

Supplemental Methods

SU-8 mold and PDMS fab

The microfluidic base and lid of the KOALA platform were fabricated using soft lithography methods with Poly-dimethylsiloxane (Sylgard 164, Dow Corning, Salzburg, MI). First, multilayer molds were created using SU-8 negative photoresist (Microchem, Newton, MA). In brief, pattern designs were created using Adobe Illustrator (Adobe, San Jose, CA) and printed on film (Imagesetter, Madison WI). A first layer was spun according to the manufacturer's specifications on a 150 mm diameter silicon wafer (WRS, San Jose, CA) using SU-8 50 to achieve 80 μm thickness or SU-8 100 for thicknesses of 180 and 270 μm . The photoresist was baked on hot plate and a Omnicure 1000 UV light source (EXFO, Quebec, Canada) was used to transfer the pattern to the photoresist. After a post-exposure baking step, the second, 400 μm thick, layer was spun on the wafer and patterned. The mold was then developed for 4 hours in SU-8 developer (PGMEA, 537543, Sigma, St Louis, MO) and washed with acetone and iso-propyl alcohol. PDMS was prepared in a ratio of 10:1 base to cross-linking agent, degassed in vacuum, and poured over the SU-8-silicon mold on a hot plate. A transparency (Cheap Joe's, Boone, NC), a layer of silicone (McMaster Carr, Eimhurst, IL), and a 5 kg weight, were placed on top of the mold, and baked at 80 degrees celsius for 4 hours. The base and lid of the KOALA device were adhered to tissue culture treated plastic from an Petri-dish (NUNC, Portsmouth, NH) prior to use. Photolithography masks used by the authors to fabricate devices is available upon request.

COMSOL modeling

A 3D model of the microchannel was developed using the COMSOL numerical simulation software (COMSOL, Burlington, MA). For the diffusion analysis a 3 mm diameter hemisphere - the size of the hydrogel bead placed in the KOALA lid - was drawn in the large port of the passive pumping microchannel and its initial concentration set to 1 with a diffusion coefficient of 100 $\mu\text{m}^2\text{s}^{-1}$, corresponding to the diffusion rate of Alexa488 in hydrogel. A diffusion coefficient of 300 $\mu\text{m}^2\text{s}^{-1}$ was used for all the low viscosity fluid-filled parts of the microchannel. Using the diffusion simulation package with the GMRES solver, a time-dependent simulation was performed over 120 min, and the value of the concentration of chemoattractant was outputted for a horizontal plane placed 10 μm above the floor of the channel. Cross-section profiles taken along the axis of the channel were quantitatively analyzed. For the shear rate analysis, the incompressible Navier-Stokes simulation package was employed with an inlet pressure of 100 Pa and an outlet pressure of 0. A steady-state solution for the flow was found using the UMF pack solver and the value of the shear stress was plotted on floor of the microchannel.

Gradient characterization

The stability of the gradient and the validity of the numerical simulation were verified experimentally using a source concentration of 1 μM AlexaFluor488 dye (Molecular Probes, Carlsbad, CA) prepared in Matrigel (356231, BD Bioscience). A 3 μL drop of gel was placed in every location of the lid of the KOALA platform and polymerized in a humidified incubator. The channels in the base were filled with PBS and the lid was placed into contact with the base. Fluorescent timelapse microscopy was performed using a 4x objective at intervals of 10 min for a duration of 90 min at 37°C in an environmental-control chamber; an IX-81 microscope (Olympus, Tokyo, Japan) was used to capture the images, which were then analyzed using the imageJ software with a line scan with a width of 70 pixels. The concentration was normalized to the concentration observed when filling the channel with stock fluorescent solution.

Preparation of hydrogel-chemoattractant mixture

N-formyl-methionine-leucine-phenylalanine (fMLP; F3506-10MG, Sigma-Aldrich, St. Louis, MO) was suspended in dimethyl sulfoxide (DMSO; D2650; Sigma-Aldrich, St. Louis, MO) at 10 mM and stored at -80°C. The hydrogel-chemoattractant (H-CA) mixture consisted of fMLP and Matrigel, mixed in a 1:1 ratio to a final gel concentration of 4 mg/mL. The H-CA mixture was prepared before each chemotaxis experiment. For all doses of chemoattractant, the fMLP dilution was performed in PBS (Invitrogen, Grand Island, NY) prior to mixing with the hydrogel.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD), and regularly cultured on tissue culture-treated flasks pre-coated with $1.5 \mu\text{g}/\text{cm}^2$ of bovine plasma fibronectin (FN) (Sigma-Aldrich, St. Louis, MO). HUVECs were maintained in EGM BulletKit media (CC-3124; Lonza) consisting of EBM-2 basal medium supplemented with 2% fetal bovine serum (FBS), bovine brain extract with heparin, hEGF, hydrocortisone, and gentamicin/Amphotericin B. HUVECs were fed every other day, passaged every 3 to 4 days at 90% confluence, and only passages 4-6 were used in microchannel experiments. To prepare HUVEC monolayers, microchannels were first primed with $10 \mu\text{L}$ PBS followed by $3 \mu\text{L}$ FN at $100 \mu\text{g}/\text{mL}$. Microchannels were incubated at 37°C for 1 h in humidified trays to allow FN adsorption to the bottom substrate. After incubation, FN was replaced twice with $3 \mu\text{L}$ HUVEC media. HUVECs were seeded at $4,000 \text{ cells}/\mu\text{L}$ $1.25 \mu\text{L}$ per microchannel, and allowed to adhere and culture until confluency (48 h). HUVEC microscale cultures were either used in neutrophil experiments when confluent. Activated HUVEC monolayers in microchannels were induced with $10 \mu\text{g}/\text{mL}$ interleukin- 1β (IL- 1β) for 4 h before performing neutrophil capture.

Neutrophil capture from whole blood on HUVECs

After obtaining consent at the time of the blood draw, whole blood was obtained from a lancet puncture on the finger of a self-reported healthy donor. The human subject protocol was approved by the University of Wisconsin Center for Health Sciences Human Subjects Committee. In brief, the skin was thoroughly cleaned with an alcohol swab and a disposable lancet (Safety Lancet, 02-675-160, Fischer Scientific) was lightly pressed against the skin and actuated. The first drop of blood was discarded and the finger pressed to obtain a drop of blood of about $5\text{-}10 \mu\text{L}$ on the bottom of a flat sterile petri-dish. $3 \mu\text{L}$ of blood was pipetted into a reservoir containing $18 \mu\text{L}$ of PBS and mixed gently. Following IL- 1β stimulation, neutrophils were captured out of dilute whole blood in preparation of performing the chemotaxis assay. $1 \mu\text{L}$ of dilute whole blood was passed through each microchannel two times, with each pass 30 seconds apart. After allowing neutrophils to capture for 4 minutes, erythrocytes were removed by performing 3 washes with $3 \mu\text{L}$ of PBS, alternating the aspiration of PBS-blood mixture between the input and output ports. The PBS was replaced twice with $3 \mu\text{L}$ of EGM BulletKit media (CC-3124, Lonza) with 20 mM HEPES (25-060-CI; Mediatech, Manassas, Va).

Modeling fluid dynamics in microchannels

Using Supplemental Equations (1) and (2), an analytical model and a numerical simulation was performed to calculate the flow rate in the microchannels of the KOALA platform. The dimensions used in the analytical model were: a volume of $3 \mu\text{L}$ of fluid dispensed on the input port of the channel of length 3 mm, width 0.8 mm and height $200 \mu\text{m}$; the input port had a diameter of 1.5 mm and the output port is assumed to have a much larger diameter than the input port.

Neutrophil chemotaxis for varying gradient slopes

In order to test neutrophil chemotaxis for different slopes of the chemical gradient, we fabricated a separate KOALA device using methods described in **SU-8 mold and PDMS fab**. The base microchannels had lengths of 6, 5, 4, 3, 2, and 1 mm in order to change the slope of the chemical gradient; the height and width of the microchannels were $80 \mu\text{m}$ and $800 \mu\text{m}$, respectively; the input and output ports had diameters of 1.5 mm and 2.5 mm, respectively, with heights of $600 \mu\text{m}$. The neutrophil sorting and image capture was performed as described in the **Washing efficiency characterization** section. Four doses of fMLP were tested: 0 M (control), 100 nM, 500 nM, 1 mM. Each dose was tested for three separate experiments ($n=3$). During each experiment, the neutrophil chemotaxis for each channel length was observed and automatically tracked using Je'Xperiment software.

Washing efficiency characterization

Different base microfluidic channels for the KOALA platform were fabricated according to the methods described in the PDMS fabrication section with channel thicknesses of 80, 180, $270 \mu\text{m}$. Each of these devices were coated for 30 min with $100 \mu\text{g}/\text{mL}$ human recombinant P-Selectin (R&D Biosystems, Minneapolis, MN). $3 \mu\text{L}$ of blood from a lancet puncture were collected and diluted in $18 \mu\text{L}$ of PBS and $1 \mu\text{L}$ of the latter solution was inserted into the microchannel base. Subsequently, the content of the

microchannel were washed 3 times with 3 μ L of PBS and images were taken on a IX-81 microscope using the 10X magnification objective.

Je'Xperiment tracking

Stacks of phase contrast images taken for each channel on the KOALA platform were exported into .tiff format and loaded into the Je'Xperiment software (available upon request). An automated algorithm was developed to track migrating neutrophils automatically. First, the user selects a representative neutrophil in one of the image stacks. Second, the algorithm performs a convolution on each image of the stack and identifies local maxima in the image produced. Maxima found are linked together into tracks by using an overall distance minimization strategy to find extensions of the tracks at each time-frame that minimize the overall displacement of the neutrophils. Possible erythrocyte contaminants, wrongfully tracked particles, and dead or immobile neutrophils, are removed using a track filtering. The final tracks were analyzed to obtain their mean speed, chemotactic index, and directional velocity.

Comparing automated Je'Xperiment and ImageJ cell tracking

The accuracy of the automated software Je'Xperiment was assessed by comparison with experiments analyzed manually using the "Cell-tracker" plugin of ImageJ (<http://rsbweb.nih.gov/ij/>). Three channels coated with E-selectin and three channels coated with P-selectin were used to capture neutrophils and record their chemotaxis to fMLP. In each channel 5 neutrophils were selected and tracked manually as well as analyzed using JEX.

HUVEC immunostaining

Immunostaining was performed to verify presence of PECAM-1 at intercellular junctions, and upregulation of E-selectin upon activation using IL-1 β . After 4 hours, HUVECs were fixed, permeabilized, and immunostained with either monoclonal anti-human E-selectin antibody (BBA1; R&D Systems Inc., Minneapolis, MN) or monoclonal mouse anti-human CD31 (MCA1738T; AbD Serotec., Oxford, UK) and Hoechst 33342 nuclear dye (H1399, Invitrogen, Carlsbad, CA). Images of the culture were acquired on a Nikon Eclipse Ti inverted fluorescence microscope coupled to a Nikon DS-QiMc CCD camera (Nikon Instruments, Melville, NY). A 10x objective (NA=0.30) was used for images shown in Figure 3a and a 20x objective (NA=0.50) was used for micrograph in Figure S3. NIS-Elements software (Nikon Instruments, Melville, NY) was used to capture and export the images in .tif format. A dry imaging medium was used for all captured images. ImageJ software was used to adjust the contrast of the images; the image processing was conducted uniformly across the entire image.

Tracking neutrophil chemotaxis on HUVECs

Stacks of phase contrast images taken for each channel on the KOALA platform were exported into .tif format and loaded into imageJ using the "import stack" tool. The imageJ plugin "Cell tracker" was used to track the migrating neutrophils on top of the endothelial cell monolayer. For each image stack, the two distinct morphologies of neutrophils were tracked using 10 cells of each phenotype tracked over at least 40 frames. 6 image stacks from 6 microfluidic channels were used for each repeat, and 3 independent repeats were performed. The tracked images were outputted using imageJ, and the quantification of the migration speed, Chemotactic Index (CI) and directional velocity was performed using Excel (Microsoft, Redmond, WA).

Determining Type II to Type I transition

Neutrophils exhibiting the Type I and Type II morphologies were counted throughout a 90 minute timelapse to determine the proportions of each cell type over time. The "Cell counter" plugin of imageJ was used for cell counting. A representative channel was analyzed for three separate experiments. The neutrophils exhibiting Type I and Type II morphologies were counted every 5 minutes for the entire 90 minute timelapse. The proportion of Type II neutrophils at each point was calculated by dividing the number of neutrophils exhibiting a Type II morphology by the total number of cells counted in that frame (sum of Type I and Type II cell counts).

Measuring the area of Type I and Type II neutrophils

The area of the cells as seen under phase contrast microscopy was measured for neutrophils with Type I and Type II morphologies; the area of erythrocytes were also measured for reference. Once the correct scale was set in imageJ, the tracer tool and measure function were used to calculate the area of the cells. Representative neutrophils and erythrocytes across 6 microchannels were measured (52 Type II, 59 Type I, 25 erythrocytes).

Capture efficiency experiments

Neutrophils (at $\sim 1 \times 10^6$ cells/mL density) were purified (details in “Human neutrophil purification”) and then tagged with calcein-AM stain (L-3224; Invitrogen, Grand Island, NY). The calcein-AM was prepared by mixing 1 μ L of calcein-AM with 1 mL of PBS. Cells were then placed into the diluted calcein-AM and incubated at 37°C for 15 minutes. 3 μ L the tagged cells were then resuspended into 15 μ L of whole blood and injected into the microchannels. Phase contrast and fluorescent images were taken of 5-6 microchannels prior to washing, and then the normal washing procedure was performed. Images of the microchannels were taken again after washing. Cells were counted manually using the imageJ plugin “Counter” for both the pre and post-wash channels. Count data from 5-6 channels were averaged, yielding an average count for a single experimental output (n=1). Three replicates (n=3) were performed. The capture efficiency was calculated by dividing the average number of neutrophils captured after washing was performed by the average number of neutrophils before washing was performed.

Mouse maintenance

TNF transgenic mice of the 3647 line were bred, cared for, and housed according to the University of Wisconsin Institutional Animal Care and Use Committee. Mice that were positive for the TNF transgene by Polymerase Chain Reaction (PCR) and littermate controls were used from 1.5 to 8 months of age.

Statistical analysis

Open source software, Mstat (<http://www.mcardle.wisc.edu/mstat/download/download.html>) was used to perform statistical analysis on the data gathered. The Wilcoxon signed rank test (two-tailed, paired) was used to determine statistical significance. This test was used to determine the significant differences between categories where normality could not be assumed. Each output (chemotactic index, speed, directional velocity, and count) was individually measured for 2-6 microchannels and each channel output value was averaged to yield a single experimental output (n=1). Three (n=3) replicates were performed for all chemotaxis experiments. Five (n=5) replicates were performed for mouse neutrophil count data.

Mouse blood draw and neutrophil capture

Prior to the blood draw, microchannels were coated with 100 μ g/mL concentration P-selectin (737-PS-050; R&D Systems, McKinley Place, Minneapolis, MN) for at least 30 minutes at 4°C. Mice were anesthetized using isoflurane until they did not experience pain from paw pinch and were then subjected to a tail snip. A 3 μ L drop of blood was removed, which was then diluted into 18 μ L PBS. 1 μ L of dilute whole blood was passed through each microchannel two times, with each pass separated by 30 seconds. A minimum of 0.5 μ L of dilute blood was tested to reliably fill the microchannel for neutrophil capture. After allowing neutrophils to capture for 4 minutes, erythrocytes were removed by performing 3 washes with 3 μ L of PBS, alternating aspiration of PBS-blood mixture between the input and output ports. The PBS was replaced twice with 3 μ L of Hanks Buffered Salt Solution (HBSS; 21-023-CV; Mediatech, Manassas, Va) with 20 mM HEPES.

Supplemental Equations

We used an analytical model for estimating the flow rate in a microchannel generated by passive pumping. An important assumption of the model is a negligible pressure at the output port, which we can reasonably assume in this case given the much larger output port diameter. The pressure drop generated by surface tension is determined using the Laplace equation (1), where γ is the interfacial energy of water and air, $R(t)$ is the radius of curvature of the input drop through time, and ΔP is the pressure drop between the input and output of the channel. The flow rate generated by a given pressure drop in a microfluidic channel of rectangular cross-section can be written as a function of ΔP using the Washburn

equation (2), where η is the viscosity of the fluid, L is the length of the channel, λ is the aspect ratio of the channel, w is the width of the channel, h is the height of the channel, and $Q(t)$ is the flow rate through time.

$$(1) \quad \Delta P_{in \ channel} = \frac{2\gamma}{R(t)}$$

$$(2) \quad \Delta P_{in \ channel} = \frac{8\eta Lg(\lambda)}{w^3h} Q(t)$$

The speed, chemotaxis index (CI), and directional velocity of a tracked neutrophil were calculated using Equations (3-5), where n is the number of frames of the timelapse image, t_i is the time interval between frames $i-1$ and i , δx_i and δy_i are the displacements along the x and y axis respectively between times $i-1$ and i , and ΔT is the time interval between the first and last frame of the timelapse.

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$$(3) \quad Speed = \sum_{i=1}^n \frac{\sqrt{\delta x_i^2 + \delta y_i^2}}{n\delta t_i}$$

$$(4) \quad CI = \frac{\sqrt{(x_n - x_0)^2 + (y_n - y_0)^2}}{\sum_{i=1}^n \sqrt{\delta x_i^2 + \delta y_i^2}}$$

$$(5) \quad Directional \ velocity = \frac{1}{n\Delta T} \sum_{i=1}^n \delta x_i$$

Table S1: Neutrophil migration speed ($\mu\text{m}/\text{min}$) for different channel lengths and chemoattractant doses

n	Source [fMLP] (nM)		6 mm	5 mm	4 mm	3 mm	2 mm	1 mm
3	100	AVG.	2.92	3.66	4.49	6.72	8.86	10.99
		S.E.	0.46	1.16	1.20	1.69	0.82	2.19
	500	AVG.	2.79	5.65	7.96	8.42	9.54	11.21
		S.E.	0.31	0.97	0.26	0.28	0.44	0.85
	1000	AVG.	3.17	5.28	7.97	9.61	11.13	11.49
		S.E.	0.61	1.17	1.41	0.64	0.41	0.40

Table S2: Neutrophil chemotactic index for different channel lengths and chemoattractant doses

n	Source [fMLP] (nM)		6 mm	5 mm	4 mm	3 mm	2 mm	1 mm
3	100	AVG.	0.12	0.16	0.15	0.27	0.29	0.33
		S.E.	0.03	0.04	0.03	0.01	0.04	0.02
	500	AVG.	0.10	0.22	0.29	0.29	0.28	0.29
		S.E.	0.01	0.01	0.00	0.02	0.03	0.02
	1000	AVG.	0.16	0.26	0.28	0.29	0.28	0.28
		S.E.	0.03	0.02	0.01	0.01	0.02	0.01

Table S3: Neutrophil chemotaxis velocity ($\mu\text{m}/\text{min}$) for different channel lengths and chemoattractant doses

n	Source [fMLP] (nM)		6 mm	5 mm	4 mm	3 mm	2 mm	1 mm
3	100	AVG.	0.14	0.35	0.33	0.72	1.49	2.18
		S.E.	0.09	0.25	0.14	0.11	0.27	0.05
	500	AVG.	0.22	1.12	1.41	1.38	1.12	1.34
		S.E.	0.03	0.31	0.02	0.03	0.03	0.20
	1000	AVG.	0.35	1.00	1.43	1.12	1.18	1.08
		S.E.	0.12	0.21	0.13	0.24	0.16	0.25

Supplemental Figures

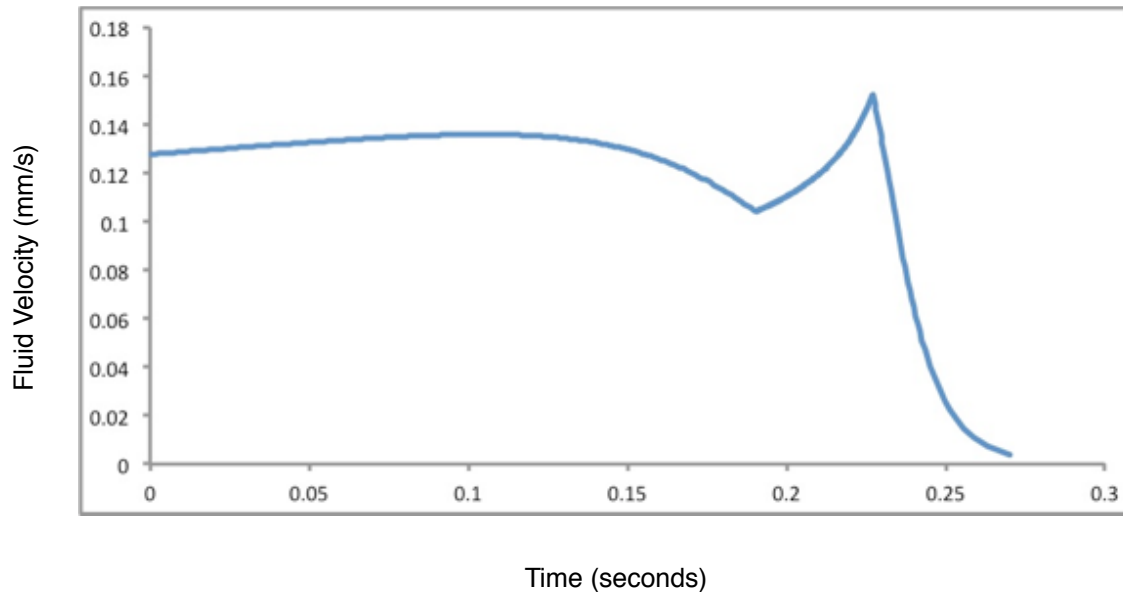


Figure S1. Velocity profile at the center of the microchannels for the base component of the KOALA device. The fluid velocity at the center of the microchannel was approximately 0.12 mm/s, yielding a flow rate of 26 $\mu\text{L/s}$. The velocity profile is stable for the most part of the flow, which lasts for about $\frac{1}{4}$ of a second, with a brief spike towards the end. The spike occurring at the end of the analysis window is the result of a transition between two phases of the analytical model.

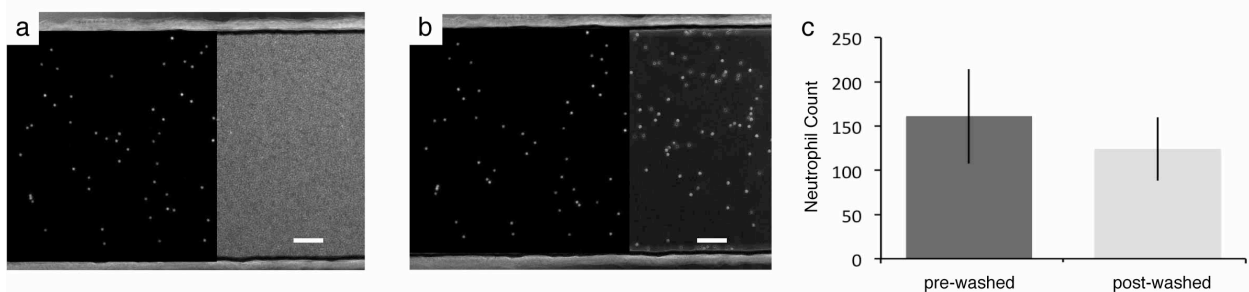


Figure S2. Capture efficiency of human neutrophils using the KOALA technique. (a,b) Phase contrast Micrographs of whole blood in the base microchannels prior to the washing step (a) and after the washing step (b). Fluorescent neutrophils within the blood are superimposed on the phase contrast images to show the location of neutrophils. (c) Average neutrophil counts pre and post washing within the microchannels. Neutrophils capture on average with 80% efficiency on the P-selectin substrate. Scale bars 100 μm ; phase contrast and fluorescent images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”

Je'Xperiment workflow

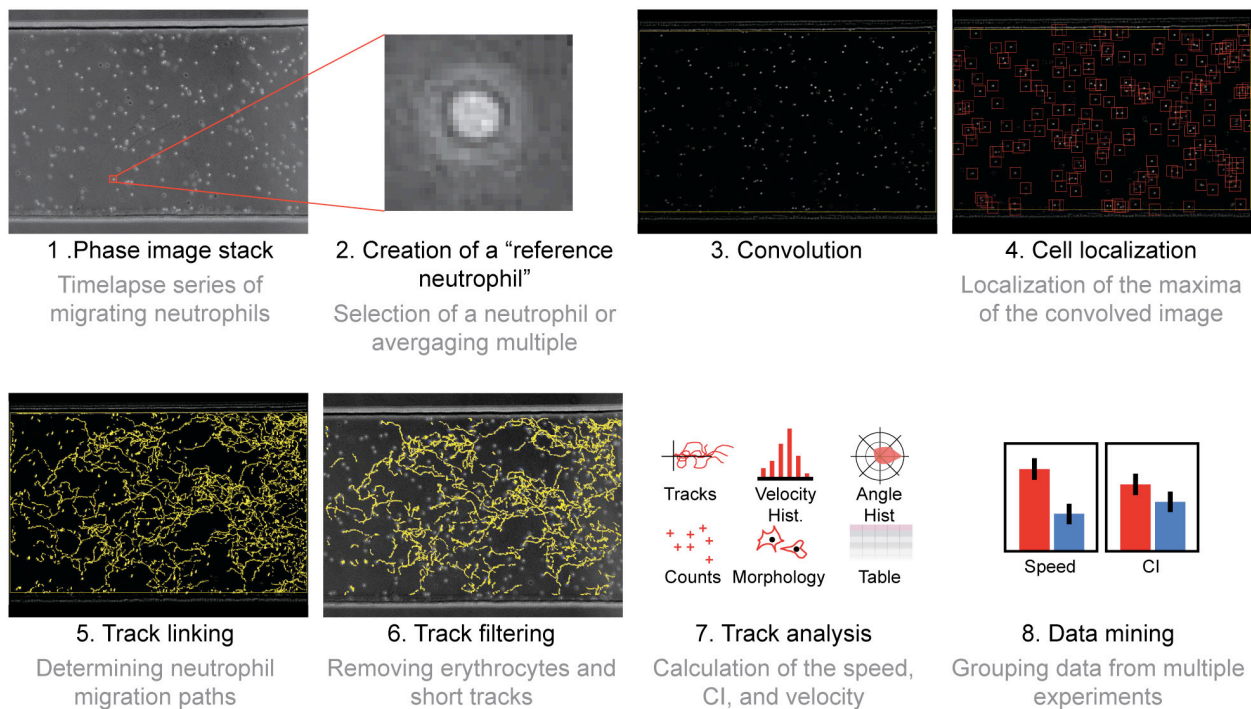


Figure S3. Je'Xperiment analysis workflow. JEX begins with the initial image in the timelapse (1); then the user selects a target cell (2); then the software performs a convolution (3) and locates the cells based on the convolved image (4). The cells are tracked from frame to frame (5), and filtering can be applied to further remove erythrocytes or cells that were not well-tracked (6). The software outputs a list of position vectors for each track and automatically yields outputs of interest (7), and has an interface to compare data over multiple samples and outputs (8).

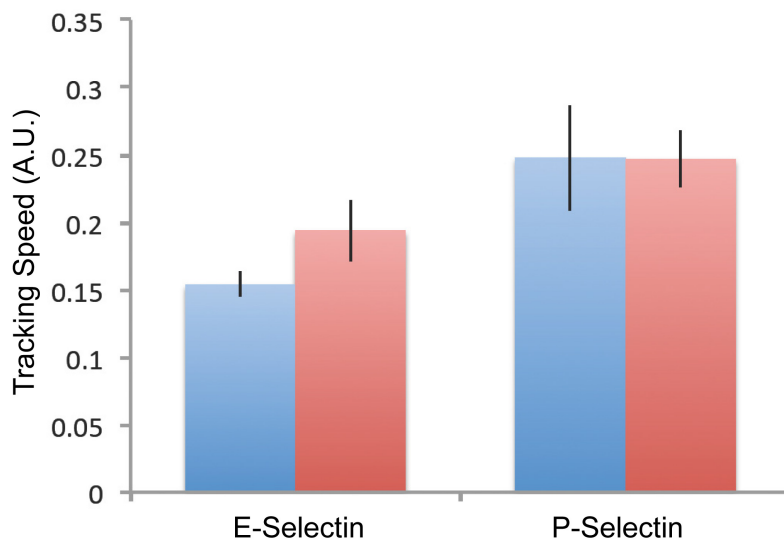
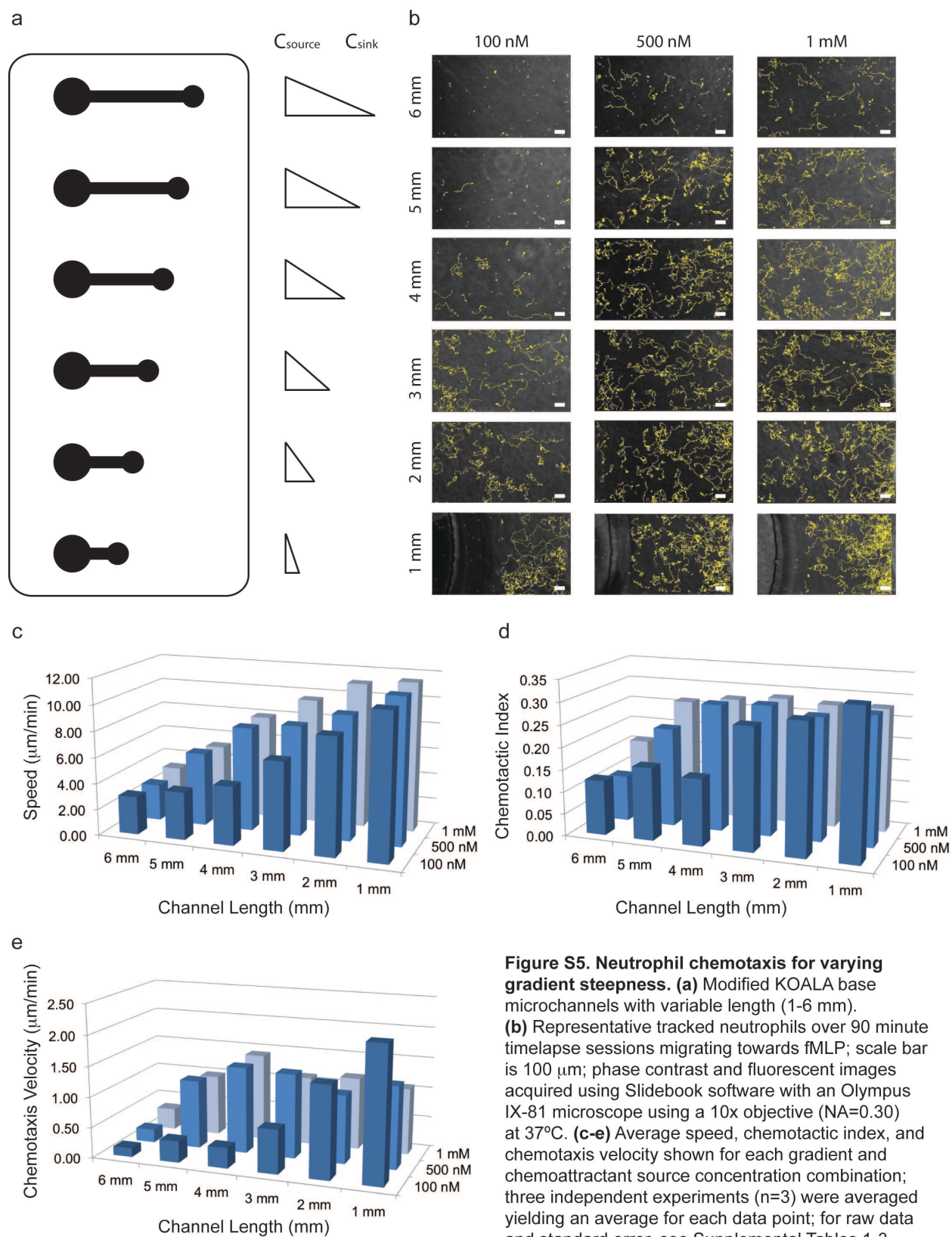


Figure S4. Je'Xperiment vs. ImageJ. Comparison of neutrophil chemotaxis speed from tracking data obtained automatically using Je'Xperiment and manually using the Cell-tracker plugin of ImageJ for neutrophils captured on a P/E-selectin coated substrate. Results show that automated tracking produces

results equivalent to manual tracking for P-selectin. The difference for the E-selectin can reside in the decreased capture efficiency, and therefore a higher erythrocyte count that be tracked by JEX, which can lead to erroneous tracking.



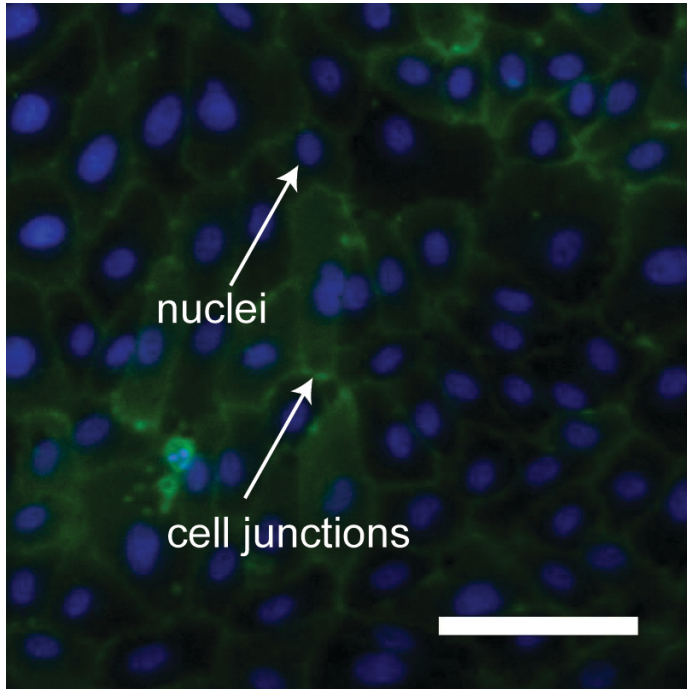
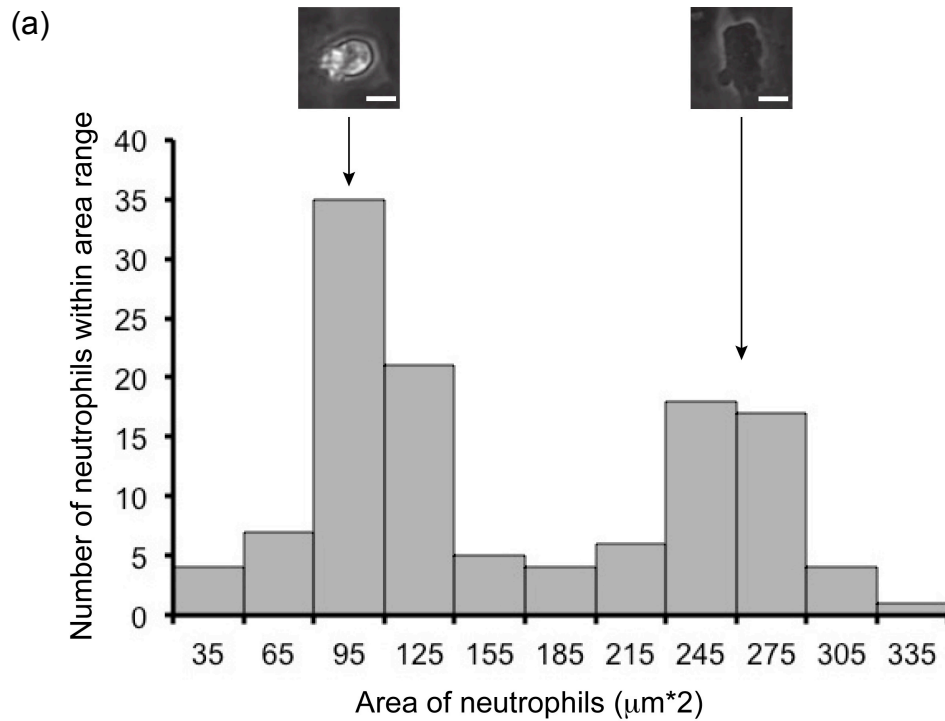


Figure S6. PECAM-1 expression on HUVECs. Micrograph of staining for PECAM-1 showing that a confluent monolayer was achieved with intercellular junctions expressing PECAM-1. Reliable formation of a confluent monolayer was critical for the successful integration of endothelial cells in the intraluminal crawling embodiment of KOALA. Scale bar 50 μm . See HUVEC immunostaining in Supplemental Methods for details on image acquisition.



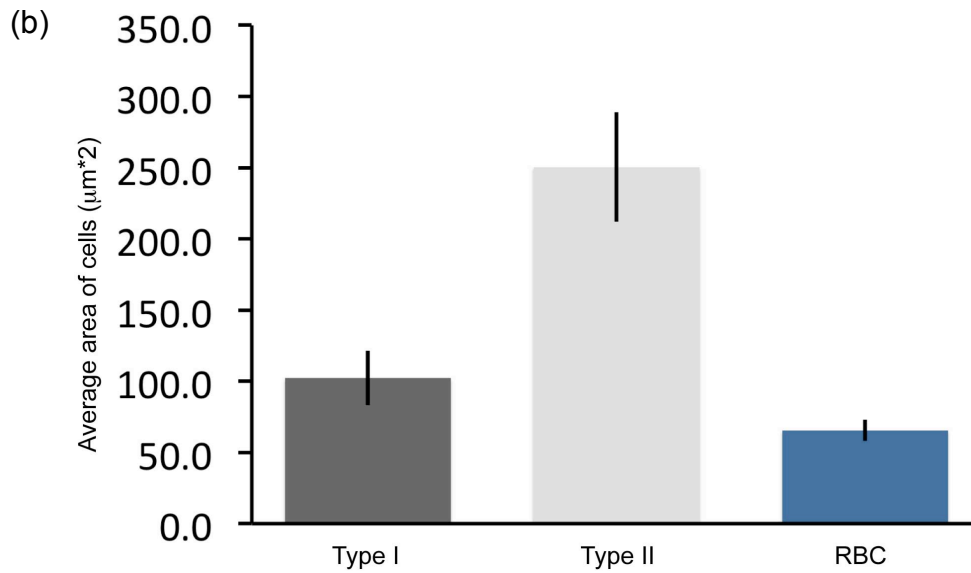


Figure S7. Area of Type I and Type II neutrophils as they appear under phase contrast microscopy.

(a) Area histogram of randomly selected neutrophils showing a bimodal distribution of area the area of neutrophils; at each mode we observe two distinct neutrophil morphologies, which we termed Type I and II for reference. **(b)** The average area for the different neutrophil morphologies are shown, along with the average area of red blood cells (RBC) for reference. The standard deviation is reported for each area measurement. The area was measured for 59 Type I, 52 Type II, and 25 RBC's over 6 microchannels.

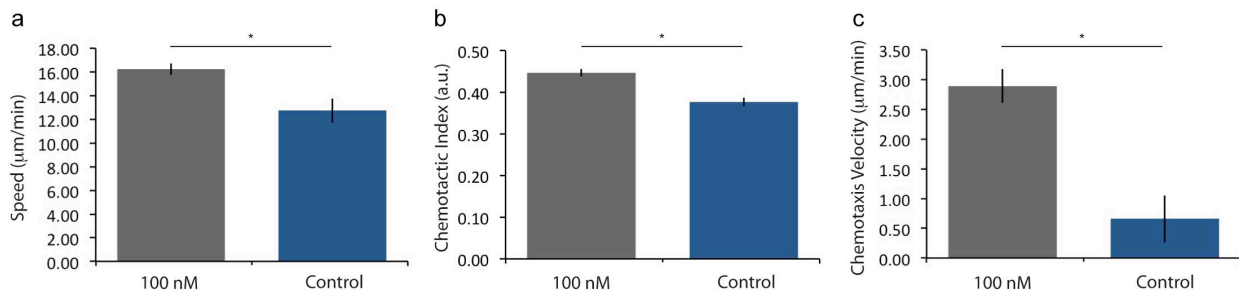


Figure S8. Comparing migration for neutrophils on ECS with a chemical gradient and control. (a-c)

Average speed, chemotactic index, and chemotaxis velocity for neutrophils migrating on a monolayer of activated HUVECs with 100 nM gradient of fMLP and control ($n=3$). HUVECs were activated with IL1- β for 4 hours prior to neutrophil capture and purification from whole blood. All three chemotaxis outputs were significantly higher for neutrophils in a chemical gradient compared to controls, where slower, less directional migration was observed ($*p<0.05$).

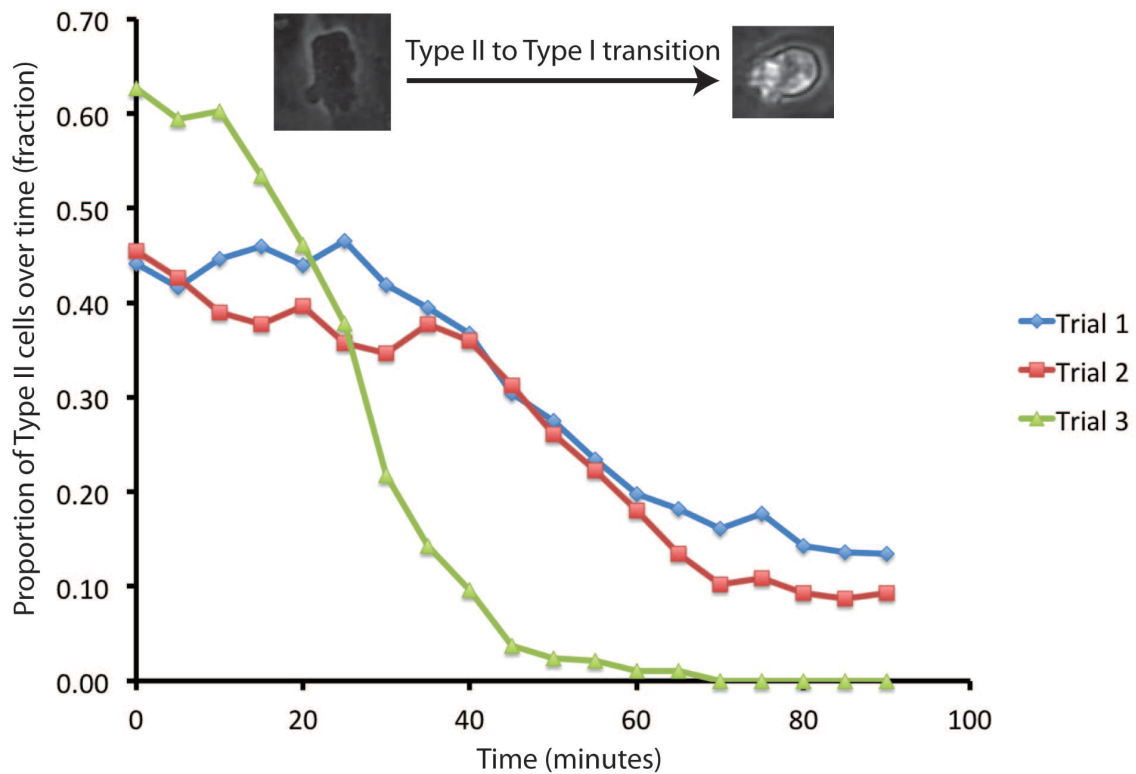


Figure S9 Characterizing the transition of neutrophil morphology from Type II to Type I over time.

The neutrophils begin roughly evenly split between Type I and Type II morphologies; however, Type II cells transition to Type I after about 30-45 minutes into the timelapse session. Slight increases in proportion of Type II cells are not due to neutrophils changing from Type I to Type II, but rather due to neutrophils of each type entering and leaving the analysis window. Each trial shows analysis on one representative microchannel for a 90 minute timelapse session (n=3 total).

Supplemental Videos

Video 1 Demonstration of the intraluminal crawling assay in KOALA. Human neutrophils migrating towards 100 nM fMLP on a HUVECs monolayer, tracked automatically with JEX. The timelapse is 90 minutes long. Note that the erythrocytes along the edge of the channel remain relatively static, showing the KOALA method enables a static flow, diffusion-dominant microenvironment; the erythrocytes are not completely washed because the culture of HUVECs in the microchannel requires taller microchannels that do allow for efficient cell sorting. The source of chemoattractant is located on the left side of the video. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details in on imaging Materials and Methods section under “Microscopy.”

Video 2 Example of human neutrophils migrating in a gradient of chemoattractant. Human neutrophils migrating towards 500 nM fMLP on P-selectin-coated polystyrene, tracked automatically with JEX. A channel length of 3 mm is shown, which represents the geometry used for all the embodiments of KOALA shown in this work. The timelapse is 90 minutes long. The source of chemoattractant is located on the left side of the video. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”

Video 3 Example of control (no chemoattractant) for 2D chemotaxis in KOALA. Human neutrophils in 2D on P-selectin-coated polystyrene, with hydrogel and media (no chemoattractant mixed in the hydrogel). Neutrophils show virtually no movement throughout the timelapse. Note that some erythrocyte movement is observable over the course of the timelapse, likely due to a temperature gradient in the microscope incubator causing slight fluid convection. The timelapse is 120 minutes long. The source, which in this case does not contain chemoattractant, is located on the left side of the video. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”

Video 4 Demonstration of the intraluminal crawling assay in KOALA with no chemoattractant. Human neutrophils migrating towards 0 nM fMLP (control) on a HUVECs monolayer, tracked automatically with JEX. The timelapse is 90 minutes long. The source of chemoattractant is located on the left side of the video. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details in on imaging Materials and Methods section under “Microscopy.”

Video S5 Transient Type I and II neutrophil phenotypes on HUVECs. Zoomed in view of neutrophils migrating on HUVECs showing the cells switching between the transient Type I and II phenotypes; the video is 45 minutes long. The source of chemoattractant is located on the left side of the video. In most cases, neutrophils that adopted a Type II phenotype eventually adopted the behavior and morphology of originally Type I cells. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”

Video S6 WT mouse neutrophils in a gradient of chemoattractant. WT mouse neutrophils migrating on P-selecting coated polystyrene surface towards 1 mM fMLP, automatically tracked with JEX. The timelapse is 120 minutes long. The source of chemoattractant is located on the left side of the video. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”

Video S7 TNF-Tg mouse neutrophils in a gradient of chemoattractant. TNF-Tg mouse neutrophils migrating on P-selecting coated polystyrene surface towards 1 mM fMLP, automatically tracked with JEX. The timelapse is 120 minutes long. The source of chemoattractant is located on the left side of the video.

Note that significantly more cells have been captured on the P-selectin coated surface than in Video S3. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”

Video S8 3D human neutrophil chemotaxis. Neutrophils obtained from a human using a lancet puncture, migrating towards 100 nM fMLP in a 3D collagen matrix [5 mg/mL]. The source of chemoattractant is located on the left side of the video. Note that several cells are in and out of the focal plane throughout the time-lapse session, however JEX software analyzes the cells’ 2D projected path. The timelapse is 90 minutes long. Phase contrast images acquired using Slidebook software with an Olympus IX-81 microscope using a 10x objective (NA=0.30) at 37°C. Additional details on imaging in Materials and Methods section under “Microscopy.”