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# Three-dimensional modulation of cortical plasticity during pseudopodial protrusion of mouse leukocytes

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ABSTRACT

Leukocytes can rapidly migrate virtually within any substrate found in the body at speeds up to 100 times faster than mesenchymal cells that remain firmly attached to a substrate even when migrating. To understand the flexible migration strategy utilized by leukocytes, we experimentally investigated the three-dimensional modulation of cortical plasticity during the formation of pseudopodial protrusions by mouse leukocytes isolated from blood. The surfaces of viable leukocytes were discretely labeled with fluorescent beads that were covalently conjugated with concanavalin A receptors. The movements of these fluorescent beads were different at the rear, central, and front surfaces. The beads initially present on the rear and central dorsal surfaces of the cell body flowed linearly toward the rear peripheral surface concomitant with a significant collapse of the cell body in the dorsal–ventral direction. In contrast, those beads initially on the front surface moved into a newly formed pseudopodium and exhibited rapid, random movements within this pseudopodium. Bead movements at the front surface were hypothesized to have resulted from rupture of the actin cytoskeleton and detachment of the plasma membrane from the actin cytoskeletal cortex, which allowed leukocytes to migrate while being minimally constrained by a substrate.



## 1. Introduction

Migrating cells *in vivo* exhibit several basic processes, including cytoplasmic protrusion at their leading edge, adhesion to the extracellular matrix (ECM), and generating force against this adhesion to move the cell body forward. Although the same basic processes are involved, different cell types exhibit different modes of migration related to their specific functions. Inflammatory cells utilize rapid, flexible migration strategies to mount an effective immune response [1]. In particular, leukocytes can move virtually within any substrate found in the body at speeds up to 100 times faster than mesenchymal cells that remain firmly attached to the ECM even when migrating [2].

Because the physical characteristics of cytoplasmic protrusion at the leading edge and cell body translocation are major determinants of the mode of cell migration [3], studies from the mechanical viewpoint are essential for understanding the rapid, flexible migration strategy utilized by leukocytes. Leukocyte migration is characterized by pseudopodial amoeboid type movement [2]. Cells that migrate by amoeboid type movement do not have bundled actin stress fibers or focal adhesions. This is in contrast to mesenchymal cell movement that is typically exhibited by fibroblasts with developed stress fibers that contribute to retracting the cell body and focal adhesions that firmly link actin stress fibers and the ECM. During amoeboid type movement, a contractile actomyosin cortex under the lipid membrane and loose contacts between a cell and a substrate are responsible for force generation [4]. These differences in physical characteristics determine the migration strategy of whether a cell can migrate only on a defined substrate or flexibly interact and migrate on any substrate, as leukocytes do [5]. Thus, determining cell cortex mechanics is essential for understanding the rapid, flexible migration strategies utilized by leukocytes [1].

Although significant progress has been made in elucidating the molecular mechanisms that underlie cell migration [6], quantitative characterizations of the associated cellular mechanical properties remain largely incomplete, particularly for amoeboid type migration. The physical behaviors of lamellar and lamellipodial protrusions that are typical of mesenchymal cell movement and keratocyte-like movement are well understood based on subcellular observations of the dynamics of the actin cytoskeleton using fluorescent speckle microscopy [7–10]. Actin cytoskeletal dynamics in thin lamellipodia and lamellae can be investigated by tracking markers on them based on time course images in only one focal plane [11]. In contrast, cell cortex dynamics during amoe-

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boid type migration are three-dimensional (3D). Technical difficulties with 3D tracking of markers have been a drawback for clarifying cell cortex mechanics.

The aim of our study was to characterize those cell cortex physical behaviors that underlie rapid, flexible migration strategies utilized by leukocytes during their amoeboid movement. To investigate cortex physical behaviors three-dimensionally, we used carboxylate-modified fluorescent latex beads to discretely label the cell surface. These carboxylate-modified beads were covalently conjugated to a known cell surface receptor, concanavalin A (Con A) [12,13]. The movements of many kinds of surface receptors involving Con A have been shown to reflect the interactions between the cell membrane and the underlying cytoskeleton [14]. Furthermore, when Con A is present on the membrane of a cell with an actin rich cortex, the movement of Con A is tightly coupled to the motility of the actin cortex [12]. Using this method with mouse leukocytes isolated from blood, we successfully determined the 3D modulation of cortical plasticity during pseudopodial protrusion at subcellular resolution.

## 2. Materials and methods

### 2.1. Cell isolation

A drop of fresh mouse blood was obtained from a tail puncture and mixed with Ringer's acetate solution (Physio140 Injection, Otsuka Pharmaceutical). Leucocytes were then isolated by density gradient centrifugation at 500×*g* for 15 min using Lymphosepar II (*d* = 1.090; Immuno-Biology Laboratories). The fraction containing leucocytes was diluted with modified Ringer's acetate solution supplemented with $1.48 \times 10^{-3}$ mol/l $MgCl_2 \cdot 6H_2O$ and 0.2% fetal bovine serum. The cell suspension was then placed on a glass bottom dish (MatTek) that had been precoated with fibronectin (5 μg/cm$^2$) and the cells were allowed to settle and adhere to the bottom glass.

### 2.2. Labeling viable leukocyte surfaces with fluorescent beads

Yellow–green carboxylate-modified beads (diameter = 0.1 μm, 2% suspension in water; Molecular Probes, Inc., Eugene, OR) were diluted to 1:1000 with modified Ringer's acetate solution. Cells that had adhered to the glass bottom dish were incubated with this solution for 10 min, after which the cells were washed several times with modified Ringer's acetate solution.

### 2.3. Microscopy

To induce chemotaxis, 2 nM formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was filled in a glass micropipette (tip diameter = 3 μm), and then applied by pressure ejection from the micropipette placed at 100 μm from a cell.

To detect a cell's outline and whole cell cortex dynamics, a time series of image sets was acquired consisting of a differential interference contrast (DIC) image of the cell (Fig. 1A) in one focal plane and fluorescent bead images (Fig. 1B) in different focal planes (0.5 μm intervals) using a confocal microscope (FV1000D, Olympus). For some experiments, a time series of image data sets was acquired with an inverted microscope with a 100× 1.4NA Plan objective lens (Olympus) and an iXonEM EMCCD camera (Andor Technology) controlled with live cell imaging software (Andor iQ, Andor Technology). Image sets were acquired every 20 s for 6 different leukocytes.

To acquire images focusing on the rapid beads movements on a leukocyte pseudopodium, bead images in a single focal plane were acquired by wide-field fluorescent microscopy every second using an inverted microscope with a 100× 1.4NA Plan objective lens (Olympus) and an iXonEM EMCCD camera (Andor Technology) controlled with live cell imaging software (Andor iQ, Andor Technology). Image acquisition was performed for 3 different leukocytes.

### 2.4. Image analysis for bead movements

Using the method illustrated in Fig. 1C, the 3D position ($x_i$, $y_i$, $z_i$) of a fluorescent bead was manually acquired in a Cartesian coordinate system for the *i*th image data set at some time, $t_i$, based on the criteria that the center of the fluorescent signal was the *xy*-position of the bead and the position of the focal plane with maximum bead fluorescent intensity corresponded to its *z*-position.

To characterize the dynamics at the rear, center, and front surfaces of a cell, as illustrated in Fig. 1D, we defined a relative coordinate system, ($x'$, $y'$, $z$), which was fixed to the movement of the cell's center of mass. Then, the relative velocity, $v'_i = \left(v'_{x'i}, v'_{y'i}, v'_{zi}\right)$, with reference to the cell's center of mass for a time interval, $\Delta t = t_{i+5} - t_i$, was calculated. To quantitatively define the rear, center, and front surfaces of a cell, the $x'$-position was normalized as $x'^*$ by dividing the $x'$-position with reference to the cell diameter, $R = 2\sqrt{\bar{S}/\pi}$, where $\bar{S}$ was the mean $x'y'$-projected area of the cell to be analyzed. The rear, central, and front surfaces were those regions with $-1 < x'^* \leq -0.5$, $-0.5 < x'^* \leq 0$, and $0 < x'^* \leq 0.5$, respectively.

Rapid, random movements of the beads on the surface of a newly forming pseudopodium were characterized by a diffusion coefficient. The diffusion coefficient was estimated based on the mean-square displacement [14]:

$$\mathrm{MSD}(\Delta t_n) = \frac{1}{N-1-n}\sum_{j=1}^{N-1-n}\left[(x_{j+n}-x_j)^2 + (y_{j+n}-y_j)^2\right],$$

where $\Delta t_n = t_{i+n} - t_i$, $N$ was the total number of image sets in a time sequence, $n$ and $j$ were positive integers, and $n$ was the time increment.

## 3. Results

Fluorescent carboxylate-modified beads on the surfaces of 6 different leukocytes during pseudopodial protrusion were tracked. A representative time sequence of bead movements during pseudopodial protrusion is shown in Fig. 2. As seen in the DIC image in Fig. 2A (left column), at $t$ = 4.4 s, the leukocyte exhibited a round shape. Subsequently, the cell began to extend two pseudopodia toward the bottom left and right of the figure at $t$ = 97.9 s. Then, the bottom left pseudopodium retracted toward the main cell body, whereas the bottom right pseudopodium remained extended from $t$ = 97.9 s to 359.7 s. During pseudopodial protrusion, the integrated distance that the cell travelled was 17.3 μm for 374 s; the average speed was 17.3 μm/374 s = 0.05 μm/s. The mean speed of 6 different leukocytes was 0.04 ± 0.01 μm/s.

The movements of the fluorescent beads were observed with a confocal microscope (middle and right columns in Fig. 2A). The spatial and temporal intervals of the confocal optical slice images were 0.5 μm and 1.1 s, respectively, and 17 optical slice images were required to three-dimensionally observe all the beads on a whole single cell. This image acquisition condition allowed us to three-dimensionally track bead movements on a whole single cell cortex. For the leukocyte in Fig. 2, the positions of 28 beads could be determined. The positions of the beads projected on the *xy*-plane and *xz*-plane are shown in Fig. 2B and C, respectively. The movements of 8 randomly selected beads were tracked and are shown by colored symbols in Fig. 2B and C.

A bead that was on the rear peripheral surface of the cell body at $t$ = 0 (bead No. 1 in Fig. 2B and C) remained on the rear periph-