Published in final edited form as:
Lab Chip. 2006 February ; 6(2): 191–198. doi:10.1039/b511877h.

Microfluidic system for measuring neutrophil migratory responses to fast switches of chemical gradients†

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Abstract

Experimental systems that provide temporal and spatial control of chemical gradients are required for probing into the complex mechanisms of eukaryotic cell chemotaxis. However, no current technique can simultaneously generate stable chemical gradients and allow fast gradient changes. We developed a microfluidic system with microstructured membranes for exposing neutrophils to fast and precise changes between stable, linear gradients of the known chemoattractant Interleukin-8 (IL-8). We observed that rapidly lowering the average concentration of IL-8 within a gradient, while preserving the direction of the gradient, resulted in temporary neutrophil depolarization. Fast reversal of the gradient direction while increasing or decreasing the average concentration also resulted in temporary depolarization. Neutrophils adapted and maintained their directional motility, only when the average gradient concentration was increased and the direction of the gradient preserved. Based on these observations we propose a two-component temporal sensing mechanism that uses variations of chemokine concentration averaged over the entire cell surface and localized at the leading edge, respectively, and directs neutrophil responses to changes in their chemical microenvironment.

Introduction

Neutrophils are an essential component of the first line of defense against infections and are capable of orienting themselves and migrating in complex environments of continuously changing chemical signals. Although many details of the molecular signaling networks inside the neutrophils have been identified, little is known about how all pieces work together and how individual cells measure the chemotactic gradients in their environment. A debate is still ongoing about whether spatial and/or temporal mechanisms regulate neutrophil chemotaxis.[1,2] Spatial sensing mechanisms are favored and suggest that neutrophils respond to chemotactic gradients by comparing the chemotactic concentrations at two distinct locations on their body and then amplifying the differences through positive and negative feedback loops.[3,4] Additional arguments supporting a spatial mechanism are

†Electronic supplementary information (ESI) available: Device characterization using fluorescent dyes (movies 01–05) and neutrophil migration in response to chemokine gradient switches (movies 06–10).

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the observation of pseudopod formation in a stationary neutrophil subsequent to gradient exposure[1] and the ability of neutrophils to follow a moving source of chemoattractant.[5] Temporal mechanisms for probing the gradients on the other hand, are based on comparisons of the concentrations in the same location at two successive time points. This mechanism, comparable to gradient sensing in prokaryotes, is supported by observations of neutrophil responses to waves of chemoattractant[6] and reversal of the direction of migration following concentration decrease of uniform chemotactic fields.[7]

One of the major obstacles in precisely identifying the eukaryotic sensing mechanism has been the lack of an experimental system that could provide controlled and reproducible changes of the chemical microenvironment around the cells during chemotaxis. Although several of the existing techniques today can generate relatively stable spatial chemical gradients,[8,9] most of these approaches have limitations on the control and reproducibility of temporal changes of these gradients. Assays using the Boyden chamber,[10] agarose or collagen gels, or shallow layer chambers, like the Dunn[11] or Zigmond[12] chambers, require several minutes for gradient stabilization and consequently are not appropriate for fast gradient changes. Previous attempts to gradient changes using microfluidic chemotaxis devices[13,14] also resulted in long switching times that limited their use to the generation of stable gradients only.[15] Micropipettes and photoactivated release of caged chemicals can generate new gradients in seconds, but these gradients can be easily perturbed by external factors and reproducibility of experimental conditions is generally a concern.[16,17] While the first group of techniques is generally used to study steady state cellular behavior, the second is usually employed in exploring fast cellular responses. Because the use of techniques for long-term observations cannot be extrapolated to faster changes and *vice versa*, there is currently little or no correlation between the two types of experimental observations.

We observed neutrophil migration and quantified neutrophil responses to four different types of gradient switches of the potent chemokine and neutrophil chemoattractant, Interleukin 8 (IL-8, CXCL8), in a microfluidic device. We incorporated microstructured membranes in two microfluidic gradient generators to achieve fast and accurate switches between any two stable gradients, with different directions and/or average concentrations. We evaluated the individual contribution of the temporal and spatial changes on neutrophil directional migration and found that neither the temporal nor the spatial sensing mechanisms alone can explain neutrophil behavior after the fast switches. Thus, we propose an alternative explanation based on a unitary model for neutrophil responses to gradients, in which a two-component temporal sensing mechanism directs neutrophil responses to gradient changes. Negative temporal variations of chemokine concentration localized at the leading edge or averaged over the entire cell are integrated to determine a stop of neutrophil migration and morphological depolarization.

Materials and methods

Device design, microfabrication and characterization

A microfluidic device was built of two layers of polydimethyl siloxane prepolymer (PDMS, Corning, Corning, NY) that were cast on two separate silicon wafers with photopatterned negative photoresist (SU8, Microchem, Andover, MA). One 250 μm thin PDMS layer incorporated the gradient generator network and the main channel. The thickness of the layer was controlled by spinning uncured PDMS on top of the corresponding wafer. A second, thicker PDMS layer (4 mm) incorporated the valve actuation chambers and a second channel network. The thick and the thin layers were bonded together after curing and exposure to oxygen plasma. Communications between the microfluidic networks on the two levels were accomplished *via* a series of channels. The double layer PDMS construct was then selectively bonded to a glass slide patterned with a thin (50 Å) chrome film (Fig. 1).

The lack of bonding between the PDMS and the metal film patterns prevented the immobilization of the microstructures on the diaphragm, while the irreversible bonding of the rest of the device to unprotected glass provided effective sealing of the fluidic networks.

Neutrophil preparation

Whole blood was drawn in the morning from healthy male volunteers by venipuncture into tubes containing sodium heparin (Becton Dickinson, San Jose, CA). Neutrophils were isolated by density centrifugation in Lymphocyte Separation Medium (ICN Pharmaceuticals, Aurora, OH) and purified by hypotonic lysis, as previously described.[18] Neutrophils were stored in media at 37 °C before being used in experiments.

Gradient switches and control experiments

Four 1 ml syringes were filled with human Interleukin-8 (IL-8; PeproTech, Rocky Hill, NJ) in Iscove's Modified Dulbecco's Medium (IMDM; Mediatech, Herndon, VA) with 0.5% (w/v) fetal bovine serum (FBS; Mediatech) at concentrations defining the lowest and highest level of the desired gradient (0, 12.5 nM, or 25 nM). A 1 μL min^{-1} flow rate was achieved for each syringe (0.74 mm s^{-1} linear velocity in the center of the main channel) by using two electromechanical pumps (Harvard Apparatus, Holliston, MA). Initially, all four valves were opened to create a steady-state condition. Flow into the main channel (100 × 450 μm) was then blocked and the output from both gradient networks directed to waste. The main channel was flushed with media through the cell-loading channel to clear residual chemokine. Neutrophils at an approximate concentration of 5×10^6 cells ml^{-1} were loaded uniformly across the migration channel and allowed to adhere for 1 min. The gradient was initiated by opening the valve from the desired network into the main channel and closing the drain valve. Cells were allowed to migrate for 10–15 min after which a switch in the gradient was performed. Only one gradient switch after the initial exposure was performed during each experiment. The formation, stability, and temporal evolution of the spatial gradients were assessed in separate experiments by flowing distilled water and fluorescein (Sigma Aldrich, St Louis, MI) solutions through the device. Gradient evaluation was always performed at the same set point along the migration channel. Cell migration following four different experimental and control gradient switches was examined. One "initial switch" was used to suddenly expose neutrophils to a 0 to 12.5 nM gradient of IL-8. Following the "step-down switch" a 12.5 to 25 nM gradient was replaced with a 0 to 12.5 nM IL-8 gradient. In a "step-up switch", a lower average concentration gradient (0 to 12.5 nM) was followed by a higher average concentration gradient (from 12.5 to 25 nM). In a "gradient flip" switch, the initial 0 to 12.5 nM gradient was switched to a gradient of the same slope and average concentration but in the opposite direction (12.5 to 0 nM). Finally, "control" switches to an identical gradient (0 to 12.5 nM IL-8) were used to test if eventual perturbations of flow during the switch were not affecting the neutrophil migration.

Image analysis

Migration was observed through a Nikon Eclipse TE2000-S microscope (Nikon, Japan). Brightfield images (10X) were taken every 10 s using a digital camera (Hamamatsu, Japan) controlled by IPLab 3.6.1 (Scanalytics, Fairfax, VA) and saved on a hard drive. Migrating cells were tracked using the automated tracking feature in MetaMorph (Universal Imaging, Downington, PA) and data further analyzed using Matlab software (The MathWorks, Natick, MA). Only neutrophils that migrated more than 20 μm (equivalent to 2 cell lengths) in the initial gradient and after the gradient switch were taken into account in quantification of the response time and were defined as exhibiting a directionally biased migratory response to the chemokine gradient. Cells that remained within a 20 μm radius of their original position were excluded from subsequent analysis, as well as cells that migrated against the gradient persistently or stopped moving along the gradient before the switch.

Individual cell response time to the initial gradient was the time measured for a migratory neutrophil to reach a relative displacement threshold equal to 20% of normalized displacement from the beginning of the experiment to the time of the switch (15 min). Of the directionally migrating neutrophil at the time of the switch, only those that displaced more than 20 μm along the direction of the new gradient were considered when measuring the response time after switch. Neutrophils that either stopped, moved less than the set threshold (20 μm), moved against the gradient, or de-adhered from the surface were excluded from analysis. Similar to the initial response time, the response time after the switch was measured by taking 20% of the normalized displacement from the time of the switch to the end time point of experiments (15 min).

Statistical analysis

Neutrophil response times after switches and chemotactic indexes were calculated as mean and standard deviation. Initial response times and response times after switches were compared using one-way ANOVA.

Results

Microfluidic gradient switch

Precise temporal and spatial control over chemical gradients was achieved in a microfluidic device by implementing a complex network of channels and valves. Two autonomous and completely separated chemical gradients were produced in two gradient networks and alternatively directed through valves 2 and 3 (Fig. 2a) into the 450 μm wide main channel where cell motility was directly observed. Because the generation of the gradient in this type of networks is dependent on continuous fluid flow, waste channels, also controlled by valves 1 and 4 (Fig. 2a) were required for maintaining the gradient when a network was not used. To switch between gradients, the waste and main channel valves were operated in tandem. With valves 1 and 3 opened and valves 2 and 4 closed, the cells in the main channel were exposed to the gradient produced in the lower network. Alternatively, with valves 1 and 3 closed and valves 2 and 4 opened the gradient from the upper network was directed to the main channel (Fig. 2b). Because the two networks were independent, switching between any two gradients of choice was possible. In addition, with valves 2 and 3 towards the main channel closed, the cells could be loaded in the main channel completely isolated from the chemicals flowing through the networks.

The design and positioning of the valves were critical for the functioning of the device. The outlets of each gradient generator network were split symmetrically towards two pairs of nine outlet channels. A design for a microstructured membrane was developed to simultaneously control the four pairs from the two gradient generator networks (Fig. 2c). A membrane with dead-end channels in continuation of the outlet channels, each having rectangular cross-sections, 50 μm wide and 100 μm tall, could be deformed by changing the pressure in the actuation chamber above it (Fig. 2d). In closed position, the separating walls were pressed against the glass slide, forming a very effective seal between the soft PDMS and the flat metal valve seat, completely blocking the flow. When the pressure in the actuation chamber was decreased, the membrane and separating walls were lifted, the dead end outlet channels were opened in a common chamber and flow was established.

The valves controlling the flow in the main channel were positioned very close to the proximal end of the main channel such that the effect of their opening and closing was very fast on the gradient profile at the main channel level. We measured, using combinations of fluorescent dyes and distilled water, that for a flow rate of 1 μL min^{-1}, a complete flip of the direction of the gradient across the main channel is achieved in less than 5 s (Fig. 3 and

supplementary videos 1–5, see ESI†). Less than 5 s were needed for the formation of a gradient in a channel initially containing no chemical. Minimal perturbations of the gradient were observed following control switches involving changes between identical gradients from the two networks.

Cellular responses

The response of neutrophils to gradient switches was characterized based on cell morphology and quantitative measurements of displacement (Fig. 4). Individual cell tracks from representative cells are presented for each type of gradient switch (Fig. 5a and supplementary videos 6–10, see ESI†). Population averages of chemotropic index (CI) for an 8 min time window were also measured (Fig. 5b).

The response of resting, primary human neutrophils to the newly established gradient was gradual. Spherically shaped, resting neutrophils began forming ruffles on their entire surface immediately after the exposure to a gradient. With time, the protrusions on the cell surface decreased in number while increasing in size, until morphology characteristic of full polarization, represented by a leading edge and uropod, was achieved. Neutrophils that moved more than 20 μm along the axis of the generated gradient were defined as exhibiting a directionally biased migratory response to the chemokine gradient. Using these criteria, 15 neutrophils on average, representing between 40 and 70% of the total number of neutrophils in the field of view in each experiment, were tracked and included in subsequent quantitative analysis. The initial response time of resting neutrophils to gradients was defined on the basis of significant cellular displacement in the direction of the gradient. In order to account for the heterogeneity in cellular migration velocities, a relative threshold, equal to 20% of the total displacement over 15 min in the direction of the initial gradient, was established for each cell. The initial response time was defined as the time required for one resting neutrophil to reach that threshold. For neutrophils in the 0 to 12.5 nM IL-8 gradient (n = 424) this initial response time was 5.3 ± 2.5 min. No significant differences were observed between this and the response time for neutrophils in a 12.5 to 25 nM initial gradient (4.6 ± 2.6 min, p = 0.46).

Following step-up and control switches, neutrophils maintained their polarization and continued to migrate in the direction of the gradient. Brief gradient perturbations during the control switch (Fig. 3) did not alter neutrophil migration. Neutrophil directional polarization and average displacement following control switches were maintained, as well as the directional bias (CI 0.28 ± 0.41). After a step-up switch, a temporary depression in chemotactic response, reflected in a reduction of average CI from 0.34 ± 0.40 to 0.16 ± 0.35, occurred 70 s after the concentration increase (Fig. 5b).

In contrast, neutrophil responses were very different following the step-down and flip switches. Initially, neutrophils maintained their polarized morphology and continued to migrate in the direction of the previous gradient. After this delay, the morphology of the cells changed dramatically to a rounded, unpolarized shape. Neutrophils then progressed from the unpolarized state, through intermediary states presenting multiple and temporary protrusions, to the final polarized, migratory state, in a manner comparable to the initial response to the presentation of a chemokine gradient. Following a step-down switch, neutrophils transiently became unpolarized (Fig. 4 and Fig. 5a), and demonstrated a brief depression in chemotactic motion, from average CI 0.27 ± 0.36 to 0.08 ± 0.33, occurring 20 s after the switch. At 4.7 ± 2.2 min after the step-down switch chemotactic motion was resumed with an average CI of 0.34 ± 0.41 (Fig. 5b). Cells exposed to a gradient inversion also demonstrated a transient loss of polarization. Reorientation of cells and development of a chemotactic bias up the oppositely oriented gradient, from average CI 0.30 ±0.41 to 0.29 ±0.36, occurred 5.6 ±2.3 min after the gradient flip (Fig. 5b). No reversals of direction

without cell depolarization were observed following the gradient flip in the microfluidic device.

Similar to the initial response, a response time for migrating neutrophils after switches has been defined as the time required for one neutrophil to reach the 20% relative displacement threshold. The response time for the step-down gradients (4.7 ± 2.2 min) and flip gradients (5.6 ± 2.3 min) were statistically comparable to the time for the initial response to a gradient ($p = 0.37$). The response time for step-up gradients (3.2 ± 2.0 min) and control switches (3.6 ± 2.4 min) were significantly shorter ($p < 0.001$) than for the other switches, and were comparable to the average time required for migrating neutrophils to reach the 20% displacement threshold in the absence of a switch ($p = 0.32$).

Discussion

We developed a microfluidic device that allowed us to observe and quantify neutrophil directional motility in extremely stable spatial gradients and through precise temporal gradient changes. We used two separate microfluidic networks to independently generate two chemical gradients. While only one network was used at a time to generate a gradient over the neutrophils in the main channel, the flow was maintained through both microfluidic networks at all times during the operation of the device, to assure the availability of a pre-formed second gradient with no delay at the time of valve switching. Simultaneous draining of the second network to waste prevented uncontrolled diffusion and the extinction of the concentration gradient in the outlet channels when a network was not in use. Another technical aspect worth mentioning is the integration of the microstructured membranes in the device where they function as valves. This valve design allowed for simultaneous control of fluid flow through each set of nine individual channels with large (2: 1) height to width aspect ratio. This particular design allowed easy passage of cells and the gating of the cell-seeding channel very close to the main channel wall, and was an important advantage over other types of microfabricated elastomeric valves that could only be used to close shallow channels, with an aspect ratio less than 1: 2.[19–21]

We observed and quantified neutrophil responses to precise changes of the average concentration and direction of chemotactic gradients. The presented microfluidic device is the first experimental system where the loss of polarization after the gradient flip was observed systematically for all of the responsive neutrophils. Although the collapse of cell polarization following the change in the direction of the gradient has been observed before,[3,22] such events were usually less frequent compared to neutrophils turning around in the direction of the new gradient, in the absence of depolarization. Previously reported results described 38% rounded cells and 72% U-turns following gradient reversal in the Zigmond chamber,[23] and 60% rounded and 40% U-turns in micropipette displacement experiments.[3] By contrast, no U-turns were observed during fast gradient reversals using our device.

Our observations, supported by quantitative analysis of averaged chemotactic index and mean displacement for the cell population, revealed that when the gradient remained in the same direction and the average concentration went up, moving neutrophils adapted to the new conditions and continued their directed locomotion without interruption (Fig. 6). The only comparison we can make between our observation and previous reports is with neutrophil responses in a uniform chemokine environment following increases in chemokine concentration. In the absence of a gradient, neutrophils were reported to stop for 30 s and then resume random, chemokinetic motion after the concentration increased.[23,24] When the average concentration of the chemokine was reduced to half, neutrophils stopped migrating, depolarized and then eventually repolarized and resumed migration after a delay. Previous

reports using uniform-field concentration decrease reported a time of 150 s for the resumption of locomotion[24] or even reversing the direction of their polarization.[7] The absence of a chemokine gradient in previous experiments and the very fast development of the new gradient in our experiments are two important differences contributing to the observed discrepancies. When the direction of the chemokine gradient was flipped, we observed neutrophil depolarization regardless of average concentration increase or decrease.

The measured times for the initiation of persistent directional migration in the direction of the gradient (5.3 ± 2.5 min) were significantly shorter than the 15 min reported for neutrophil response in Zigmond chambers[12] or in filters.[25] The response time we observed is, however, longer than the polarization times between 80 s[24] and 2 min[26,22] reported for neutrophils responding to chemokines released from micropipettes. The wide range of results can be explained by differences in the speed of gradient formation and the precision of the criteria for evaluating the response time. While previous reports used cell spreading[24] or changes of morphological characteristics[26,22] to assess cell polarity, we made precise measurements of cell migration in the direction of the gradient and used these as the threshold criteria for neutrophil polarization. This approach allowed us to quantify the time for functional polarization of a neutrophil based on image analysis alone. The use of normalized displacement in the direction of the gradient also minimized the errors due to nonspecific effect of flow on neutrophil migration. For the slow flow rate used in our experiments the neutrophil trajectory deviations were about 10%, in concordance with previously reported observations.[27] Other potential sources of errors, like the distortion of the gradient due to the alterations of the laminar flow by the cells themselves, were avoided by the use of channels an order of magnitude larger than the cells.

One question arises as to the mechanisms of neutrophil responses following fast concentration and direction switches. Our present study suggests that neither the temporal nor the spatial gradient sensing mechanism alone could explain the particularities of these responses. The spatial sensing mechanism can justify the neutrophil response to step-up and gradient flip changes but fails to explain the depolarization of the neutrophil following the step-down switch. In this situation, the difference in chemokine concentrations at different locations on the neutrophil surface is preserved and consequently, according to the spatial sensing mechanism, neutrophil polarization should have been maintained despite decreasing average concentration. Similarly, the temporal sensing mechanism explains the neutrophil depolarization following concentration decreases, but cannot account for the collapse of neutrophil polarization after concentration increase with simultaneous change in the direction of the gradient. According to the temporal sensing mechanism, increasing concentrations should have sustained the pre-existent direction of migration.

Although a combination of the spatial and temporal mechanisms cannot be definitely excluded, we consider a unitary mechanism more likely. Based on our observations of neutrophil responses to the four types of switches, we hypothesize that a two component temporal sensing mechanism directs neutrophil responses to microenvironment changes. One component is the averaged chemokine concentration over the entire cell body. While increasing averaged chemokine stimulation over the entire cell surface reinforces neutrophil polarization and directional migration, decreasing stimulation leads to polarization collapse and neutrophil stop. The second component is the averaged chemokine concentration at the dynamic leading edge of a polarized cell. Decreasing chemokine concentration over time at the developing leading edge may indicate an unfavorable direction for migration and can also stop neutrophil motion and determine neutrophil depolarization. The integration of the two components may explain neutrophil depolarization after either step-down or flip switches through the activation of one or the other of the two components described before. The depolarization through either the decrease of average concentration over the entire cell

body or the decrease concentration at the leading edge is usually complete, with full reset of morphological asymmetry characteristic for polarized neutrophils. Statistically similar time-periods necessary for the initial response after the first exposure to chemokine gradient or the recovery of directional migration after step-down or flip switches provide additional support to our hypothesis.

The transition from a setting in which neutrophils are exposed to one chemokine gradient or no chemokine at all, to one in which cells are suddenly exposed to a new spatial gradient of chemoattractant, may be relevant to physiological situations. This would include the transition that a neutrophil makes from rolling along a vessel wall to a firmly attached cell migrating across vascular endothelium[28] and exposure to the chemokine gradient across the vessel wall and within the tissue.[29] Ultimately, understanding the mechanisms of neutrophil migratory responses to rapid changes in chemokinetic gradients may lead to new approaches to the control of acute or chronic inflammation in human disease.

Conclusions

A microfluidic system for studying cell migration and allowing quick and precise switching between stable chemotactic gradients is presented. Neutrophil responses to such switches are described, and unexpected behaviors are revealed. This novel technique enables the quantitative measurement of critical components of neutrophil migration. It also provides support for a new, two-component temporal sensing mechanism that uses variations of chemokine concentration averaged over the entire cells and localized at the dynamic leading edge to direct neutrophil responses. Further studies enabled by the temporal and spatial control of chemotactic gradients have the potential to lead to the elucidation of fundamental mechanistic elements involved in directional neutrophil migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Biomedical Imaging and Bioengineering (BioMEMS Resource Center, P41 EB002503 to MT) and Public Health Service (RO1 A149757 to MCP).

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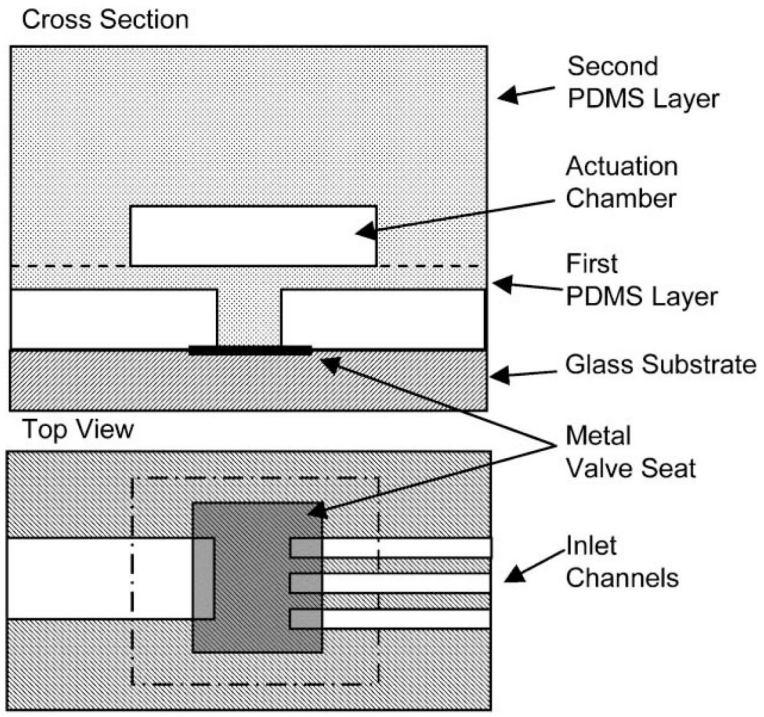

Fig. 1.
Schematics of microfabrication of the microstructured membrane and valve.

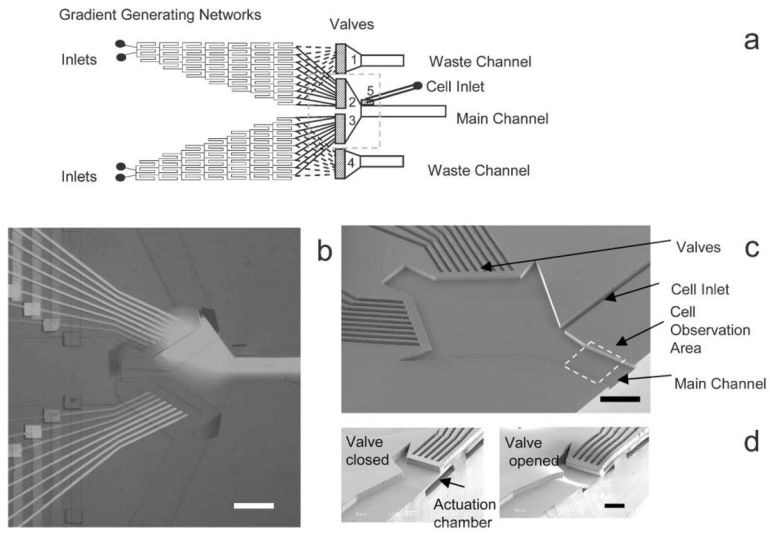

Fig. 2.

Schematics and details of the microfabricated device. (a) Two gradient generator networks were connected through a system of valves to one main channel and two waste channels. The flow in the upper gradient network could be directed either to the main channel (through the valve marked 2) or to a waste channel (valve 1). Similarly, the flow in the lower network could be directed to the main channel (valve 3) or a second waste channel (valve 4). Cells that were introduced into the device through the cell inlet channel, controlled by valve 5, and were observed in the main channel during exposure to the soluble gradients produced in either one of the gradient networks. (b) Spatial chemical gradients were formed in the main channel from one of the two pairs of nine streams of different concentrations from the gradient generator networks. For the example shown, the upper waste valve (valve 1, using the same notation as in panel a—not shown) and the lower main valve (valve 3) were closed, while the upper main valve (valve 2) and the lower waste valve (valve 4 not shown) were opened. Valve 5 was closed. Scale bar is 500 μm. (c)–(d) Schematics of the microstructured valves controlling the flow to the main channel. (c) Scanning electron micrograph (SEM) shows a cross section of the device through the valves 2 and 3. The device was placed upside-down and the glass substrate removed. Each valve simultaneously controls nine inlet channels. Scale bar is 500 μm. (d) SEM of the opened and closed microstructured valve. The valve is normally closed in the absence of actuation. The contact between the structures on the membrane and the thin metal film on the glass slide provides a good seal. Decreasing the pressure in the actuation chamber deforms the membrane and distances the valve from the glass. The valve can open about 100 μm, allowing the unobstructed passage of fluid from the nine inlet channels into the outlet channel. A similar valve allows the passage of cells through the cell inlet channel into the main channel. Scale bar is 200 μm.

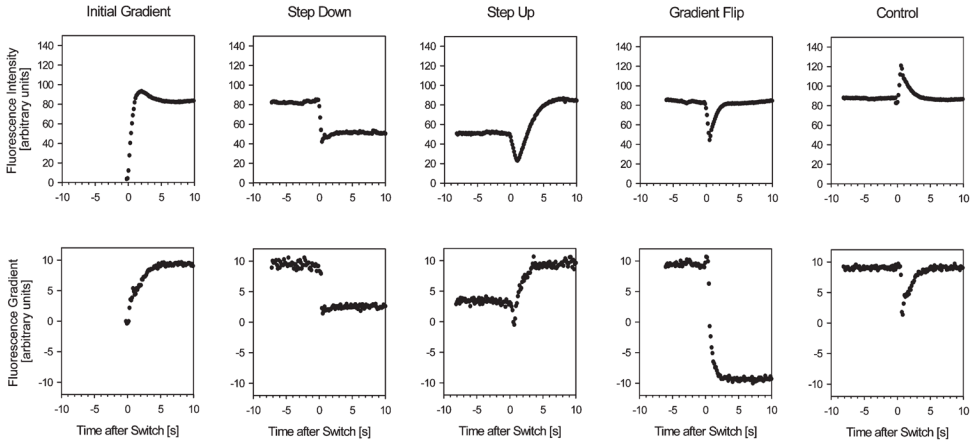

Fig. 3.

Temporal and spatial control of chemical gradients. The formation of the initial gradient and step down, step up, gradient flip, and control switches are presented in detail. Quantitative measurements using fluorescein dye were performed at 7.5 frames per second in the middle stream of the main channel. The fluorescence intensity *versus* time is presented in the upper series of plots, while the difference in fluorescence intensities between streams at 10 microns above and below the middle stream is presented in the lower series. The formation of the initial gradient was complete in approximately 4 s. A comparable time was needed for the stabilization of a lesser gradient steepness following a step down switch or a steeper gradient following a step up switch. The formation of a gradient of the same slope and average concentration but in the opposite direction was accomplished in approximately 4 s. Control switches to an identical gradient were used to test if the short perturbations of flow during the switching were not affecting the neutrophil migration.

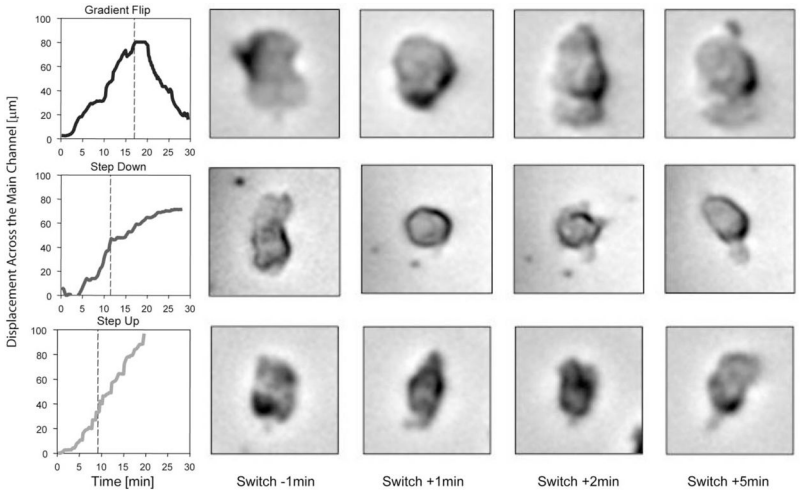

Fig. 4.

Examples of neutrophil displacement and morphology after gradient switches. Displacement is measured in the transversal direction starting from the initial position of the neutrophil before exposure to gradient. The time of the switch is indicated by the dashed vertical line. Representative brightfield images of neutrophils 1 min before and at 1, 2 and 5 min after the gradient flip, step down and step up switches are displayed.

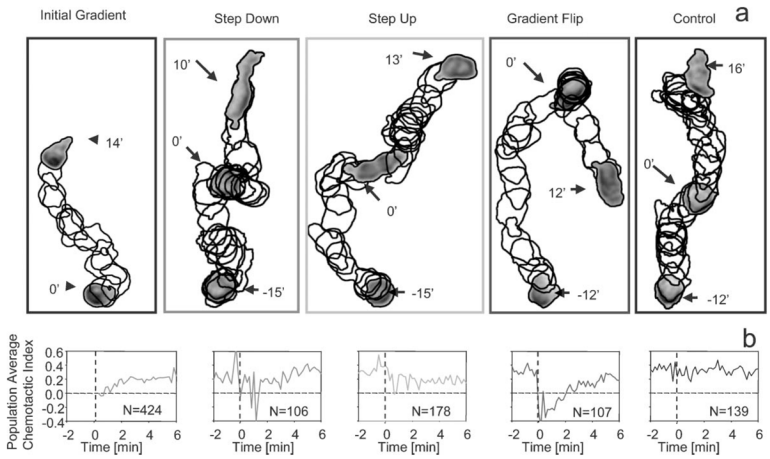

Fig. 5.

Individual neutrophil and average population responses to gradient changes. (A) Sequential cell images showing the contour of the neutrophil during the gradient change. Neutrophils show regular migration patterns before the gradient change (time 0). Following a flip or step down neutrophils depolarize and repolarize after few minutes. Cell outlines correspond to cell position at 1-minute time intervals. (B) Average chemotactic index (CI) for all neutrophil observed during each type of gradient switching event.

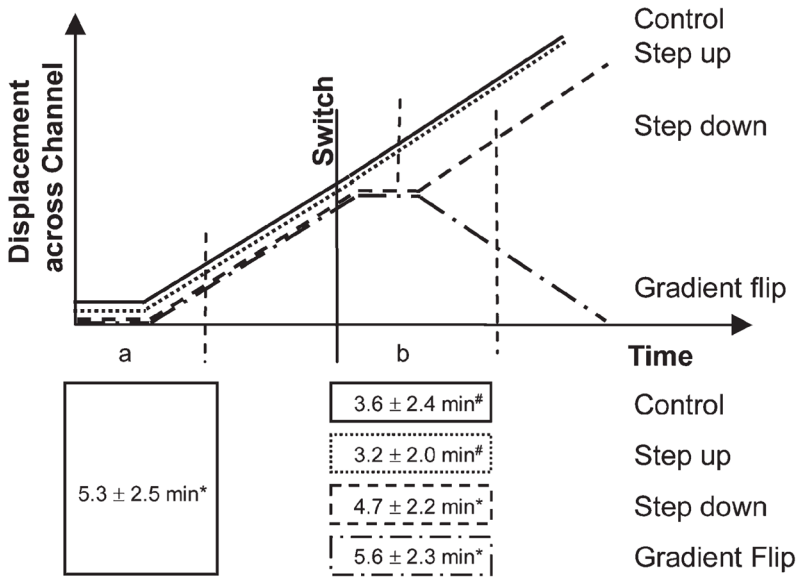

Fig. 6.

Summary of neutrophil responses to changing chemical gradients. The time to reach the displacement threshold after initial gradient exposure (a) or after gradient switches (b) is outlined by vertical dashed lines and indicated in minutes (average and standard deviation) in the boxes below the plot. After the initial exposure to chemokine gradient cells showed a delay in initiating the chemotaxis. Following the switch, cells continued their motion for an average of 30 s. If after the switch neutrophils were exposed to increased concentrations and gradients in the same direction as before the switch, they maintained their migration. If the concentration was decreased or the gradient was reversed, neutrophils stopped before moving in the new direction of the gradient. Shorter response times were measured for neutrophils exposed to step-up (dotted line) and control (solid line) switches than following the initial exposure to gradient and step-down (dashed line) and flip (dot-dashed lines) switches. Two groups of statistically similar response times were outlined (* $p = 0.37$ and # $p = 0.32$).

Table 1

Temporal and spatial gradient requirements for neutrophil asymmetry and migration

	Matching Gradient	**Reversed Gradient**
Increasing Concentration	Polarization Reinforcement	Polarization Collapse
Decreasing Concentration	Polarization Collapse	Polarization Collapse