Semi-Automated Cell Tracking and Quantification of Neutrophil Swarming to MRSA on a Bone Implant in a Murine Femur Model

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Disclosures: None of the authors have any disclosures.

Introduction: Implant-associated osteomyelitis remains a major orthopaedic problem. As neutrophil swarming to the surgical site is a critical host response to prevent infection [1], elucidation of this dynamic behavior in vivo is central to understanding the host response. To this end, we developed a longitudinal imaging of bone marrow (LIMB) system to visualize bacteria and host cell dynamics proximal to a transfemoral implant in a murine model. While descriptive data from this model provided insights on "the race for the surface", quantitative outcomes of neutrophil swarming are needed for hypothesis testing research and product development. Although user-assisted automated tracking approaches exist, they are labor-intensive and suffer from inter-user variability. Therefore, we aimed to develop a robust semi-automated protocol to analyze LIMB videos of fluorescent bacteria and leukocytes for the quantification of neutrophil swarming behaviors.

Methods: We modified the previously developed LIMB system [4] to image a fixed region of interest proximal to a transfemoral implant. Catchup mice [5] with tdTomato expressing neutrophils received a transfemoral pin with or without EGFP-expressing USA300 methicillin-resistant *Staphylococcus aureus* (MRSA). At 2-, 4-, and 6-hours post-implantation, real-time videos (30 minutes long) were obtained using intravital two-photon laser scanning microscopy. The semi-automated cell tracking protocol was adapted from Trainable Weka Segmentation (TWS) [2] and TrackMate [3] (Fiji/Image J), and customized MATLAB code for data integration (**Figure 1**). In brief, image stacks were drift-corrected using the Image Stabilizer plugin in Fiji. Minimum intensity projection was subtracted from the stack to remove stationary artifacts. To avoid inter-user variability primarily introduced by inaccurate cell identification during the tracking process, the neutrophils were first segmented using TWS, which uses a library of machine learning training features to generate a probability score of the foreground (cells). The probability maps generated were used as input for TrackMate to track moving cells based on cell diameter and total displacement. The tracks were manually verified and then quantitated using a custom MATLAB code. Data are presented as median ± interquartile range. Mann-Whitney tests adjusted for multiple comparisons by the Holm-Šídák method were used to test if the tracking metrics differed between individuals and the study groups.

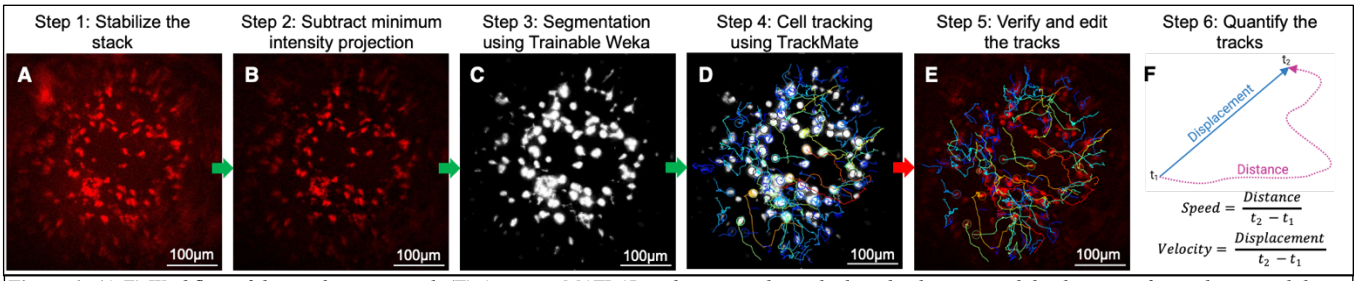

Figure 1: *(A-E) Workflow of the tracking protocol. (F) A custom MATLAB code was used to calculate the distance and displacement for each neutrophil track. Speed and velocity were calculated as the ratios of distance and displacement to track duration, respectively. Directionality, a measure of the straightness of a track was calculated as the ratio of displacement and distance.*

Results: To determine if the protocol yields reproducible results, two users independently analyzed three LIMB time-lapses and compared the tracking metrics. Inter-reader reliability was excellent as ICC > 0.98 for all neutrophil swarming outcome measures ($p \geq 0.05$). Further, when Catchup mice were challenged with a MRSA-contaminated or sterile implant, we observed that the neutrophils proximal to infected pins traveled farther and faster. Specifically, the distance and displacement of the neutrophils were greater at 6 hours, while the speed and velocity were greater at all measured time points in infected animals compared to uninfected animals (**Figure 2**).

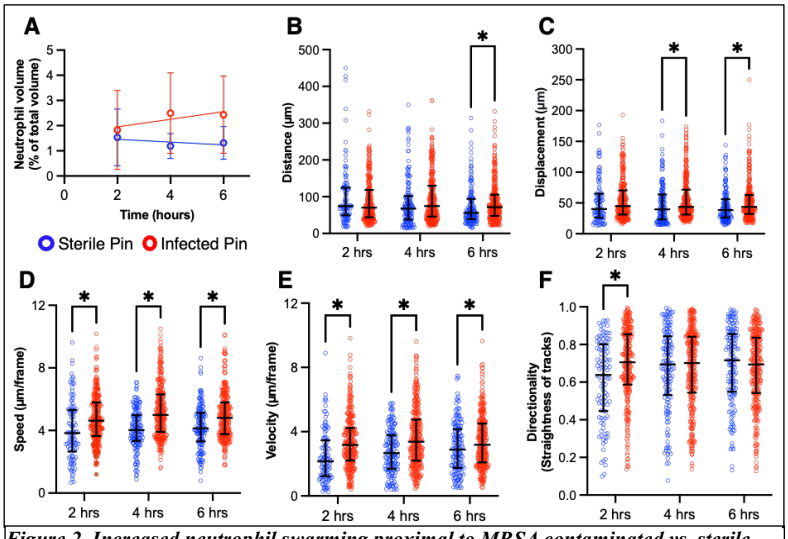

Figure 2. Increased neutrophil swarming proximal to MRSA contaminated vs. sterile bone implant. *(A) Change in neutrophil volume with time proximal to an infected and a sterile implant. Semiautomated quantification of neutrophil distance traveled (B), displacement (C), mean speed (D), mean velocity (E), and directionality (F). (n=105-316 tracks/group/time point, N=3 mice/group)).*

Discussion: The key innovation in our protocol is the use of TWS to segment the neutrophils, which eliminates the need for arbitrary user-defined thresholding. This step also resulted in the tracking algorithm detecting tracks with high accuracy. The protocol can be easily adapted to study the kinematics of different cells by changing the TWS classifier and the tracking parameters. Of note, distance and displacement can be influenced by the number of frames the cell was tracked, cell density, etc. In contrast, speed and velocity are normalized by the frame count (time), thus providing unbiased measures of cell behavior. An inherent limitation of all cell tracking protocols is that they perform less optimally when the density of cells is high. Future studies focusing on track connection rules and penalties will help address this issue.

Significance/Clinical Relevance: Our reproducible cell tracking protocol allows quantitative investigation of immune mechanisms such as neutrophil swarming, pathogen evasion strategies, and the effects of different drugs on the kinematics of immune cells. Additionally, these metrics can serve as benchmarks for assessing the efficacy of antimicrobial implants.

Acknowledgments: This work was supported by grants from the AO Trauma Clinical Priority Program and NIAMS (P30 AR069655, P50 AR072000).

References: [1] Kienle, K. et al. *Immunological Reviews*. 273 (1), 76–93 (2016). [2] Arganda-Carreras, I. et al. *Bioinformatics*. 33 (15), 2424–2426 (2017). [3] Tinevez, J.Y. et al. *Methods*. 115, 80–90 (2017). [4] Reismann, D. et al. *Nature Communications*. 8, 2153 (2017). [5] Hasenberg A. et al. *Nat Methods* 12, 445-52 (2015).