Measuring the Effect of IL-8 and fMLP on Neutrophil Chemokinesis Using Artificial Intelligence

Joshua J. Shin, Jonathan S. Reichner

SUPPORTING INFORMATION

MATERIALS AND METHODS

Reagents.

Histopaque 1077, dextran (400-500 kDa), N, N, N′, N′-Tetramethylethylenediamine (Temed), and formyl-methionyl-leucyl-phenylalanine (fMLP) were purchased from Sigma-Aldrich; 3-Aminopropyltriethoxysilane (APTS) were purchased from Aldrich; Glutaraldehyde was purchased from Polysciences; Leibovitz L-15 medium, Dulbecco's Phosphate Buffered Saline (DPBS 1x), Human Plasma-derived Fibronectin, and Hanks Balanced Salt Solutions were purchased from Gibco by Life Technologies; Recombinant human endothelial cell-derived IL-8 was purchased from R&D systems; Sulfo-Sanpah, 10% Ammonium persulphate solution (APS) was purchased from Fisher Scientific; EasySep™ Magnet was purchased from Stem Cell Technologies; MACSxpress Erythrocyte

Depletion Reagent was purchased from Miltenyi Biotec.

Cell Preparation.

Neutrophils were isolated from healthy human volunteers by collection into ethylenediaminetetraacetic acid–containing Monoject tubes (Covidien). A histopaque underlay (15ml) was used for the initial cell separation through centrifugation and was followed by gravity sedimentation through 3% dextran for 40 minutes at room temperature. Contaminating erythrocytes were removed by hypotonic lysis using 0.2% and 1.6% salt solution, and an additional removal was performed by using the MACSxpress Erythrocyte Depletion Reagent and EasySep™ Magnet. Finally, we obtained an approximately 99% neutrophil purity, resulting in reduced interference in AI cell tracking. Neutrophils were suspended in Hanks Balanced Salt Solution (without Ca2+/Mg2+) on ice until use in the experiments. A hemocytometer was used to quantify the neutrophil count both before and after the erythrocyte depletion process in order to ensure an accurate and consistent neutrophil count within each experimental well.

Institutional Review Board approval was obtained from the Rhode Island Hospital's Committee on Protection of Human Subjects to allow the donation of venous blood from normal, healthy donors for isolation of peripheral blood neutrophils. In addition, informed consent was obtained in accordance with the Declaration of Helsinki.

The optimal neutrophil density for the Ibidi µ-Slide 2 Well chamber was established at 250,000 neutrophils per chamber. This configuration was found to yield an optimal balance, ensuring that approximately 50 neutrophils were tracked within each time-lapse field of view (2560x2160 px). This precise neutrophil density maximized the number of cells tracked in each experimental run while effectively minimizing errors arising from AI tracking, specifically the challenge of overlapping neutrophils.

Each chemokinetic migration assay was conducted in an Ibidi µ-Slide 2 Well chamber with 1kPa gels at 37°C and 5% CO2. Approximately 250,000 neutrophils were resuspended into 37°C L-15 with 5 mg/ml glucose, alone as vehicle control, with 100-nM concentration of fMLP with L-15g, 7-nM IL-8 with L-15g, or both 100-nM concentration of fMLP and 7-nM IL-8 with L-15g to create a total volume of 1 ml.

To prevent the inadvertent generation of a chemotactic gradient, all chemokines were thoroughly mixed by vortexing them together in a conical tube before their introduction into the well. Notably, neutrophils were the final component added to the well and were given a 10-minute settling period in an incubator and an additional 10 minutes on the microscope before each time-lapse, ensuring a consistent and uniform exposure to chemokines for the neutrophils within each experiment.

Substrate Preparation.

Polyacrylamide gels were made by mixing a solution of acrylamide and bis-acrylamide and polymerized using tetramethyl ethylenediamine (T-med) and ammonium persulfate (APS). Gels were made in glass bottom Ibidi µ-Slide 2 Well chambers and were treated with 3-Aminopropyltriethoxysilane (APTS) hydrophilic silane and glutaraldehyde fixative (adequately washed) using Chemglass Life Sciences 15mm Round Cover Slips as molds. Gel stiffness was regulated by varying the percentage of bis-acrylamide in relation to the percentage of acrylamide in the initial mixture. 1 kPa gels were prepared using a mixture of 40% acrylamide and 2% bis-acrylamide in a ratio of 37.5:50, with the remaining portion consisting of di H2O. Gel polymerization was initiated by adding 1 µL of T-med and 2.5 µL of 10% APS. Gels were allowed to polymerize at room temperature for 15 minutes to a final size of approximately 15mm in diameter and 2 mm thick and were submerged in water for an additional 10 minutes.

Using the chemical crosslinker Sulfo-Sanpah, gels were coated with fibronectin. Two coats of Sulfo-Sanpah were allowed to covalently bond to the acrylamide gel for approximately 30 minutes at room temperature in both dark conditions and under UV. Fibronectin, at 20 µg/mL, was then added to the gel and allowed to saturate overnight, cross-linking the protein. Gels were washed three times with Hanks Balanced Salt Solution before starting preparation for experiments.

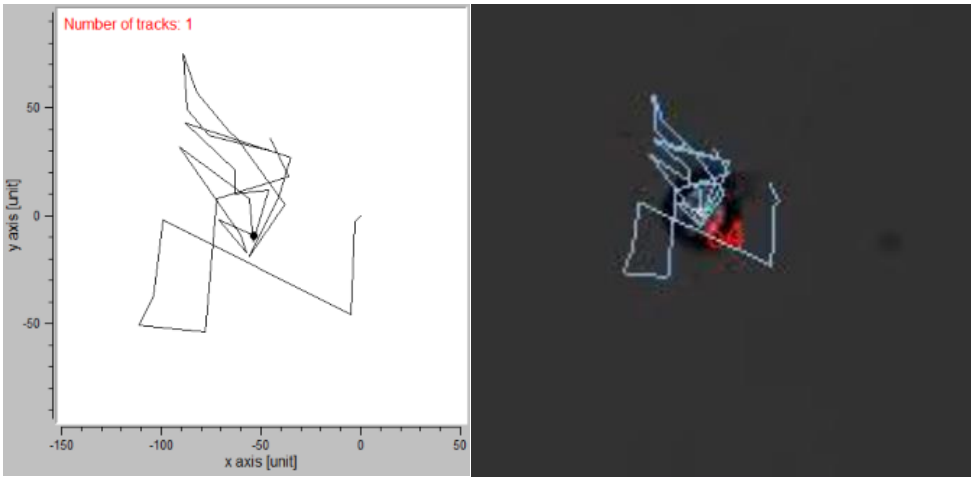

Figure S1. Extracted and Cleaned Data from AI Tracker.

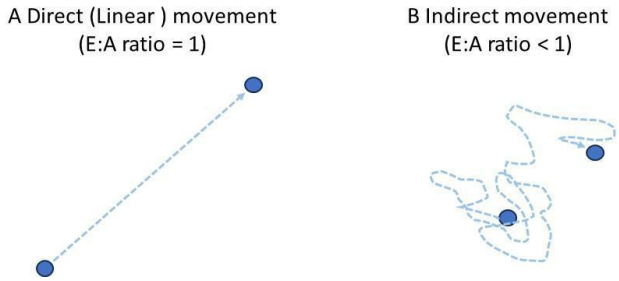

Figure S2. Linear Movement vs. Indirect Movement. E:A stands for Euclidian distance/Accumulated distance, also known as the CI

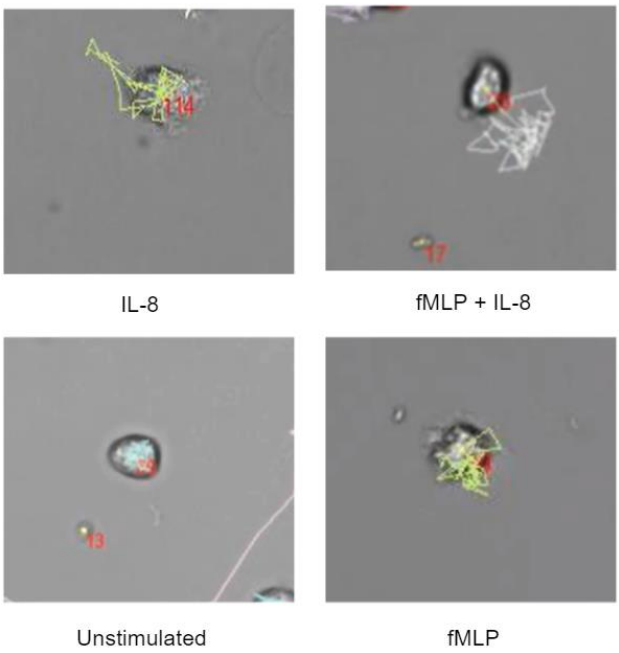

Figure S3. Extracted and Cleaned Data from AI Tracker. Exposure of neutrophils to different chemoattractants (IL-8, fMLP + IL-8, fMLP, and unstimulated on 1kPa polyacrylamide gels. Bright-field (BF) images show neutrophils at the end of the 45-minute timelapse video. Colored lines representing neutrophil tracks (their accumulated distance) were generated by Fast Track AI Chemotaxis Analysis software and illustrate cell movement during the imaging period.