{m}$) followed by quantization to the imaging pixel resolution ($0.5 \mu\text{m}$ in xy , $1.0 \mu\text{m}$ in z). Simulated tracks are analyzed using the same pipeline applied to experimental data.

L. Statistical analysis

Custom code written in Python is 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.

ACKNOWLEDGMENTS

We thank Rose Sockol and the University of Oregon Zebrafish Facility staff for fish husbandry and care. We thank Catherine D. Robinson for performing gut dissections and bacterial plating, and Emily P. R. Avey for generating gut lumen masks for bacterial distribution analysis. This work was supported by the National Science Foundation under Award No. 2310570 and the National Institutes of Health under Award No. P01GM125576. The funders had no role in study design, data collection and analysis, decision to publish, or preparation.

P.A. performed experiments and analyzed data. P.A. and R.P. designed the experimental and analysis methods and wrote the manuscript.

DATA AVAILABILITY

The data that support the findings of this article are openly available as Supplemental Material [38], and are uploaded to the Zenodo repository [70].

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