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Image-based analysis of primary human neutrophil chemotaxis in an automated direct-viewing assay

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Abstract

Multi-well assays based on the Boyden chamber have enabled highly parallel studies of chemotaxis – the directional migration of cells in response to molecular gradients – while direct-viewing approaches have allowed more detailed questions to be asked at low throughput. Boyden-based plates provide a count of cells that pass through a membrane, but no information about cell appearance. In contrast, direct viewing devices enable the observation of cells during chemotaxis, which allows measurement of many parameters including area, shape, and location. Here we show automated chemotaxis and cell morphology assays in a 96-unit direct-viewing plate. Using only 12,000 primary human neutrophils per datum, we measured dose-dependent stimulation and inhibition of chemotaxis and quantified the effects of inhibitors on cell area and elongation. With 60 parallel conditions we demonstrated 5-fold increase in throughput compared to previously reported direct viewing approaches.

Keywords

Chemotaxis; Neutrophils; High-content Analysis; Microfluidics

1. Introduction

While modified Boyden chambers (Boyden, 1962) in multiwell format are the current standard for highly parallel studies of chemotaxis (Frevert et al., 1998; Schepetkin et al., 2007; Vishwanath et al., 2005), direct-viewing approaches, where cells migrate on a horizontal surface, have particular advantages for quantitative studies, such as perturbation of function by drug candidates. The concentration profile experienced by cells in a Boyden chamber is unknown and may be influenced by cells as they traverse membrane pores (Zicha et al., 1991). In contrast, the concentration gradient produced in direct-viewing devices can be verified using fluorescent dyes (Zigmond, 1977; Zicha et al., 1991; Abhyankar et al., 2006). Furthermore, the geometry of modified Boyden chambers is not conducive to microscopic study of cells during chemotaxis, which precludes the use of high content analysis.

Direct-viewing methods for *in vitro* cell migration studies have a long history covering a wide variety of approaches, each with its own set of trade-offs in gradient parameters, optical clarity, ease of use, and cost. Zigmond (Zigmond, 1977) and Dunn (Zicha et al., 1991) chambers improved on the optics of the under agarose assay (Nelson et al., 1975),

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while all three are more precise than the glass capillaries used by Ketchel and Favour (Ketchel and Favour, 1955). Microfluidic approaches (Jeon et al., 2002; Wang et al., 2004; Tharp et al., 2006; Heit et al., 2008; Keenan and Folch, 2008; Meyvantsson and Beebe, 2008), in turn, have enabled a new level of spatiotemporal control, including defined gradient profiles of various shapes (Wang et al., 2004; Tharp et al., 2006) and fast switching between profiles (Irimia et al., 2006b). However, none of the direct-viewing approaches reported to date are suitable for highly parallel studies; the largest number of parallel conditions reported to date is twelve (Kanegasaki et al., 2003).

Many microfluidic gradient generators rely on continuous flow to maintain a constant gradient profile over time (Jeon et al., 2002; Irimia et al., 2006a; Mosadegh et al., 2007; Atencia et al., 2009). Since this demands a unique set of input sources for each experimental condition, highly parallel studies using continuous flow are intractable. Furthermore, exposing cells to flow can influence experimental results (Walker et al., 2005; Beta et al., 2008). Devices that do not employ flow must provide means to protect the concentration gradient from disturbances; nanoporous membranes (Abhyankar et al., 2006), hydrogels (Diao et al., 2006), valved compartments (Frevert et al., 2006), and fluid-level equilibration (Kanegasaki et al., 2003) are among those that have been used successfully.

The objective of our work was to develop a method that would provide the rich information of direct-viewing chemotaxis studies in an automated format with throughput comparable to Boyden-based plates. To this end a device was developed where the gradient was kept stable by shunting disturbing flows through a low resistance path. This simple design enabled the construction of large arrays of microfluidic units in which cell and reagent addition could be automated via surface tension-driven passive pumping (Walker and Beebe, 2002; Meyvantsson et al., 2008). Automation allowed highly parallel experiments to be run to study dose-dependent stimulation and inhibition of chemotaxis as well as chemoattractant and inhibitor effects on cell morphology.

2. Methods

2.1. Design, fabrication, and general operation

Each microfluidic unit has five components as shown in Figure 1b. The attractant and cell port are both 2 mm deep; their volumes are approximately 2.85 μl and 8.86 μl , respectively. The gradient channel is 85 μm deep, 1 mm long, and 2 mm wide, but fans out slightly on the source end; the gradient channel volume is around 209 nl. The source and shunt channels are 3.42 μl and 9.38 μl , respectively. The microfluidic channels were injection molded in cyclo-olefin polymer, and sealed with a 0.25 mm thick film of the same material via laser welding. The internal surfaces of the microfluidic channels were made hydrophilic by tissue culture treatment. Unless otherwise stated a 96-tip CyBiWell (CyBio, Jena, Germany) automated liquid handling system was used for dispensing. The plate had reservoirs in between the microfluidic units and at the edges that were filled with phosphate buffered saline (PBS, Thermo Fisher Scientific) to minimize evaporation.

The plate is generally used as follows: Starting with a dry plate, 20 μl of cell culture media (RPMI + 10% FBS) containing inhibitors is dispensed at the attractant port. The media fills the source, gradient, and shunt channels via capillary action (see Figure 1c–f). A cell suspension is dispensed to the cell-addition port where they settle adjacent to the gradient channel. The plate is incubated for 30 minutes for compound treatment (otherwise 10 minutes to ensure cells have settled). To introduce chemoattractant, 3 μl solution is added to the attractant port and flows into the source channel via surface tension-based passive pumping (Walker and Beebe, 2002). The plate is incubated for 2.5 hours and subsequently

imaged using an automated inverted microscope. This protocol is often modified for specific applications, such as for morphology analysis as described below.

2.2. Gradient characterization

The formation of a gradient was characterized by wick-filling each channel with 20 μ l cell culture media (EGM2MV BulletKit from Lonza), dispensing 3 μ l microbead suspension to the cell port, and 3 μ l of a 10 μ M Alexa-Fluor[®]-594 (Life Technologies; MW 760 Da) to the attractant port. The lid was sealed with vacuum grease (Dow Corning) and images captured on an inverted microscope (Nikon TE2000) with a heated stage (37°C) at a 30 minute interval (Figures 2a–c). For this analysis 13 channels were chosen: one on each corner (A1, A12, H1, H12), one mid-way on each side (D1, D12, A6, H6), and five internal (B3, F3, D6, B9, F9); in the plate shown in Figure 1a, rows are labeled with letters A–H, and columns are labeled with numbers 1–12.

Octave software (www.gnu.org/software/octave) was used for image analysis. The gradient channel was automatically detected in each image and a 1.472-by-0.699 mm² region was selected such that it was centered on the gradient channel width and bordered with the cell port. The average intensity along the width was calculated for each pixel location along the length (see axis in Figure 1h). An average of two six-point standard curves was used to convert intensity to concentration for each whole plate measurement. A least squares line fit (Gnuplot, www.gnuplot.info) was calculated to determine the slope of the gradient as well as the concentration at the border between the gradient channel and cell port. To aid visualization of concentration vs. distance profile the data was binned in 36.8 μ m segments (average of 20 adjacent points) before calculating the average and standard deviation, and plotting the data.

To assess plate-to-plate repeatability we performed the above described analysis on four different plates (Figure 2d), except that only one time-point was captured. Each plate was kept in the incubator (37°C, 5% CO₂) for 2.5 hours and then brought to the microscope for imaging. To test the influence of external forces on gradient stability a plate was subjected to maximum speed motion, both vertically and horizontally, on a liquid handling platform and gradient parameters were quantified before and after.

2.3. Chemotaxis assays

Chemotaxis assays were run in Phenol-Red Free RPMI-1640 (Sigma) with 10% heat-inactivated either fetal bovine serum (FBS, Gibco) or 1% bovine serum albumin (BSA, Equitech Bio). Human-recombinant IL-8 was obtained from R&D systems, Wortmannin from MP Biomedicals, and Latrunculin B from Calbiochem. Polymorphonuclear (PMN) leukocytes (expected to be at least 90% neutrophils) were isolated from healthy donor blood (protocol approved by an institutional review board) using Polymorphprep (Accurate Chemical and Scientific Corp.) and labeled with calcein AM (Life Technologies). A 96-well cell source plate was prepared by adding 9 μ l of cells at a concentration of 4×10^6 cells/ml to each well. This is sufficient cell suspension for two chemotaxis assay plates to be seeded in series. 20 μ l media with or without inhibitor (or containing IL-8 for uniform concentration experiment) was added to each attractant port filling the microfluidic units via capillary action. The cell suspension was mixed in the source plate and 3 μ l transferred to the chemotaxis assay plate and the plate lidded immediately. The plate was placed in a humidified secondary container (Bioassay dish, Corning) and the cells allowed to settle for 10 minutes at room temperature. If treating with inhibitors, the cells were pre-incubated in the plate for 30 minutes (37°C, 5% CO₂). 3 μ l of chemoattractant was added to the attractant port and the plate incubated for 2.5 hours.

Image acquisition of each channel was done on an inverted microscope with an automated stage. The gradient region of each image was cropped manually, and the number of cells that had migrated into the gradient channel was quantified using Metamorph's count nuclei application module (Molecular Devices). Curve fits, box-whiskers analysis, IC₅₀-, and EC₅₀-values were calculated using Prism (GraphPad Software).

2.4. Morphology analysis

Cell suspension, media and inhibitors were prepared the same way as described above for chemotaxis assays. Neutrophils were seeded in the gradient channel by adding 3 μ l to the attractant port of a dry plate followed by 17 μ l media added to the attractant port; where uniform cytokine concentration was desired this contained IL-8 at concentrations 0.4 or 4 nM; two sets contained inhibitors: 1 μ M Latrunculin B, and 10 μ M Wortmannin respectively. After 30 minute incubation (37°C, 5% CO₂), 3 μ l were added to the attractant port; for conditions where a gradient was desired this was a 62 nM IL-8 solution; where uniform concentration was desired this matched the IL-8 or inhibitor concentration already present. Ten replicates were included for each condition. After 1.5 hour incubation the plate was transferred to a heated stage (37°C) and imaged immediately. Imaging was completed in 10 minutes.

Using image analysis software (CellProfiler, www.cellprofiler.org) objects ranging 10–100 pixels in diameter (automated threshold determined using Otsu's global method) were identified. For each object, the area and elongation (the ratio of the distance between the foci to the major axis of an ellipse whose second moments are equal to those of the object) were found, and the average reported for each image. The average number of cells per image was 82. Statistical comparisons were made using the two-tailed student's T-test (normal distributions with unknown means and unknown equal variances). The motorized microscope stage was used to calibrate pixels to microns and convert area values from pixels to μ m².

3. Results

3.1. Design and operation

To facilitate automation an array of 96 microfluidic units was produced in a plate dimensioned according to microplate standards (Figure 1a). As shown in Figure 1b, each feature had two access ports (attractant port and cell port) and three channels (source channel, gradient channel, and shunt channel). In forming a molecular gradient the source channel and cell port served as source and sink, respectively. The gradient was established in the gradient channel, which had a high fluidic resistance compared to the shunt channel – a ratio of approximately 40:1. Thus only 2.5% of any volume displacement between the two access ports would be predicted to occur through the gradient channel.

Chemotaxis and morphology assays were set up through a series of dispensing and incubation steps. The inside surfaces of each microfluidic feature were hydrophilic. Through capillary action (Figure 1c–f), the channels were filled with media containing biomolecules and compounds for treatment. The sharp expansion of the geometry at the border between the gradient channel and cell port ensures that wick-filling the shunt channel is energetically favorable over filling the cell port (Figure 1e); this prevents a bubble from being trapped in the shunt channel. For chemotaxis assays, cell suspension was added to the cell port, where the cells settled to the bottom adjacent to the gradient channel. Figure 1i shows a histogram of cell distribution (mean and standard deviation; N=13) at t=0 as a function of location in the cell port (see axis in Figure 1h). After a period of incubation the chemoattractant solution was dispensed to the attractant port to initiate the gradient (Figure 1g–h). For cell

morphology assays, the cells were added prior to filling with media such that they were present in the gradient channel at the beginning of the assay. Surface tension-based passive pumping (Walker and Beebe, 2002; Berthier and Beebe, 2007; Meyvantsson et al., 2008) ensured that the chemoattractant flowed from the attractant port into the source channel, while minimal flow occurred when the cell suspension was added to the cell port. The chemoattractant diffused through the gradient channel to form a gradient.

3.2. Gradient characterization

A fluorescent dye was used to model the chemoattractant gradient and the gradient profile was analyzed in one dimension with the origin at the border between the gradient channel and the cell port and positive direction toward the source (Figure 1h). Figure 2a shows the gradient profile at 0, 0.5, 2.5 and 10 hours. The gradient was established – i.e. reached linearity with maximal steepness – in less than 0.5 hours and then dissipated slowly over time. A least squares line fit was found for the concentration vs. distance data and the average slope and concentration at the origin plotted for each time point as shown in Figures 2b and 2c, respectively. The concentration at the origin is that experienced by cells as they enter the gradient channel. A comparison of the experimental data to the fitted lines indicated that the gradient was linear.

For a 10 μM source, the slope of the gradient decreased from 1.49 to 1.19 $\mu\text{M}/\text{mm}$ between 0.5 and 2.5 hours. In this period the concentration at the origin rose from 0.28 to 0.38 μM . After another 7.5 hours ($t = 10 \text{ h}$) the slope and concentration at the origin were 0.63 $\mu\text{M}/\text{mm}$ and 0.52 μM , respectively. Data from the 0.5–2.5 hour time period represents the incubation time in neutrophil chemotaxis experiments described below; the slope dropped by less than 21% and the concentration at the cell entrance to the gradient channel rose by less than 37% during this period. Over the following 7.5 hours, the slope dropped to 42% of its original value, while the origin concentration rose to 85% above the initial value. On average during the 10 hour time course, the concentration at the gradient-cell port junction was 3.9 % of the source concentration. The same analysis applied to four different plates shown in Figure 2d indicates good plate-to-plate repeatability.

To assess the influence of external forces on gradient stability gradient profiles were quantified before and after vigorous motion both horizontally and vertically. The slope dropped from 1.04 to 0.95 $\mu\text{M}/\text{mm}$ and the concentration at the origin dropped from 2.75 to 2.18 μM .

3.3. Chemotaxis assays

Chemotaxis can be quantified by counting the cells in the gradient channel after incubation; migration can bring neutrophils across the full 1 mm channel length in less than two hours. To determine the experimental window for this method the number of neutrophils that enter the gradient channel with an interleukin-8 (IL-8) gradient formed by a 500 nM source (generally produces maximum effect, EC_{100}) was compared to that with no chemoattractant present. These conditions yielded 784 ± 152 (s.d., $N=28$) and 0.3 ± 0.5 cells in the channel, respectively. This corresponds to a Z' – a statistical measure of screening data quality (Zhang et al., 1999) – of 0.42. Representative images with and without IL-8 are shown in Figures 3a and 3b, respectively.

Neutrophils can be stimulated to migrate non-directionally (chemokinesis) by a uniform IL-8 concentration. The response of neutrophils to uniform IL-8 concentrations was tested as shown in Figures 3c, 3d, 3e, and 3f (■); the graph in Figure 3f (■) shows the number of cells that had reached the gradient channel at the end of the incubation period. The strongest response to uniform IL-8, observed at 4.38 nM IL-8, was 79 ± 16 cells (s.d., $N=4$). In

contrast, a 500 nM IL-8 gradient control included in the experiment yielded 861 ± 193 cells (s.d., $N=24$). This suggests that the chemotactic index – the ratio between the number of responding cells in a gradient to that under uniform concentration (Arai et al., 1997) – was at least 10.9 under these conditions. In a separate experiment the dose dependent chemotactic response to interleukin-8 (IL-8) gradients was determined as shown in Figure 3f (●). The half maximal effective concentration (EC_{50}) was determined to be 155 nM (95% confidence interval spans 0.46 log-molar units). These data were also analyzed on the basis of cell position in the gradient channel. A box-whiskers plot is shown in Figure 3g. The greatest average distance reached relative to the gradient-cell port border was observed at 24 nM source concentration.

Using 500 nM IL-8 gradients neutrophils were exposed to the phospho-inositide 3-kinase inhibitor Wortmannin, and the actin monomer stabilizer Latrunculin B (Lat B), and the dose dependent inhibition of chemotaxis studied (Figures 2h–i). The half-maximum inhibition concentrations (IC_{50}) were determined to be $2.32 \mu\text{M}$, and 10.8 nM for Wortmannin and Latrunculin B, respectively; the 95% confidence intervals for these determinations spanned 0.84 and 0.29 log-molar units, respectively (three separate experiments yielded similar IC_{50} values; data not shown).

3.4. Morphology assays

During chemotaxis neutrophils undergo pronounced changes in morphology (Stephens et al., 2008). The cells make firm attachments to the substrate, spread out and form protrusions at the front and narrow appendages at the trailing edge, which give the cells an elongated appearance. Inhibitors can influence cell shape in different ways depending on their mechanism. The morphology of cells was studied in the absence of IL-8, with a uniform concentration, and with an IL-8 gradient in the presence and absence of inhibitors.

The results from an analysis of cell morphology are shown in Table 1 and Figure 4. In the absence of IL-8 (control) the cells remained round with a small average area, while in the presence of IL-8 they acquired the flattened and elongated morphology characteristic of migration to varying extents. With increasing stimulus a greater fraction of cells responded. A relatively low uniform concentration of IL-8, 0.4 nM, caused no detectable increase in average cell area, while a uniform 4 nM concentration – equal to, and two-fold higher than the K_d of CXCR1 and CXCR2, respectively (Schnitzel et al., 1994) – resulted in a slight increase in area. In the presence of an IL-8 gradient (62 nM source concentration) the average area was 15.6% greater than that of the no chemoattractant control. On the other hand, cells exposed to the same gradient together with inhibitor Wortmannin were indistinguishable from the control. Interestingly, the largest increase in cell area was observed when the inhibitor Latrunculin B was added in the presence of an IL-8 gradient: 35.6 % greater than the control.

Although the cells treated with Latrunculin B spread out to a greater degree than those under any other condition, their shape was distinctly different from other spread cells. To capture this difference we studied elongation. While minimal for cells in the absence of chemoattractant (control) and with uniform 0.4 nM IL-8, the average elongation was substantial with 4 nM uniform IL-8. The elongation observed among cells exposed to an IL-8 gradient was more than double that of the control. When inhibited with Latrunculin B and Wortmannin, the average elongation was reduced significantly relative to the gradient condition ($p = 1.82 \times 10^{-7}$, and $p = 2.00 \times 10^{-14}$, respectively). However, the morphological effects of the two inhibitors were different. In the case of Wortmannin, most of the cells show the round morphology of the controls, and only a small fraction are fully elongated. This is reflected in average area and elongation statistically indistinguishable from the

control. In contrast, the Lat B-treated cells have a larger, round shape that is distinct from the control cells.

4. Discussion

The direct-viewing chemotaxis plate described above provides a simple and reproducible way to perform high content analysis of neutrophil chemotaxis. Compared to previously reported direct-viewing approaches, this plate enables an unprecedented level of parallelism; 60 conditions were tested in parallel using automated dispensing and imaging. This is a 5-fold improvement over the 12 replicate system reported by Kanegasaki et al. (Kanegasaki et al., 2003), which to our knowledge is the largest number of direct-viewing assay replicates reported to date using a single device. While Boyden-based plates can be automated, they do not allow imaging during chemotaxis. Cell consumption is also significantly lower; we used 1.2×10^4 cells per datum, compared to 7.5×10^4 to 2×10^6 cells required for Boyden-based assays (Kawohl et al., 1980; Frevert et al., 1998; Schepetkin et al., 2007). Given appropriate imaging equipment, the full 96 channels of the plate can readily be used for analysis. This is routinely done with cell count-based readouts at magnification lower than that used in the morphology analysis.

It is important to distinguish between randomly oriented migration response (chemokinesis) and that directed according to a molecular gradient (chemotaxis). The characterization of the gradient profile (Figure 1) suggests that the chemoattractant concentration close to the junction between gradient channel and cell port would differ by 5.3, 3.1, and 1.2 % across the approximate length of a neutrophil ($10 \mu\text{m}$) at 0.5, 2.5, and 10 hours, respectively. These values correspond well to the reported sensitivity of neutrophils, which can sense gradients where concentration varies only 1–2 % across the length of the cell (Servant et al., 2000). The observation that neutrophil migration in the presence of IL-8 gradients was at least 10.9-fold greater than that in the presence of uniform IL-8 (chemotactic index) across a large range of concentrations (Figure 3f and Supplementary figure 1) demonstrates that the response observed was mainly due to chemotaxis.

The results from chemotaxis assays depend on a variety of factors including donor variability, media composition, and readout method. Based on the gradient characterization shown in Figure 1, we would expect cells entering the gradient channel to see only around 3.9 % of the source concentration, which would, for the half-maximal effect of IL-8 put the concentration at the gradient-cell port border at 6 nM. This is somewhat higher than previously reported values, which range from 0.3 to 3 nM (Smart and Casale, 1993; Frevert et al., 1998) for Boyden-based studies, depending on assay protocol (e.g. readout method). This disparity may be explained in part by the assay format differences: the assay presented here requires cells to migrate several hundred microns horizontally, while Boyden-based experiments depend on movement across tens of microns vertically – in the direction of gravity.

While cell count readouts provide data analogous to that from modified Boyden chambers, the box-whiskers analysis reveals a different facet of the response. The EC_{100} for the greatest average distance (Figure 3g) is around 24 nM (source concentration), which is significantly lower than the EC_{100} for cell number (Figure 3f). Interestingly, the greatest average distance from the gradient-cell port junction occurs at 24 nM source concentration; this corresponds to 0.936 nM at the junction, which falls within the range of reported values for IL-8 half maximal effect.

For inhibition with Lat B, our results are consistent with reports that 100 nM Latrunculin A (similar, but slightly less potent than Lat B (Spector et al., 1989)) almost completely inhibits

chemotaxis in a Boyden-based assay (Pring et al., 2002). For inhibition with Wortmannin, reports using Boyden-based assays range from no inhibition at 1 μ M (ref. (Thelen et al., 1995)) to $IC_{50} = 26$ nM (ref. (Knall et al., 1997)); in these studies different media recipes and IL-8 concentrations were used, which may have caused the discrepancy. We found the IC_{50} to be two orders of magnitude higher than that reported by Knall et al. (Knall et al., 1997). Our findings demonstrate that the approach presented above is suitable for robust quantification of neutrophil chemotaxis, and our results fall within the range of results reported from Boyden-based studies.

Cell migration is associated with well characterized morphological changes (Stephens et al., 2008). The simple two-parameter morphology analysis we performed revealed important differences in the effects of Lat B and Wortmannin on neutrophil chemotaxis. Both inhibitors were used at concentrations that fully inhibited the migration of cells from the cell port into the gradient channel (Figures 2h and 2i). At this concentration Wortmannin caused cells to adopt a shape that was indistinguishable from the no chemoattractant control. Lat B on the other hand caused the cells to expand to a larger diameter with some elongation. This is consistent with disruption of polarization, which relies on actin polymerization inhibited by Lat B. Notably, the difference in elongation between 4 nM uniform chemoattractant and untreated control was statistically significant ($p = 4.69 \times 10^{-10}$), while the cell area under these two conditions was not significantly different. Taken together, these findings underscore the advantages of using image-based analysis to quantify multiple parameters – i.e. area and elongation in addition to cell count.

Future work will include development of small molecule and immunological staining protocols for the direct-viewing assay plate. Automated chemotaxis assays with such readouts will enable a vast amount of information to be gathered which can be utilized to explore the molecular mechanisms involved in chemotaxis as well as inhibition of this cellular process.

5. Conclusion

Full compatibility with conventional laboratory automation infrastructure, such as automated microscopy and 96-tip liquid handling, allows immediate use of the direct-viewing assay plate in cell biology laboratories in both academia and industry. Combined with the rapid progress in high content analysis instrumentation and software, this plate opens new possibilities in highly parallel quantitative analysis of chemotaxis. These improvements may be useful in efforts to elucidate the role of neutrophils in diseases such as rheumatoid arthritis and chronic obstructive pulmonary disorder, as well as in the discovery of improved therapies targeting inflammation (Mackay, 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Direct-viewing plate supports wick-filling and cell patterning

a) The plate (bottom view) has 96 microfluidic units located in a rectangular grid pattern with 9 mm $\times$ 9 mm spacing. b) Each unit has five components: an attractant port and source channel where a chemoattractant is provided, a gradient channel connected to the source into which the chemoattractant diffuses to create a gradient, a sink (cell port) located at the other end of the gradient channel, and a shunt channel that helps ensure a stable gradient by diverting flow away from the gradient channel. “s-s” shows a cross-section along the dashed line. c–f) A time sequence showing wick-filling. c–d) Since the internal surfaces are hydrophilic, aqueous solutions are drawn into the channels via capillary action. e) Due to the sharp increase in height at the junction between the gradient channel and cell port, the lowest energy path for filling is around the shunt channel. f) Once the shunt channel has been filled, the cell port is filled up from both ends. g–h) A schematic representation of a chemotaxis assay. For morphology assays the cells were seeded in a different way (see text). g) After wick-filling each unit with cell culture media, cells were seeded in the cell port and a chemoattractant solution was loaded in the source channel. h) After a period of time the chemoattractant diffused into the gradient channel and formed a gradient. Chemotactic cells moved into the gradient channel toward increasing concentration. The origin was set to the junction between the cell port and gradient channel. The location of the cells was recorded relative to the origin. i) At the beginning of the assay the cells are distributed in a repeatable pattern in the cell port (N=13).

Figure 2. The plate provides a repeatable linear gradient

a–d) Characterization of a molecular gradient (AlexaFluor® 594) at 37°C. The graphs show a) gradient profile at 0, 0.5, 2.5 and 10 hours with best fit lines for the last three, b) the slope, and c) the concentration at the origin as determined by least squares line fit for each replicate. Graphs show mean values and standard deviations (N=13). d) The bar graph shows the slope (red) and concentration (green) at 2.5 hours in four different plates run at the same time.

Figure 3. Dose-dependent stimulation and inhibition of chemotaxis

a–b) Cell distribution at the end of the assay after 2.5 hour incubation. a) 500 nM interleukin-8 (IL-8) gradient. b) no chemoattractant control. c–e) Cell migration in the presence of uniform IL-8 at c) 0.27 nM, d) 4.38 nM, and e) 560 nM. f) Dose dependant response to IL-8 gradients. The graph shows the mean and standard deviation for replicates of 4, except where indicated (“*” denotes replicate of 3). g) A box-whiskers plot (10%, 25%, 50%, 75%, 90%) indicating the distribution of cell positions at the assay endpoint as a function of IL-8 gradient source concentration. h–i) Dose-dependent inhibition of IL-8 mediated chemotaxis (500 nM source). Half-maximum inhibition concentrations (IC_{50}) were found to be 10.8 nM for Latrunculin B and 2.32 μ M for Wortmannin.

Figure 4. High content analysis reveals inhibitor effects on cell morphology

Inhibition of neutrophil chemotaxis is associated with inhibitor-specific changes in area and elongation. Neutrophils were placed under experimental conditions with various concentration profiles of IL-8: a) none, b) uniform 0.4 nM, c) uniform 4 nM, or d-f) gradient with 62 nM source. The last condition was tested with d) no inhibitor, e) 10 μ M Wortmannin, and f) 1 μ M Latrunculin B. g) Scatter plot showing area and elongation. Each point represents one of ten replicates and the rectangles are centered at the mean and sized according to the mean $\pm$ one standard deviation. Conditions shown are: control (+), uniform 0.4 nM IL-8 ($\blacktriangle$), uniform 4 nM IL-8 ($\triangle$), 62 nM IL-8 gradient ($\times$), gradient and 10 μ M Wortmannin ($\bullet$), gradient and 1 μ M Lat B ($\circ$), N=10 for each condition.

Table 1

Average area and elongation $\pm$ standard deviation for cells treated with chemoattractants and inhibitors (N=10). Data labeled with “*” and “**” were significantly different ($p < 10^{-4}$) from no attractant control and gradient without inhibitor conditions, respectively.

Attractant	Inhibitor	Area [μm^2]	Elongation []
-	-	169.4 $\pm$ 9.8	0.309 $\pm$ 0.025
Uniform 0.4 nM IL-8	-	162.3 $\pm$ 2.8	0.267 $\pm$ 0.016
Uniform 4 nM IL-8	-	177.6 $\pm$ 5.9	0.452 $\pm$ 0.028*
Gradient 62 nM IL-8	-	195.8 $\pm$ 13.3*	0.637 $\pm$ 0.034*
Gradient 62 nM IL-8	1 μM Lat B	229.7 $\pm$ 9.8*,**	0.526 $\pm$ 0.026*,**
Gradient 62 nM IL-8	10 μM Wort	169.8 $\pm$ 4.1**	0.330 $\pm$ 0.028**