Non-monotonic relationships between cell adhesion and protrusions

Dawid Szewczyk\textsuperscript{1,2}, Tetsuya Yamamoto\textsuperscript{1,2} and Daniel Riveline\textsuperscript{1,2,3}

\textsuperscript{1} Laboratory of Cell Physics, ISIS/IGBMC, Université de Strasbourg and CNRS (UMR 7006), 8 allée Gaspard Monge, F-67083 Strasbourg, France
\textsuperscript{2} Development and Stem Cells Program, IGBMC, CNRS (UMR 7104), INSERM (U964), Université de Strasbourg, 1 rue Laurent Fries, BP10142, F-67400 Illkirch, France
E-mail: riveline@unistra.fr

\textit{New Journal of Physics} 15 (2013) 035031 (21pp)
Received 24 June 2012
Published 27 March 2013
Online at http://www.njp.org/
doi:10.1088/1367-2630/15/3/035031

Abstract. Cells probe their environments by extending protrusions: this process is mediated by the polymerization of actin gels at the edge of cells. Although their molecular components have been widely studied, their mesoscopic properties remain to be characterized. In this paper, we show that cell adhesion modulates actin gel dynamics. By changing the grafting density of fibronectin on a surface, we changed the adhesion strength of a cell on this surface. We found that the length of filopodia, the speeds of their growth and the speeds of retrograde flows were non-monotonic functions of the grafting density of fibronectin. The minima of the length and speeds of filopodia and the maximum of the speeds of retrograde flows are found at the same fibronectin density; this implies that there are strong correlations between these parameters. We used a simple model to predict that retrograde flows show non-monotonic behaviors because integrin–fibronectin binding mediates actomyosin and friction forces applied to actin gels. This model also predicts that connectivity of actin gels is responsible for the strong correlations between retrograde flows and filopodial growth. Altogether, our study investigates how actomyosin forces and friction with the substrate influence actin gel dynamics in living cells.

\textsuperscript{3} Author to whom any correspondence should be addressed.

Content from this work may be used under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.
1. Introduction

Interactions between adherent cells and extracellular matrices (ECM) play an important role in several cellular processes, e.g. cell migration [1, 2] and differentiation [3, 4]. Migrating cells adhere and spread on two-dimensional (2D) surfaces via ECM proteins, e.g. fibronectin (FN), and extend protrusions, filopodia and lamellipodia, to sense their environment. These protrusive structures are driven by the polymerization of actin cytoskeleton at the spreading front of cells. Actin cytoskeleton is often modeled as a polymer gel that performs active processes—the polymerization of actin filaments and the generation of actomyosin contractile forces. The general hydrodynamic theory of active gels has been developed in an extension of the hydrodynamic theory of liquid crystals by Kruse et al [5–7] and this theory predicts the formation of asters, vortices and spirals that were observed in reconstituted systems [8]. It is thus of interest to study the protrusive activities of cells on the basis of the mechanical properties of active gels.

The forces arising from actin polymerization and the actomyosin contractile forces generated by stress fibers drive retrograde flows that transport actin gels to the center of cells [9, 10]. In many cases, (one side or both of) the ends of stress fibers are mechanically connected to ECM proteins on 2D surfaces via integrin (in cell membranes) and focal contacts (in cytoplasm). Actin gels in lamellipodia are also mechanically connected to ECM proteins via integrin and focal contacts with stick-slip processes (sometimes called clutch mechanisms) and this applies friction forces to retrograde flows [11, 12]. It has been proposed that these friction forces may be the driving forces to move cell fronts forward [12]. Previously, the effects of interactions between cells and substrates on the speed of migrating cells [1] and the spreading area [2] were studied by measuring these quantities as functions of the grafting density of ECM proteins and the rigidity of substrates. However, to the best of our knowledge, the effects of

---

**Contents**

1. Introduction .......................... 2
2. Experimental results ................. 4
3. Theoretical model ..................... 9
   3.1. Non-monotonic behaviors of retrograde flows .......... 9
   3.2. Dynamics of filopodial growth ... 12
4. Discussion ............................. 16
5. Materials and methods ............... 18
   5.1. Micropattern fabrication .......... 18
   5.2. Cell culture ..................... 18
   5.3. Time lapse experiments .......... 19
   5.4. Cell areas ....................... 19
   5.5. Length and velocity of filopodia . ......... 19
   5.6. Retrograde flow ................... 19
   5.7. Focal contacts staining and fibronectin intensity 19
   5.8. Statistical analysis ............... 20
Acknowledgments ...................... 20
References ............................. 20

---

*New Journal of Physics* 15 (2013) 035031 (http://www.njp.org/)
Figure 1. Forces applied to the actin gel of a cell during cytoskeletal reorganization that leads to filopodial/lamellipodial protrusions. Stress fibers (that are mechanically connected to the substrate via FN/integrin cluster/linkage proteins binding) apply actomyosin forces $F_{\text{act}}$ to the actin gels, and integrin molecules that are bound to the actin gels via linkage proteins (but not via stress fibers) apply friction forces $F_{\text{fri}}$ that are directed to the spreading front of the cell because the flows of actin gels are retrograde. Forces $F_{\text{pol}}$ arising from actin polymerization are applied at the ends of filopodia and are balanced with the elastic forces $F_{\text{mem}}$ of the membrane.

these interactions on protrusive activities of migrating cells have not been studied yet. It is thus of interest to study how interactions between actin gels and 2D surfaces via integrin–ECM protein binding affect the protrusive activities and retrograde flows of migrating cells.

In the present study, we measured the spreading area, the length and speed of filopodia growth and the speed of retrograde flow, of fibroblasts, NIH3T3, plated on micropatterned surfaces as functions of the density of FN grafted on the glass substrate. To suppress cell polarization, these cells are plated on circular adhesive regions that are fabricated by the microcontact printing method, where the other part is passivated by non-adhesive materials. On these adhesive regions, cells do not migrate from these regions, but show retrograde flows and filopodial protrusions [13]. In these experiments, we found that the spreading area, the length and speed of filopodia growth and the speed of retrograde flows are non-monotonic functions of the density of FN. The fact that the minima and maxima are found at the same FN density suggests that these parameters have strong correlations. To elucidate the physical
mechanisms involved in these non-monotonic behaviors and the correlations between retrograde flows and filopodial growth, we used a simple model that is an extension of a hydrodynamic theory of active gels for lamellipodia [7]. Our model predicts that the non-monotonic behaviors of retrograde flows are because of the fact that interactions between actin gels and adhering surfaces (where FN is grafted) have a dual role; these interactions mediate both actomyosin contractile forces via stress fibers and friction forces via stick-slip-type interactions between focal contacts and actin gels. Our theory also predicts that connectivity of active gels at the interface between filopodia and lamellipodia is responsible for the strong correlation between the speeds of retrograde flows and the speeds of filopodial growth.

2. Experimental results

Cells are known to adhere to substrates that are coated with FN [19]. We therefore prepared circular adhesive regions that were coated with FN by the microcontact printing method and we grafted poly-L-lysine-g-poly(ethyleneglycol) (PLL-g-PEG) polymers on the other regions of the surface (figures 2(A) and (B) and section 5). In this geometry, cells do not migrate from these adhesive regions (figure 2(C)) and show steady retrograde flows and stochastic filopodial protrusions. Selections of adhesive area and geometry of pattern were justified as follows: (i) the area of printed pattern was corresponding to the mean cell area on a homogeneously coated surface in conditions similar to the usual cell culture, ∼1590 μm²; (ii) cell morphology affects the reorganization of cytoskeleton and we therefore used an isotropic circle as the shape.
of adhesive regions to suppress the anisotropy of protrusion activities. Finally, the distance between motifs was set at 100 µm to suppress any interactions between neighboring cells.

We changed the density of FN on adhesive patterns by changing the concentrations $C_{FN}$ of FN in the solutions used for microcontact printing, fixing the stamping time and pressure, see also section 5. We quantified the changes in the grafting density of FN on the surface by stamping fluorescently labeled fibronectin. As shown in figure 2(D), the grafting density of FN increased linearly with the FN inking concentration.

We deposited cells, allowed them to spread for 30 min and then analyzed their behaviors within 5 h after plating. For all the cases of FN concentrations that were used in our experiments, cells adhered preferentially on the adhesive regions. Many of the cells adjusted their morphology to the shape of the motifs. However, by visual inspection, 10–40% of these cells were polarized depending on conditions and both polarized and unpolarized cells were included in averaging. Strikingly, the phenotypes of cells were qualitatively changed with increasing the FN surface density (figure 3); for lower densities, cells presented larger areas than for higher densities. In addition, the number of filopodia for lower concentrations was less than that for higher concentrations. These findings imply that the organization of the actin cytoskeleton depends on the grafting density of FN.

We characterized the effects of cell adhesions on the dynamics of protrusion activities. Cells show two types of protrusions—filopodia and lamellipodia. We mainly characterized filopodia because they have a constant width of 200 nm; this is in contrast to lamellipodia–lamella regions that have various widths and are not strictly defined. Figure 4 illustrates the selected features analyzed in our study; cell spreading (figure 4(A)), filopodia (figure 4(B)), their growth (figure 4(C)), retrograde flows (figure 4(D)) and focal contacts that mechanically connect actin cytoskeleton and FN (figure 4(E)). We measured the associated

Figure 3. DIC images of NIH3T3 fibroblasts plated on different FN concentrations. (A) 30 µg ml$^{-1}$, (B) 40 µg ml$^{-1}$, (C) 47.5 µg ml$^{-1}$, (D) 55 µg ml$^{-1}$ and (E) 70 µg ml$^{-1}$. Cells show changes in areas and morphologies. Cell contours are outlined in white.
Figure 4. Features of cell morphology used in this study. (A)—Cell area measurement. (Left) DIC image of the cell and (right) result of the image processing used for obtaining the contour. (B)—Cell filopodia length. (Left) DIC image of the cell edge showing a group of filopodia and (right) zoomed image of the highlighted filopodia. (C)—Velocity of filopodia. DIC images of a growing filopodia. (D)—Retrograde flow. DIC image of a vesicle moving radially toward the cell center. The displacement is emphasized by dashed lines. (E)—Area of focal contacts. (Left) Fluorescence image of a cell stained for paxillin on a FN spot. Only focal contacts inside the pattern were selected for the analysis. (Right) High magnification images of highlighted focal contacts (1–4).
parameters as functions of FN concentration (that is proportional to the grafting density of FN), see figure 5: the average spreading area of cells (figure 5(A)), the average length of filopodia (figure 5(B)), the average speed of elongation and retraction of filopodia (figures 5(C) and (D)), the average speed of retrograde flows (figure 5(E)) and the average focal contact coverage (figure 5(F)). The spreading area was measured to estimate how individual cells were constrained. Filopodia and retrograde flows were selected as read-outs of active gel activities at cell peripheries.

The spreading area of the cells was not a monotonic function of FN concentration (figure 5(A)). For high and low concentrations, the behavior is easily understood: for 70 µg ml⁻¹, the cell area was lower than the area of the motif, suggesting that the cell was strongly constrained. For 30 µg ml⁻¹, the cell area was slightly larger than the motif area, which suggests that cells were weakly adherent to the pattern, and could spread easily. At a first glance, one may think that these two limits were interpolated by a monotonic curve. However, the curve was not monotonic, with an apparent local minimum at 40 µg ml⁻¹ and a local maximum at 47.5 µg ml⁻¹. The non-monotonic behavior of the spreading area may be because FN affects more than one type of force in actin gels.

We next characterized the activities of actin gels as functions of FN concentration by measuring the average lengths of filopodia (figure 5(B)) and the average velocities of their growth (figure 5(C)) and retraction (figure 5(D)). The speed of the growth and retraction of filopodia was symmetric. The mean length of filopodia was 6.13 ± 0.49 µm at the lowest FN concentration (used in our experiments), whereas it was shorter (yet not minimal) at the highest concentration, 4.51 ± 0.31 µm. The velocity of filopodial growth at the lowest concentration was larger (2.77 ± 0.24 µm min⁻¹) than that at the highest concentration (2.52 ± 0.23 µm min⁻¹). These parameters saturate at the grafting densities of FN larger than 47.5 µg ml⁻¹. However, surprisingly, at the intermediate concentration (40 µg ml⁻¹), filopodia showed minimal length (3.81 ± 0.28 µm) and minimal velocity (2.05 ± 0.17 µm min⁻¹). This minimum is recognized as significant by the Mann–Whitney U test. Here we note that the FN concentration that shows the minima of these parameters is equal to the FN concentration that shows the minima of the spreading area.

Retrograde flows also reflect active processes in actin gels. We therefore measured the speeds of retrograde flows. We used vesicles that flow with retrograde flows as probes and measured their velocities as a function of FN concentration (figure 5(E)). The speeds of retrograde flows were different from the speed of filopodial growth; the speeds of retrograde flows ranged between 1.89 ± 0.12 and 2.41 ± 0.13 µm min⁻¹, whereas the speeds of filopodial growth ranged between 2.81 ± 0.28 and 2.05 ± 0.017 µm min⁻¹. Retrograde flows show the maximum speed at the same FN concentration, 40 µg ml⁻¹, as the minimum speed of filopodial growth. Our experimental results suggest that there are strong correlations between the length and speeds of filopodia and the speeds of retrograde flows.

Our experimental results suggest that the active processes of gels are correlated with the contacts between cells and the substrate. It is expected that cell–substrate contacts are stabilized via focal contacts that are discrete domains in cell membranes (figure 4(E)). We thus labeled focal contacts and measured the fraction of area occupied by focal contacts on motifs (figure 5(F)); this fraction is the sum of the area of focal contacts divided by the area of an adhesive region (for the case of the polarized cells, we used the area of the fan-shaped region where focal contacts exist). We took only the focal contacts that are elongated and have area that is larger than ∼ 1 µm in the measurements; these focal contacts are expected to be bound to
Figure 5. Measurements of cell properties and dynamics as functions of FN concentration ($C_{FN}$). (A) The average spreading area of cells ($A_c$) on the FN adhesive regions ($n = 448$ time points, $N = 75$ cells). The red line shows the
stress fibers [14, 15]. Indeed, the fraction of focal contacts was not sensitive to FN concentration. The intensity of fluorescence from focal contacts did not change significantly (data not shown).

3. Theoretical model

3.1. Non-monotonic behaviors of retrograde flows

To predict the physical mechanisms involved in the non-monotonic behaviors of the length and speeds of filopodia, and the speeds of retrograde flows, we here use a simple model that is an extension of a hydrodynamic model of active gels [7]. In this theory, we take into account the dual role of fibronectin–integrin (FN–I) binding: this binding mediates actomyosin forces generated by stress fibers and friction forces by directly binding to actin gels, where these forces are directed in the opposite direction, see figure 1. We first analyze the role played by FN–I binding in retrograde flows. Retrograde flows have been observed at two regions, lamellipodia and lamella, which are distinguished by the difference of actin density, the speeds of retrograde flows and molecular components, and matured focal contacts are usually found at the interface between these regions [9]. However, in our model, we do not take into account this difference, because this does not change the physics.

Integrin molecules at the basal part of a cell membrane bind to FN at the substrate with binding rate $\kappa$ and dissociate with rate $1/\tau_{\text{FN}}$, see figure 1. Integrin molecules that are not bound to FN freely diffuse in the cell membrane. Because only integrin molecules at the basal part of the cell membrane are immobilized by binding to FN, we consider the other part of the membrane as the reservoir of integrin molecules; the density $C^0_{\text{int},u}$ of integrin molecules that are not bound to FN is constant. We here assume that integrin molecules can bind to stress fibers or to actin gels (via linkage proteins, e.g. vinculin) only when they are bound to FN. The density $C^0_{\text{int},u}$ of integrin molecules that are not bound to FN thus follows the kinetic equation

$$\frac{\partial C^0_{\text{int},u}}{\partial t} = -\kappa \sigma_{\text{FN}}C^0_{\text{int},u} + \frac{C^0_{\text{int},b}}{\tau_{\text{FN}}}.$$  

(1)

$\sigma_{\text{FN}}$ is the grafting density of FN and $C^0_{\text{int},b}$ is the density of integrin molecules that are bound to FN. Integrin molecules that are bound to FN at their extracellular domains can bind to stress fibers or directly to actin gels at their intracellular domains. In this case, the density $C^0_{\text{int},u}$ of integrin molecules that are bound to FN follows the kinetic equation

$$\frac{\partial C^0_{\text{int},u}}{\partial t} = \kappa \sigma_{\text{FN}}C^0_{\text{int},u} - \frac{C^0_{\text{int},b}}{\tau_{\text{FN}}} - N^u \frac{\partial C^0_{\text{int},b}}{\partial t} - N^e \frac{\partial C^0_{\text{int},u}}{\partial t}.$$  

(2)
where $\partial C_{\text{gel}}^{\text{int}}/\partial t$ and $N \partial C_{\text{SF}}^{\text{int}}/\partial t$ are the rates that free integrin clusters are bound to actin gels and stress fibers, respectively.

Recent experiments have shown that integrin molecules must be cooperatively assembled to clusters to stabilize stress fibers [17] and (nascent) stress fibers are stabilized before they are bound to integrin clusters (via linkage proteins) [16]. Although experiments suggested that the size of integrin clusters are polydispersed [18], for simplicity, we here neglect the distribution of the size of integrin clusters and consider clusters that are composed of $N$ molecules. In this case, the rate equation of the density $C_{\text{SF}}^{\text{int}}$ of integrin clusters that are bound to stress fibers has the form

$$\frac{\partial C_{\text{SF}}^{\text{int}}}{\partial t} = (\phi_{\text{SF}} - C_{\text{SF}}^{\text{int}})(\chi C_{\text{int},b}^{0})^N - \frac{C_{\text{SF}}^{\text{int}}}{\tau_{\text{SF}}},$$

(3)

where $\phi_{\text{SF}}$ is the density of (the unit of) stress fibers, where integrin clusters are bound via focal contacts. $\chi$ is the rate that an integrin molecule binds to a stress fiber and $1/\tau_{\text{SF}}$ is the dissociation rate of this binding. The factor $\phi_{\text{SF}} - C_{\text{SF}}^{\text{int}}$ in equation (3) implies that the number of stress fibers does not depend on the grafting density of FN. This assumption is motivated by the fact that nascent stress fibers are assembled before these stress fibers are bound to integrin clusters via linkage proteins (and thus the number of stress fibers does not depend on the grafting density of FN) [16]. Another experiment suggests that cells spread on different micropatterns seem to conserve the number of stress fibers [19]. For simplicity, we do not take into account the kinetics of linkage proteins between integrin and stress fibers.

Integrin molecules (that are bound to FN at their extracellular domains) are bound to actin gels (probably via linkage proteins) with rate $\lambda$ and are dissociated with rate $1/\tau_{\text{gel}}$ at their intracellular domains. In this case, the rate equation for the density $C_{\text{gel}}^{\text{int}}$ of integrin clusters that are bound to the gel has the form

$$\frac{\partial C_{\text{gel}}^{\text{int}}}{\partial t} = \lambda C_{\text{int},b}^{0} - \frac{C_{\text{gel}}^{\text{int}}}{\tau_{\text{gel}}},$$

(4)

Solving equations (1)–(3) in the stationary state leads to the form of the density $C_{\text{int},b}^{0}$ of free integrin molecules (that are bound to FN) and the density $C_{\text{int}}^{\text{gel}}$ of integrin molecules that are bound to actin gels: $C_{\text{int},b}^{0} = \kappa \tau_{\text{FN}} C_{\text{int},u}^{0} \sigma_{\text{FN}}$ and $C_{\text{int}}^{\text{gel}} = \lambda \tau_{\text{gel}} C_{\text{int},b}^{0}$. The density $C_{\text{SF}}^{\text{int}}$ of integrin clusters that are bound to stress fibers has the form

$$C_{\text{SF}}^{\text{int}} = \frac{(\chi \tau_{\text{SF}} C_{\text{int},b}^{0})^N}{1 + (\chi \tau_{\text{SF}} C_{\text{int},b}^{0})^N} \phi_{\text{SF}}.$$

(5)

Stress fibers that are bound to the substrate via integrin clusters apply actomyosin contractile forces to actin gels [9] and integrin molecules that are directly bound to actin gels apply friction forces to these actin gels. We thus assume that actomyosin forces $F_{\text{act}}$ are proportional to the density of integrin clusters that are bound to stress fibers and friction forces $F_{\text{fri}}$ are proportional to the density of integrin molecules that are directly bound to actin gels (see figure 1, bottom left);

$$F_{\text{act}} = -\xi_{0} \Delta \mu C_{\text{int}}^{\text{SF}},$$

(6)

$$F_{\text{fri}} = \xi_{0} C_{\text{int}}^{\text{gel}} v_{\text{act}}.$$
These forces are applied at the basal boundary of the actin gel. In this treatment, we implicitly neglected the distribution of the length and thickness of stress fibers. $\Delta \mu$ is the change of chemical potential arising from ATP hydrolysis and $\xi_0$ is a proportional constant that relates the chemical potential difference to actomyosin forces (per integrin cluster), where $\xi_0$ is negative when actomyosin forces are contractile (following [7]). $v_{act}$ is the velocity (fields) of retrograde flows arising from actomyosin and friction forces, and is directed to the center of the cell when $v_{act}$ is positive. $\xi_0$ is the friction coefficient per integrin. Integrin molecules that are bound to FN are only in the circular adhesive region and thus actomyosin and friction forces are applied only in this adhesive region; the speeds of retrograde flows are uniform in the region between the spreading front of the cell and the boundary of adhesive region because of the balance of stresses (it is also shown by the discussion below for $F_{fri} = F_{act} = 0$).

The velocity and force profiles in active gels on the frictional substrate have already been calculated by Kruse et al [7] for the case that actomyosin forces are uniformly applied in these gels. Here, we extend this theory by taking into account the dual role of integrin—these molecules mediate actomyosin and friction forces, see equations (6) and (7). Because the formal calculations of the velocity of retrograde flows are not very different from those performed in [7], we here show a simple scaling argument. Forces $F$ applied at a cross section of actin gels of viscosity $\eta$ have a linear relationship with strain rate; $F \sim \eta \frac{\partial v}{\partial x}$ (because active gel theories assume that these gels are Newtonian fluids at low Reynolds number in a long time scale [5–7]), where $h$ is the height of the cross section. The balance of the viscous stresses and friction forces leads to $\frac{\partial F}{\partial x} = F_{fri}$. This leads to the scale of distance $\beta^{-1}$ that active gels flow until they lose velocity by friction forces in the form $\beta^{-1} = \sqrt{\frac{\eta h}{\xi_0 C_{int}}}$. For length scales that are larger than $\beta^{-1}$, viscous forces $\eta \frac{\partial v}{\partial x} \sim -\eta \beta v_{act}$ balance with actomyosin forces $F_{act}$. This force balance equation leads to the form of the velocity $v_{act}$ of retrograde flows arising from actomyosin and friction forces. Retrograde flows are also arising from actin polymerization at the ends of lamellipodia [9]. The velocity $V_R$ of retrograde flows are the sum of the velocity $v_{act}$ arising from actomyosin and friction forces and the velocity $v_{pol}$ of actin polymerization at the spreading front (see figure 1):

$$V_R \sim v_{pol} - \frac{\xi_0 \Delta \mu}{\eta \beta} C_{SF}^{int}.$$  

Equation (8) predicts that retrograde flows are non-monotonic functions of the grafting density $\sigma_{FN}$ of FN (that are proportional to the concentration $C_{FN}$ of FN in the solutions to prepare adhesive patterns). For the regime of small FN density, actomyosin contractile forces arising from stress fibers increase with increasing the grafting density of FN. For the regime of larger FN density, it is difficult for integrin clusters to bind to stress fibers because of the limitation of the number of stress fibers and thus friction forces dominate actomyosin forces.

The fitting parameter of equation (8) is the (average) number $N$ of integrin that cooperatively binds to a stress fiber, the rescaling factor of FN concentration $\alpha$ ($\equiv \chi_{SF}^k T_{FN} C_{int, u}^{0} \sigma_{FN} / C_{FN}$) and the magnitudes of retrograde flows arising from actin polymerization $v_{pol}$ and those arising from actomyosin and friction forces $v_{mag}$ ($\equiv -\xi_0 \Delta \mu / \sqrt{\eta_0} \sqrt{\chi_{SF} T_{gel} h} / (\lambda \tau_{gel})$). We used vesicles as probes to measure the speeds of retrograde flows; these vesicles are usually localized at lamella [21] and the retrograde flows in lamella are mainly driven by actomyosin forces [9, 10]. We thus assumed that actin polymerization $v_{pol}$ is
negligibly small\(^4\). The curve fitting of our experimental results with equation (8) suggests that \(N = 10.5\), \(\alpha = 0.034\, \text{ml} \, \mu\text{g}^{-1}\), \(v_{\text{pol}} = 0 \, \mu\text{m} \, \text{min}^{-1}\) and \(v_{\text{mag}} = 3.0 \, \mu\text{m} \, \text{min}^{-1}\), see figure 6(A) and summary in table 1.

The size of integrin clusters is in the order of 0.1–1 \(\mu\text{m}\) [18] and because the size of integrin monomer is in the order of \(\sim 10\, \text{nm}\), \(N \sim 10\) is a reasonable estimate. However, equation (8) is not sensitive to the values of \(N\) for \(N > 6\) and thus more precise estimate of \(N\) by fitting our experimental results with equation (8) is difficult. The rescaling factor \(\alpha\) is a phenomenological parameter that depends on the binding and unbinding rates for the coupling between integrin and FN, the coupling between stress fibers and integrin (via plaque proteins) and the proportional coefficient of the grafting density of FN versus the concentration of FN solutions used for the preparation of the sample and thus it is difficult to estimate from independent experiments. The magnitude \(v_{\text{f}}\) of retrograde flows arising from actomyosin and friction forces was 3.1 \(\mu\text{m} \, \text{min}^{-1}\). This corresponds to the case that actomyosin forces \(-\xi_0 \Delta \mu\) are of the order of 1 \(\mu\text{nN}\), the friction coefficients \(\xi_0\) are of the order of 10 \(\text{kPa} \cdot \text{s} \, \mu\text{m}^{-1}\) (estimated from the data in [12]) and the viscosity of actin gels is 40 \(\text{mN} \cdot \text{s} \, \mu\text{m}^{-2}\) (an estimation \(\sim 50\, \text{nN} \cdot \text{s} \, \mu\text{m}^{-2}\) is shown in [20]); the curve fitting gives parameters that are at least consistent with the values measured in other independent experiments in the order of magnitude.

### 3.2. Dynamics of filopodial growth

In our experiments, we found strong correlations between retrograde flows of actin gels in lamellipodia and the length and speeds of filopodia. Theoretically, the Brownian ratchet model is used to treat the dynamics of filopodial growth [22, 23]. The diffusion limited processes and buckling transitions in the growth of filopodia were studied by Mogilner and Rubinstein [23]. These theories assume that actin bundles are clamped at the interface between the filopodia and the lamellipodia–lamella region. We extend the theory of the diffusion limited growth of filopodia for the case of our experimental geometry that filopodia are dragged by retrograde flows at lamellipodia due to the connectivity of actin gels at the interface between these two regions. Because formal calculations are not very different from those performed in [23], we here show a simple scaling argument.

We consider that actin gels in filopodia are relatively hard and their natural length is \(l\). The time development of natural length follows the kinetics of actin polymerization and thus has the form [22]

\[
\frac{d l}{d t} = \delta \left( k_\text{on}^* a(L_f, t) - k_{\text{off}} \right),
\]

where \(k_\text{on}^*\) is the polymerization rate of actin and \(k_{\text{off}}\) is the depolymerization rate at the end of filopodia, \(y = L_f\) (see figure 1, bottom right for the definition of \(l\) and \(L_f\)). \(\delta\) is the size of actin monomer. \(a(L_f, t)\) is the concentration of actin monomers at \(y = L_f\). The net polymerization speeds \(dl/dt\) at filopodia should be distinguished from the polymerization speed \(v_{\text{pol}}\) at the

\(^4\) We also performed curve fitting for the case where actin polymerization affected the speeds of retrograde flows that were measured in our experiments. This did not change the values of other fitting parameters in the order of magnitude, e.g. for the case of \(v_{\text{pol}} = 0.5 \, \mu\text{m} \, \text{min}^{-1}\), \(N = 22.6\), \(\alpha = 0.036\, \text{ml} \, \mu\text{g}^{-1}\) and \(v_{\text{mag}} = 2.5 \, \mu\text{m} \, \text{min}^{-1}\), see table 2 for expressions of these symbols. However, the \(\chi^2_\sigma^2\) value increased with increasing \(v_{\text{pol}}\); the values of \(\chi^2_\sigma^2\) (the sum of the square of residuals) are 0.10 and 0.44 for \(v_{\text{pol}} = 0\) and 0.5, respectively. This implies that \(v_{\text{pol}} = 0\) is most probable as estimated by a (nonlinear) least mean square curve fitting.
Figure 6. The curve fitting of our experimental results in figure 5 with the prediction of our model. (A) The curve fitting of the speeds of retrograde flows with equation (8). The values of fitting parameters are $v_{\text{pol}} = 0 \mu\text{m min}^{-1}$, $v_1 = 3.0 \mu\text{m min}^{-1}$, $\alpha = 0.034 \text{ ml g}^{-1}$ and $N = 10.5$ (with $v_1 \equiv -\zeta_0 \Delta \mu \sqrt{\chi \tau_{\text{SF}} / (\lambda \tau_{\text{gel}} \eta \xi_0)}$ and $\alpha = \chi \tau_{\text{SF}} \kappa \tau_{\text{FN}} \xi_0 \gamma_{\text{FN}} / \tau_{\text{int}}$). (B) The sum of the speeds of retrograde flows and the speeds of filopodial growth that were measured in our experiments, see figure 5. Our scaling theory predicts that this does not depend on the grafting density $\sigma_{\text{FN}}$ of FN, see equation (13). (C) The curve fitting of the length of filopodial with equation (11), where we neglected $k_{\text{off}} \delta$ because this is estimated as a small value. The values of fitting parameters are $D \delta a_0 / n = 12.5 \mu\text{m}$ and $D / (n k_{\text{on}}) = 1.8 \mu\text{m}$. 

New Journal of Physics 15 (2013) 035031 (http://www.njp.org/)
Table 1. List of fitting parameters and their values. The curve fitting was performed for the case of $v_{pol} = 0 \mu m \text{ min}^{-1}$ that actin polymerization does not contribute to retrograde flows that were measured in the lamella region (see figure 5(E)) (see footnote 4).

| Parameter | Value | Expression |
|-----------|-------|------------|
| $N$       | 10.5  |            |
| $\alpha$  | 0.034 ml $\mu g^{-1}$ | $\chi \tau_{SF} \kappa \tau_{FN} C_{m}^{0} \sigma_{FN}^{0}/C_{FN}$ |
| $v_{mag}$ | 3.1 $\mu m \text{ min}^{-1}$ | $-\zeta_{0} \Delta \mu / \sqrt{\eta \xi_{0}} \sqrt{\chi \tau_{SF} h}/(\lambda \tau_{gel})$ |
| $L_{df}$  | 1.8 $\mu m$ | $D/(nk_{on}^{*})$ |
| $D\delta a_{0}/n$ | 12.5 $\mu m$ | |

Table 2. List of estimated values of physical quantities. $a_{0}$ is the one-dimensional (1D) concentration of actin monomers and is estimated from the volume concentration 10 $\mu M$ by using the estimate that the area of the cross section of the filopodia is 0.03 $\mu m^2$ [23]. Some of the estimates are extracted from experimental works, and the others are used in other theory.

| Meaning                                           | Symbol | Estimation | Reference |
|---------------------------------------------------|--------|------------|-----------|
| Actomyosin forces (per stress fiber)              | $-\zeta_{0} \Delta \mu$ | 1 nN | [15] |
| Friction coefficient (per integrin)               | $\xi_{0}$ | 10 kPa $\mu m^{-1}$ | [12] |
| Height of lamella                                  | $h$    | 1 $\mu m$ | [7] |
| Viscosity of actin gels                           | $\eta$ | 50 nN s $\mu m^{-2}$ | [20] |
| Depolymerization rate constant                     | $k_{off}$ | 1.4 s$^{-1}$ | [22] |
| Size of actin monomer                              | $\delta$ | 2.7 nm | [23] |
| Concentration of actin monomers                   | $a_{0}$ | 200 $\mu m^{-1}$ | [23] |
| Polymerization rate constant                       | $k_{on}^{*}$ | 11 $\mu m \text{ min}^{-1}$ | [23] |
| The number of actin filaments per filopodium      | $n$    | 20 | [23] |
| Diffusion constant of actin monomers              | $D$    | 300 $\mu m^2 \text{ min}^{-1}$ | [23] |

lamellipodia–lamella region because the type of nucleators and the orientation of actin filaments are different. Because these gels are connected to actin gels in the lamellipodia–lamella region and thus dragged by retrograde flows $V_{R}$, the net advance of filopodia has the form

$$\frac{dL_{f}}{dt} = \frac{dl}{dt} - V_{R},$$

where the length $L_{f}$ of filopodia is defined as the length of active gels that form membrane tubules.

Because the growth speed $\delta k_{on}^{*} a(L_{f}, t)$ of filopodia and the speed $V_{R}$ of retrograde flows are much slower than the speed $D/L_{f}$ of the diffusion of actin monomers, see also table 2, the concentration $a(y, t)$ of actin monomers is determined by the stationary solution of diffusion equations with two boundary conditions: lamellipodia play a role in the reservoir of actin monomers with constant concentration $a_{0}$ at $y = 0$. In stationary states, the fluxes of actin monomers arising from diffusion (at the end of filopodia) are equal to the rate of actin polymerization $nk_{on}^{*} a(L_{f})$, where $n$ is the (average) number of actin filaments in a cross section.

New Journal of Physics 15 (2013) 035031 (http://www.njp.org/)
of a filopodium. With these boundary conditions, the solution of the diffusion equation leads to the concentration \(a(L_t)\) of actin monomers at the protruding ends of filopodia. Solving equation (10) with (9) and the form of \(a(L_t)\) leads to the form of the length \(L_t(t)\) of filopodia as a function of time. For \(L_t(t) \simeq L_t^\infty\), this length has an asymptotic form

\[
L_t(t) = L_t^\infty \left(1 - e^{-k t}\right)
\]

with

\[
L_t^\infty = \frac{D}{nk_{on}^*} \left(\frac{\delta k_{on}^* a_0}{k_{off}^* + V_R} - 1\right),
\]

\[
\kappa = (k_{off}^* + V_R)/\tilde{D}, \quad \gamma_0 = L_t^\infty /\tilde{D}, \quad \text{and} \quad \tilde{D} = D a_0^* / (n(k_{off}^* + V_R)).
\]

\(D\) is the diffusion constant of actin monomers. \(L_t^\infty\) is the solution of equation (10) in the stationary state. Equation (11) suggests that the time scale \(\tau_f\) of filopodial growth is \(\tau_f = (1 - \gamma_0)/\kappa\). The average speed \(V_*\) of filopodial growth scales as

\[
V_* \sim \frac{L_t^\infty}{\tau_f} = \delta k_{on}^* a_0 - k_{off}^* - V_R.
\]

This implies that the sum of the speeds \(V_*\) of filopodial growth and the speeds \(V_R\) of retrograde flows is independent of the density of integrin molecules. This prediction, indeed, agrees with our experiments, see figure 6(B). The slight deviation of \(|V_*| + |V_R|\) from a constant value implies that the speeds of retrograde flows at the spreading front may be slightly larger than the speeds of retrograde flows that were measured in our experiments. This may be because the passivation outside adhesive regions was not perfect and the small number of stress fibers in non-adhesive regions may have increased the speeds of retrograde flows. Our theory predicts that the strong correlations between retrograde flows and filopodial growth are because of connectivity of actin gels in the lamellipodia–lamella region and those in filopodia.

The depolymerization speed \(k_{off}^* \delta\) for the case of actin filaments that bind ATP is estimated as \(0.22 \mu m min^{-1} in vitro\) [22, 23]; this speed is an order of magnitude lower than the speeds of retrograde flows \(V_R \sim 3 \mu m min^{-1}\). We thus neglect this contribution in the following discussion. Equation (13) implies that the sum of the speeds \(V_R\) of retrograde flows and the speeds \(V_*\) of filopodial growth leads to (filopodial) polymerization speed \(a_0^* \delta k_{on}^*\) that is independent of the grafting density of FN. To avoid confusion, we again note that polymerization speeds \(a_0^* \delta k_{on}^*\) at the end of filopodia should be distinguished from polymerization speeds \(v_{pol}\) at the lamellipodia–lamella region because the type of nucleators and the orientation of actin filaments are different. In our experiments, the polymerization speed is estimated to be \(4.7 \mu m min^{-1}\) from the value of \(|V_R| + |V_*|\), see figure 6. In [23], the polymerization speeds are estimated as \(6.0 \mu m min^{-1}\), where the polymerization rate \(k_{on}^*\) is \(11 \mu m min^{-1}\), the 1D concentration of actin monomer is \(200 \mu m^{-1}\) (this is estimated from the volume concentration of actin monomers, \(\sim 10 \mu M\), and the area of the cross section of filopodia, \(\sim 0.03 \mu m^2\)), and the size \(\delta\) of actin monomer is \(2.7 nm\).\(^5\) The curve fitting of our experimental results with equation (12) gives the same order of magnitude as the values estimated in [23]. The slight difference may be because of the fact that the speeds of retrograde

---

\(^5\) The polymerization rate \(k_{on}^*\) is calculated from the estimated values of membrane resistance force of 30 pN, actin polymerization rate (when there are no applied membrane resistance forces) of \(30 \mu m min^{-1}\) and the number of actin filaments in filopodia of 20, see [22, 23]. Membrane resistance forces estimated in [23] range between 10 and 50 pN.
flows measured in our experiments only have the contributions of actomyosin forces, as we have discussed in section 3.1; when retrograde flows arising from actin polymerization are not sensitive to the grafting density of FN, the retrograde flows at the spreading front are larger than the speeds of retrograde flows that we measured by tracking vesicles in our experiments by a constant value.

The fitting parameters of equation (12) are depolymerization speed $k_{off \delta}$, the length scales determined by the diffusion $D/(nk_{on}^*)$ and $D \delta a_0/n$, see figure 6(C) and table 1. Because depolymerization speed $k_{off \delta}$ is much slower than the speeds of retrograde flows, we fitted the filopodia length measured in our experimental results with equation (12) neglecting depolymerization speeds (see also the discussion above). The curve fitting gives the values of length scales $D/(nk_{on}^*)$ and $D \delta a_0/n$ as 1.8 and 12.5 $\mu$m, respectively, see figure 6(C). This corresponds to the case where the diffusion constant of actin monomers is 400 $\mu$m$^2$ min$^{-1}$, where this is the same order of magnitude as the value of the diffusion constant 300 $\mu$m$^2$ min$^{-1}$ estimated in [23]. The value of $D \delta a_0/n$ divided by the values of $D/(nk_{on}^*)$ leads to another estimate of polymerization speeds $\delta a_0 k_{on}^*$ as 6.9 $\mu$m min$^{-1}$, which is only slightly different from the value estimated above (by using the parameters estimated [23]). These estimates imply that our prediction is at least consistent with the results of our experiments. However, this does not rule out other possibilities as we discuss in the next section.

4. Discussion

We have experimentally shown that actin dynamics shows non-monotonic behaviors as functions of the grafting density of FN and that there are strong correlations between the speeds of retrograde flows, the length of filopodia and the speeds of filopodial growth. Motivated by these experimental results, we take into account interactions between actin gels and substrates in an extension of a hydrodynamic theory of active gels. Our theory predicts that the non-monotonic behaviors of the speeds of retrograde flows (as a function of the grafting density of FN) are because of the dual role played by focal contacts—mechanical connections that apply actomyosin and friction forces to actin gels. Moreover, this theory predicts that the connectivity of actin gels in lamellipodia and filopodia are responsible for the strong correlations between the speeds of retrograde flows and the speeds of filopodial growth. This ‘mesoscopic’ picture (on the basis of the physical properties of actin gels) may be useful in bridging the molecular scale pictures, e.g. proteins and genes, that have been elucidated in biology and the dynamics of cells that have been quantitatively measured in physics.

Retrograde flows that are directional flows in cells were discovered in the late 1970s [24]. At that time, molecular motors had not been identified yet. There was a long standing controversy on the driving force of retrograde flows; it was considered to be either driven by lipid flows or by actin dynamics. This controversy ends with a consensus that retrograde flows are driven by actin dynamics. It was experimentally demonstrated by the quantitative tracking of single gold particles in retrograde flows [25]. Retrograde flows are driven by actin polymerization at the spreading front of cells in lamellipodia and are driven by actomyosin contractile forces in lamella. Lin and Forscher [26] experimentally showed that retrograde flows are inversely correlated to the speeds of filopodial growth. In that work, they measured the speeds of retrograde flows at the axis of the filopodia (growth cones) of neurons and the speeds of the growth of these filopodia, where these speeds show fluctuations for different cells. They speculated that friction forces arising from direct interactions between actin gels and the...
substrate may be the origin of this inverse relationship. In our experiments, we showed the strong correlation between the speeds of retrograde flows and the speed of filopodial growth by systematically changing the grafting density of FN; this shows that interactions between actin gels and the substrate are indeed involved in the correlation.

In our experiments, the speeds of retrograde flows increased and the spreading area of cells decreased with increasing the grafting density of FN for a relatively small grafting density of FN. If the speeds of retrograde flows increased with increasing grafting density of FN because the polymerization rates of actin at the spreading front were somehow enhanced, the spreading area would increase; other mechanisms that decrease the spreading area are necessary to predict both the phenomena. If focal contacts somehow enhanced the depolymerization of actin gels at the interface between lamellipodia and lamella, this might predict the non-monotonic behaviors of retrograde flows. However, the Rho pathway that is activated by FN–I binding indeed inhibits the activity of cofillin (that accelerates depolymerization of actin filaments) [27] and thus the enhancement of actin depolymerization is probably not the mechanism that increased retrograde flows. In our experiments, we used vesicles as probes of retrograde flows and these vesicles are usually localized in lamella [21], where retrograde flows are mainly driven by actomyosin forces [9, 10]. In our model, we thus used a simple argument that actomyosin contractile forces increased with increasing the grafting density of FN, because this increases the number of stress fibers that are bound to substrates (via FN–I focal contact binding). Our theory is consistent with our experimental results (except for the fact that the area fraction of focal contacts is constant, see below). However, this does not rule out other possibilities, e.g. the Rho pathway activated by FN–I binding enhances light chain phosphorylation of the myosin molecules of stress fibers [27]; this indeed increases the actomyosin forces applied at actin gels.

Our measurements suggest that the area fraction of focal contacts did not change with changing the grafting density of FN. It was suggested that the size of integrin clusters decreases with increasing ECM proteins (but for CHO cells on fibrinogen coated surfaces) [18]. This implies that the number of integrin clusters that are bound to stress fibers increased, but the size of these integrin clusters decreased; the constant area (within the resolution of our experiments) of focal contacts may be because of these two competing effects. However, further experiments are necessary to test our prediction that the number of stress fibers that are bound to FN increases with increasing the grafting density of FN.

Palecek et al [1] have shown that the speed of cell migration is a non-monotonic (concave) function of the grafting density of ECM proteins. Many theoretical studies have elucidated the macroscopic properties of the dynamics of cell migration by extending the theory of stochastic motion of non-living matters [28]. It has been suggested that friction forces applied to actin gels in retrograde flows play an important role in protruding lamellipodia [12]. Our experiments suggest that the speeds of retrograde flows are also non-monotonic (concave) functions of the grafting density of FN. Cell type, the type of ECM proteins and the grafting density of ECM proteins used in our experiments and the experiments performed by Palecek et al are different from our experiments and thus a quantitative comparison is still not straightforward; more experiments are necessary to elucidate the relationship between cell migration and cytoskeletal dynamics. However, we believe that our experiments (that characterize the dynamics of actin gels) can be the first step towards understanding the physical mechanisms involved in the non-monotonic dependence of the speeds of cell migration (on the grafting density of ECM proteins) from the mesoscopic viewpoint.
Table 3. The ratio of FN mixtures to the final concentration for incubation.

| FN               | Final concentration (µg ml\(^{-1}\)) |
|------------------|--------------------------------------|
|                  | 30 | 40 | 47.5 | 55 | 70 |
| 10 µg ml\(^{-1}\) (fluorescent) (%) | 78 | 67 | 57 | 50 | 33 |
| 100 µg ml\(^{-1}\) (non-fluorescent) (%) | 22 | 33 | 43 | 50 | 67 |

5. Materials and methods

5.1. Micropattern fabrication

FN patterns were fabricated by the microcontact printing technique [19, 29]. Poly(dimethylsiloxane) (PDMS) stamps (Sylgard 184 kit, Dow Corning) were prepared using standard soft-lithography techniques. The mold of SU-8 resist (MicroChem Corp.) was produced on a silicon wafer (Siltronix) by irradiating it with UV light (MJB3 contact mask aligner; SUSS MicroTec) through a photolithography mask (Selba SA) and then developed by using an SU-8 developer (MicroChem Corp.). Glass coverslips were activated by immersing them into a Piranha solution (3 : 7, v/v, H\(_2\)O\(_2\) : H\(_2\)SO\(_4\)) for 5 min, thoroughly rinsed with Milli-Q and dried with N\(_2\). They were functionalized with 3-(mercaptop)propyltrimethoxysilane (3-MPTS; Fluorochem) by vapor phase for 1 h and cured for 1 h at 65°C. The PDMS stamps were obtained by mixing prepolymer and the curing agent in a 10 : 1 (w/w) ratio. Stamps were exposed to O\(_2\) plasma to make it hydrophilic. Then, they were incubated for 1 h with a mixture of 10 µg ml\(^{-1}\) rhodamine-labeled FN phosphate buffered saline (PBS) solution (Cytoskeleton Inc.) and 100 µg ml\(^{-1}\) non-labeled FN (Sigma) with the ratios shown in table 3. We used these ratios to estimate the grafting density of FN in all experiments. Then the stamp was dried with N\(_2\) and placed in contact with a glass coverslip, 25 mm in diameter, under a constant pressure for 5 min (figure 2). After removing the stamp, the sample was cleaned successively in Dulbecco’s PBS (InVitrogen, pH 7.4), Milli-Q water and 10 mM Hepes (Sigma) (pH 7.4) solutions. Afterwards, the non-functionalized areas were blocked with a 0.1 mg ml\(^{-1}\) solution of PLL-g-PEG (Surface Solutions GmbH) in 10 mM Hepes (pH 7.4) at room temperature for 30 min. Finally, the sample was rinsed with PBS twice and stored in PBS at 4°C before cell deposition.

5.2. Cell culture

NIH3T3 mouse fibroblasts were purchased from the American Type Culture Collection. They were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (InVitrogen) supplemented with 1% Pen Strept antibiotics (InVitrogen) and 10% bovine calf serum (BCS) (Sigma-Aldrich) at 37°C and 5% CO\(_2\). Afterwards, cells were trypsinised (0.25% Trypsin–EDTA) (InVitrogen) and deposited on the microcontact printed substrate at 2.3 × 104 cells ml\(^{-1}\) density for 20 min. Non-adhered cells were removed by replacing old DMEM medium with the fresh one. Cells were observed up to 5 h after plating, with no noticeable differences. For focal contact experiments, cells were incubated for 2 h before fixation.
5.3. Time lapse experiments

To reduce the deposition of ECM proteins from the serum, experiments were performed in 1% BCS L-15 medium (InVitrogen) supplemented with 1% Pen Strept antibiotics (InVitrogen). We used a Nikon Eclipse Ti inverted microscope connected to a camera (Photometrics CoolSNAP HQ2 12 bit) and an incubator (Cube and Box system, Life Imaging Services) that allows us to control the temperature at 37°C. DIC time lapse images were acquired every 15 s using a Nikon Plan Apochromat ×60 oil objective (1.4 NA). In figure 5, we showed the average values of the spreading area, the length of filopodia, the speeds of their growth and retraction and the speeds of retrograde flows that are obtained by analyzing five cells in each of three biological repeats (15 cells per condition in total). Moreover, we used the following condition to make sure that each biological repetition affects the average values in the same manner: (i) for the measurements of spreading area, microscopic images were acquired in 10 min intervals for 1 h, (ii) filopodia length was measured using the same time frames as the measurements of spreading area and we measured 15–20 filopodia per frame, (iii) we measured 9–12 filopodia to measure the speeds of filopodial growth and retraction and (iv) we measured seven to ten vesicles per cell to measure the speeds of retrograde flows.

5.4. Cell areas

Custom Matlab (MathWorks) script was developed for the measurement of cell areas. First, a Canny edge detector was applied to the initial images, which were then dilated with the linear structuring elements to delineate the outline of the object. Internal gaps were then filled in, images were smoothened and the outline was detected. Visual verification was systematically performed.

5.5. Length and velocity of filopodia

Length and velocity of filopodia were measured manually by using ImageJ built-in functions (multipoint selection and segmented line, respectively). Filopodia were mapped with numbered points and their positions were saved. The length of filopodia was calculated as the module of the vector connecting points corresponding to a given filopodia (or the sum of modules if the filopodia was marked with more than two points).

5.6. Retrograde flow

Retrograde flow was observed and mapped on the successive frames in ImageJ by using the point selection. Positions of the pixel corresponding to the tracking object were saved and retrograde flow was calculated.

The procedure was repeated for all FN concentrations and the arithmetic average for a given FN condition was calculated. The data for each FN concentration are represented as mean ± standard error.

5.7. Focal contacts staining and fibronectin intensity

Cells were fixed in 3% paraformaldehyde (Sigma) for 17 min. Afterwards, cells were treated with 0.5% Triton (Sigma) for 3 min and washed twice with PBS and once with 3% bovine
serum albumin (BSA) (Sigma) for 5 min. Then, cells were incubated with primary antibody (BD Transduction Laboratories purified mouse anti-paxillin 1/100 concentration) for 45 min and again washed twice with PBS and once with 3% BSA for 5 min. After washing, the samples were incubated for 45 min with secondary antibody (Molecular Probes Alexa Fluor 488 goat anti-mouse IgG(H+L) 1/100 concentration), and washed three times for 5 min with PBS. Finally, the samples were mounted with glycerol/PBS and secured from drying. Both measurements (area of focal contacts and intensity of patterns) were made manually by selecting the proper region of interest in ImageJ and setting needed measurements (area and mean intensity, respectively). The FC area was normalized, taking into account the FN spot area occupied by the cells edge. The data for each FN concentration are represented as mean ± standard error.

5.8. Statistical analysis

Statistical analysis was performed with the two-tailed Mann–Whitney U test and $P < 0.05$ was considered significant.

Acknowledgments

The authors are grateful to Vishwajeet Mehandia for a careful reading of the manuscript and useful comments. We thank the members of the Riveline laboratory for discussion and David Caballero for technical support. This work was supported by funds from the CNRS, the University of Strasbourg and the ci-FRC of Strasbourg.

References

[1] Palecek S P, Loftus J C, Ginsberg M H, Lauffenburger D A and Horwitz A F 1997 Integrin–ligand binding properties govern cell migration speed through cell-substratum adhesiveness Nature 385 537
[2] Engler A, Bacakova L, Newman C, Hategan A, Griffin M and Discher D 2004 Substrate compliance versus ligand density in cell on gel responses Biophys. J. 86 617
[3] Zemel A, Rehfeldt F, Brown A E X, Discher D E and Safran S A 2010 Optimal matrix rigidity for stress-fibre polarization in stem cells Nature Phys. 6 468
[4] Engler A J, Sen S, Sweeney H L and Discher D E 2006 Matrix elasticity directs stem cell lineage specification Cell 126 677
[5] Kruse K, Joanny J F, Jülicher F, Prost J and Sekimoto K 2004 Asters, vortices and rotating spirals in active gels of polar filaments Phys. Rev. Lett. 92 078101
[6] Kruse K, Joanny J F, Jülicher F, Prost J and Sekimoto K 2005 Generic theory of active polar gels: a paradigm for cytoskeletal dynamics Eur. Phys. J. E 16 5
[7] Kruse K, Joanny J F, Jülicher F and Prost J 2006 Contractility and retrograde flow in lamellipodium motion J. Phys. Biol. 3 130
[8] Nédélec F J, Surrey T, Maggs A C and Leibler S 1997 Self-organization of microtubules and motors Nature 389 305
[9] Alexandrova A Y, Arnold K, Schaub S, Vasiliev J M, Meister J J, Bershadsky A D and Verkhovsky A B 2008 Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow PLoS ONE 3 e3234
[10] Ponti A, Machacek M, Gupton S L, Waterman-Storer C M and Danuser G 2004 Two distinct actin networks drive the protrusion of migrating cells Science 305 1782
[11] Chan C E and Odde D J 2008 Traction dynamics of filopodia on compliant substrates Science 322 1687

New Journal of Physics 15 (2013) 035031 (http://www.njp.org/)
[12] Gardel M L, Sabass B, Ji L, Danuser G, Schwartz U S and Waterman C M 2008 Traction stress in focal adhesions correlates biphasically with actin retrograde flow speed J. Cell Biol. 6 999
[13] Kandere-Grzybowska K, Campbell C J, Mahmud G, Komarova Y, Soh S and Grzybowski B A 2007 Cell motility on micropatterned treadmills and tracks Soft Matter 3 672
[14] Balaban N Q et al 2001 Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates Nature Cell Biol. 3 466
[15] Riveline D, Zamir E, Balaban N Q, Schwartz U S, Ishizaki T, Narumiya S, Kam Z, Geiger B and Bershadsky A D 2001 Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an MDia-1 dependent and ROCK-independent mechanism J. Cell. Biol. 153 1175
[16] Choi K C, Vicente-Manzanares M, Zaren J, Whitmore L A, Mogilner A and Horwitz A R 2008 Actin and α-actinin orchestrate the assembly and maturation of nascent focal adhesions in a myosin II motor-independent manner Nature Cell Biol. 10 1039
[17] Arnold M, Cavalcanti-Adam E A, Glass R, Blummel J, Eck W, Kantlehner M, Kessler H and Spatz J 2004 Activation of integrin function by nanopatterned adhesive interfaces Chem. Phys. Chem. 5 383
[18] Welf E S, Naik U P and Ogunnaike B A 2011 Probabilistic modeling and analysis of the effects of extra-cellular matrix density on the sizes, shapes and locations of integrin clusters in adherent cells BMC Biophys. 4 15
[19] Théry M, Pépin A, Dressaire E, Chen Y and Bornens M 2006 Cell distribution of stress fibres in response to the geometry of the adhesive environment Cell Motil. Cytoskeleton 63 341
[20] Zimmermann J, Enculescu M and Falcke M 2010 Leading-edge–gel coupling in lamellipodium motion Phys. Rev. E 82 051925
[21] Verkhovsky A B 2012 private communication
[22] Howard J 2001 Mechanics of Motor Proteins and the Cytoskeleton (Sunderland, MA: Sinauer Associates) 2005
[23] Mogilner A and Rubinstein B 2005 The physics of filopodial protrusion Biophys. J. 89 782
[24] Abercrombie M, Heaysman J E and Pegrum S M 1970 The locomotion of fibroblasts in culture. 3. Movements of particles on the dorsal surface of the leading lamella Exp. Cell Res. 62 389
[25] Sheetz M P, Turney S, Qian H and Elson E L 1989 Nanometre-level analysis demonstrates that lipid flow does not drive membrane glycoprotein movements Nature 340 284
[26] Lin C H and Forscher P 1995 Growth cone advance is inversely proportional to retrograde F-actin flow Neuron 14 763
[27] Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P 2007 Molecular Biology of The Cell 5th edn (New York: Garland Science)
[28] Selmecezi D, Li L, Pedersen L II, Nrelykke S F, Hagedorn P H, Mosler S, Larsen N B, Cox E C and Flyvbjerg H 2008 Cell motility as random motion: a review Eur. Phys. J. Spec. Top. 157 1
[29] Théry M, Jiménez-Dalmaroni A, Racine V, Bornens M and Jülicher F 2007 Experimental and theoretical study of mitotic spindle orientation Nature 447 493