Spatiotemporal dynamics of traction forces show three contraction centers in migratory neurons

Jian Jiang, Zheng-hong Zhang, Xiao-bin Yuan, and Mu-ming Poo

1Institute of Neuroscience, State Key Laboratory of Neuroscience, CAS Center for Excellence in Brain Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China 200031
2Graduate School of the Chinese Academy of Sciences, Shanghai, China 200031

Correspondence to Mu-ming Poo: mpoo@ion.ac.cn
Z.-h. Zhang’s present address is Hussman Institute for Autism, Baltimore, MD 21201.
X.-b. Yuan’s present address is Hussman Institute for Autism, Baltimore, MD 21201.
X-b. Yuan’s present address is Hussman Institute for Autism, Baltimore, MD 21201.

Traction force against the substrate is required for neuronal migration, but how it is generated and regulated remains controversial. Using traction force microscopy, we showed in cultured granule cells the coexistence of three distinct contraction centers (CCs) that are located at the distal and proximal regions of the leading process as well as at the trailing process, regions exhibiting high-level myosin-II activities. The CC activities depended on myosin-II, actin filaments, and microtubules, as well as substrate adhesion, and exhibited apparently independent fluctuation. The difference of strain energies associated with CC activities between leading versus trailing processes tightly correlated with the displacement of the soma at any given time. Application of brain-derived neurotrophic factor (BDNF) and Slit2, factors known to guide neuronal migration, at the leading process altered CC activities by regulating the small GTPases Cdc42 and RhoA, respectively, leading to forward and rearward soma translocation. These results delineate the multiple origins and spatiotemporal dynamics of the traction force underlying neuronal migration.

Introduction

Migration of neurons from their birthplace to the designated areas is a critical step in the development of brain architecture (Hatten, 1999; Ayala et al., 2007). This migration is believed to be a directed process, in which guidance cues in the developing brain regulate neuronal contractile activities that drive neuronal migration. Much progress has been made in our understanding of the cellular and molecular basis of neuronal migration in vitro (Solecki et al., 2009) and in vivo (Tsai et al., 2007), including the role of various cytoskeleton contractile components (He et al., 2010), their regulation by intracellular signaling pathways (Feng and Walsh, 2001), and transduction mechanisms underlying cellular responses to extracellular guidance cues (Wu et al., 1999; Guan et al., 2007). However, the biomechanical aspects of neuronal migration are only beginning to be explored (Moore and Sheetz, 2011).

Mechanical forces generated by cells or tissues play important roles in a variety of biological processes, such as sensing of substrate rigidity (Plotnikov et al., 2012), assembly of focal adhesion (Balaban et al., 2001; Roca-Cusachs et al., 2013), cell fate determination (Yim and Sheetz, 2012), cell polarization (Houk et al., 2012), directional cell migration (Weber et al., 2012), epithelial spreading (Behrndt et al., 2012), and wound healing (Brugues et al., 2014). At the cellular level, traction force measurements showed that a single contraction center (CC; or “dipole”) is responsible for generating the traction force for fibroblast migration (De et al., 2007). And there is also a comprehensive model suggesting that the anterior region, soma, and posterior region play different roles in fibroblast migration (Guo and Wang, 2012). Unlike fibroblasts, the soma of migrating neurons is much smaller in size and extends long leading processes (LPs) and trailing processes (TPs) that may translocate relatively independently with respect to the soma, suggesting that the spatiotemporal pattern of traction force generation in migrating neurons may be substantially different from that of the fibroblast. At the subcellular level, actin filaments (F-actin) and the associated nonmuscle myosin-II are responsible for the contractile activity and migration of many cell types (Vincente-Manzanares et al., 2009). A high concentration of myosin-II has been found at the leading growth cone (He et al., 2010), proximal region of the leading process (pLP; Solecki et al., 2009), or TP (Martini and Valdeolmillos, 2010). By examining the relationship between myosin-II distribution and soma translocation, the main indicator of neuronal migration, previous studies have proposed disparate models of...
force generation that drive soma translocation: the soma may be pulled by the proximal region of the LP (pLPs; Solecki et al., 2009) or the growth cone of the LP (He et al., 2010), or pushed by the TP (Martini and Valdeolmillos, 2010). In addition, there is evidence that microtubules (MTs) may also be involved in regulating neuronal migration, as suggested by the finding that MT depolymerization induced by pharmacological agents or down-regulation of the motor protein kinesin-5 by RNA interference could accelerate neuron migration (He et al., 2010; Falnikar et al., 2011). In the present study, we first found that traction force may be generated simultaneously at up to three different regions in migrating neurons, including the distal region of the LP (dLP), pLP, and TP. We then performed quantitative analysis of the spatiotemporal dynamics of force generation at these three CCs, and further investigated the role of various cytoskeletal components and adhesions in the force generation.

Neuronal migration during development is regulated by guidance cues (Ayala et al., 2007), which act on the growing tip of neuritic processes at distant locations from the soma (Guan et al., 2007). The neurotrophin brain-derived neurotrophic factor (BDNF) exerts both chemokinetic (Borghesani et al., 2002) and chemotactic (Yuan et al., 2003) effects on cultured neurons (Park and Poo, 2013). Genetic depletion of BDNF impairs the migration of granule cells (GCs) to the molecular zone of developing mouse cerebellum (Borghesani et al., 2002). In developing mouse telencephalon, Slit1/2 repels neuronal precursor cells migrating from the anterior subventricular zone to the olfactory bulb (Wu et al., 1999).

Frontal application of Slit2 to the LP of cultured cerebellar GCs also induces growth cone collapse and reverses the direction of GC migration (Guan et al., 2007). The long neuritic processes of migration neurons and the existence of directional sensing mechanisms at the neuritic growth cone suggest that traction forces generated at different regions of the neuron must be properly coordinated in order to achieve effective neuronal migration.

Among many methods for measuring mechanical properties of cells or tissues, only traction force microscopy (TFM; Munevar et al., 2001) and stress-sensitive biosensors (Grashoff et al., 2010) can be used to measure the traction force of the entire cell. Two commonly used cell substrates for TFM are nanopillars (du Roure et al., 2005) and polymer sheets embedded with microbeads (Munevar et al., 2001). Fluorescent microbead-loaded polyacrylamide (PAA) gel substrate could be prepared with various degrees of stiffness suitable for studying different cell types, e.g., fibroblasts (Munevar et al., 2001), epithelial cells (Serra-Picamal et al., 2012), and neurons (Chan and Odde, 2008), by setting the PPA concentration. This method allowed us to measure neuronal traction forces that were found in this study to be much weaker than those generated by fibroblasts (Butler et al., 2002) or glial cells. By monitoring the displacement of microbeads over prolonged periods around the entire neuron, we have identified up to three distinct CCs coexisting in the same neuron, and further examined spatiotemporal dynamics during spontaneous migration and under the influence of guidance factors. Our results provide a comprehensive description of the spatiotemporal dynamics of contractile activities and their regulation by guidance molecules in migrating neurons, as well as the underlying cytoskeletal and molecular mechanisms.

Results

Three CCs identified by TFM

Cerebellum GCs were obtained by dissociating cerebella of newborn rats on postnatal day 0–2 (P0–P2) and cultured on the PAA substrate embedded with fluorescent microbeads (see Materials and methods). We measured the displacements of discrete beads in the surrounding substratum with time, relative to their substrate locations in the absence of the cells, as determined after cell removal with osmotic lysis at the end of time-lapse imaging (Fig. 1 a). There is no observable displacements of beads at a distance beyond ~30 μm from the cell (Fig. 1 b), indicating that bead displacements around the neuron reflect the deformation of the PAA gel caused by neuron-generated traction force rather than osmotic lysis. We then transformed the displacement vectors (Fig. 1 a) into a continuous map of displacements (Fig. 1 a, see Materials and methods) and stress (Fig. 1 c, see Materials and methods).

We found that the stress map is highly dynamic, exhibiting large variations with time at different regions around the cell (Video 1). For example, the relatively dark area shown in Fig. 1 c1 at the dLP behind the growth cone was a CC location at the time of observation, as indicated by convergent stress vectors. However, within minutes, two additional CCs at pLP (Fig. 1 c2) and TP (Fig. 1 c3) were observed in the same cell. Moreover, coexistence of two CCs with similar contractile activities was often observed, as illustrated in another cell in Fig. 1 c5. In GCs with more than two primary neurites, we had observed the existence of dominant CCs at all neurites at the same or different times, as illustrated in the example cell in Fig. S1, d–f. For GCs with a bifurcating LP, each branch had an independent CC (Fig. 1 c6). Since GCs with bifurcating processes or more than two primary processes were relatively rare, we focused only on nonbifurcating bipolar GCs in the present study. In 72 cells examined, we found that 9, 32, and 31 cells exhibited 3, 2, and 1 dominant CCs, respectively, at the three different regions (dLP, pLP, and TP) during the ~40-min observation period (Fig. 1 e3 and Tables S1 and S2). Thus, by identifying the sites of convergent stress vectors, we have consistently identified three locations where the CC appears most in migrating GCs, sometimes with multiple CCs simultaneously active. The CCs can also be visualized by representing the stress field in a 3D color map or a “pinwheel” color map in which directions of stress vectors are represented by color (Fig. S1, a–c).

To fully characterize the spatiotemporal dynamics of traction forces, we determined the CC location automatically by calculating the maximal change of stress directions between two adjacent points on the cell axis (darkest region in the Fig. 1 d, marked by green dots) with time over the observation period. As shown in Fig. 1 d, relative to the center of mass of the soma, the CC location moved up and down along the neuronal axis with time. Mutipeak Gaussian fitting of the cumulated frequency of the locations of dominant CC along the axis showed three distinct peaks at dLP, pLP, and TP, consistent with the three CC locations identified by convergent stress vectors (see Fig. S2 for other examples).

The stress detected in the substrate represents the resultant action of contractile activities of all CCs. The activity at one CC may be dominant so that the activities of other CCs are not clearly observed by microbead displacements in the substrate. However, the activity of nondominant CCs could be revealed by measuring the changes in stress (displacement) vectors with
Force dynamics of neurons show contraction centers • Jiang et al. 761

Figure 1. Three CCs identified by TFM. (a) Steps in determining microbead displacements around a GC. a1, image of a GC. a2, superimposed image of fluorescent beads before (red) and after (green) osmotic lysis of the cell. Insets show the boxed regions at a higher resolution (bars, 1 μm). Solid traces, cell outline. a3, discrete displacement vectors, with arrow length and arrowheads representing displacement magnitude and direction, respectively. a4, continuous displacement field map (coded in grayscale) obtained by interpolation of discrete displacement vectors in a3, marked by regularly spaced vectors. (b) Displacement of the bead at different distances from the GC. (b, left) Displacement map. (b, middle) Beads close to (cyan and purple asterisks, <30 μm) and distant from (red asterisks, ≥30 μm) the neuron. Green arrow, direction of soma translocation. Broken line, points ≥30 μm away. (b, right) Changes of displacements with time for beads at different distances. No displacement was observed for distant beads [red]. (c) Stress maps of substrates. c1–c5, stress maps computed from the continuous displacement field maps at different times for the same GC shown in a, and three distinct CCs (white circles) were observed at three different times. c1, stress map at t = 1 min (the same time as in a). c2, t = 21 min. c3, t = 38 min. c4, stress map caused by another GC showing two coexisting CCs, c5, each bifurcating process generated its own CC. (d) CC locations identified by a stress map generated by the same typical GC shown in a. (d, left) Outline (blue) and axis (red) of the GC. (d, middle) Dynamics of the stress along the axis of the migratory GC at different distances from the tip of the TP over a 60-min period, obtained from the stress map similar to that shown in c1. Line bins, 21 s. The location of the CCs (marked by green dots), determined as the site of the maximal absolute value of the angular difference between stress vectors of adjacent pixels (see Materials and methods). Cyan line, soma center. (d, right) Frequency distribution of CCs observed at different distances away from the soma during the observation period for the cell shown on the left. Red curve, best fit with a multi-peak Gaussian function (R = 0.61). Broken black line, soma center. Additionally, 18 more cases are shown in Fig. S2 and a total number of 72 GCs are summarized in e4. (e) CC locations identified by a stress differential map. e1–e3, changes in the stress (“stress differential,” ΔF over Δt = 21 s) at three different time points (t = 0, 26, and 38 min) for the same cell as in a, with ΔF color-coded. Note that three different CCs were identified [broken lines] as regions with convergent stress differential vectors. e4, percentage of cells showing CCs at three different locations. dLP, distal end of LP; pLP, proximal end of LP; TP, trailing process. Histograms, data from all 72 GCs, each for an ~40-min observation period, with the CC location determined by stress map (orange) and stress differential map (blue).
time at different locations, i.e., the stress differential ($\Delta F$ within $\Delta t = \sim 10–21$ s). As shown in Fig. 1, $e_1$–$e_3$, by calculating the changes of local stress over the previous 21-s period, we found a significant stress differential occurring at three CCs locating at dLP, pLP, and TP at three different times, as indicated by the region with convergent vectors of stress differentials. (CCs can also be identified as regions with divergent stress differentials that reflect the reduction of CC contractile activity, see Fig. 3). By using a stress differential map, we found that all 72 cells examined had at least two distinct CCs, and 51/72 had three CCs. This is more than was found using the stress map alone, as indicated by the histograms in Fig. 1 e4 and Tables S1 and S2. There-
immunofluorescence intensity along the neuron with a multipeak Gaussian function showed three peaks (Fig. 4, i and j) corresponding roughly to the location of the three CCs found in GCs cultured on PAA gel (Fig. 1, d and e4), except that higher immunostaining was found in the soma but not in the pLP. This may be attributed to the larger cellular volume at the soma.

The role of F-actin in force generation was also examined by applying pharmacological treatments that affect F-actin polymerization and stability. We found that the strain energy produced by the GC was greatly reduced (by ∼75%) after treatment with the F-actin depolymerizing drug latrunculin A (LA; 1 µM, Fig. 4, c and g). In contrast, treatment with the F-actin stabilizing drug jasplakinolide (Jasp; 250 nM, Fig. 4, d and g) resulted in stabilization of strain energy production with very little fluctuation around the same average level (Fig. 4, c and g). These results are consistent with the expectation that actomyosin contractility is the main contributor to force generation and further suggest that F-actin dynamics are required for the dynamic fluctuation of force generation.

During cell division, polymerization and depolymerization of MTs provide the pushing and pulling forces, respectively, for chromosome separation (Dogterom et al., 2005). In cultured GCs, however, we found that disrupting MTs by nocodazole (Noc; 0.5 µM) that depolymerizes MTs resulted in an enhanced stress generation (Fig. 4 e) and elevated strain energy (by approximately sixfold; Fig. 4 g), whereas treatment with

![Figure 2](https://example.com/image2.png)

**Figure 2.** Dynamic contraction activities at multiple CCs can drive soma translocation. (a, left) The typical neuron is divided into three regions (marked by colored brackets) roughly corresponding to regions around three CCs: dLP (red), pLP (green), and TP (blue). (a, right) Changes in the total local strain energy (see Materials and methods) of three regions of the GC shown on the left during a 60-min observation period. Arrows, asynchronous contraction of two or three CCs; arrowheads, synchronous contraction of three CCs. Additionally, three more cases are shown in b–d. The dynamic and asynchronous contraction can also be observed in the 18 cases in Fig. S2. (b) CC at dLP pulled the soma forward in a migrating GC. (b, top) Line scan of stress along the cell axis during the observation time. Cyan line, soma center; green dots, position of the dominant CC. (b, middle) Local strain energies at dLP, pLP, and TP. (b, bottom) Soma translocation with time. Pink shade, CC activities at dLP pulled the soma forward. (c and d) Two other migrating GCs showing rapid soma translocation when CC activities at TP and pLP were high, respectively, and strain energies at TP (SdLP + SpLP) versus TP (SdLP) were highly unbalanced (black bar vs. gray bar). More detailed analysis of five cases similar to b–d are shown in Fig. 3 and Fig. S3, and a total number of 28 GCs were summarized in e. (e) Cross-correlation of the velocity of soma translocation with [SdLP + SpLP] (marked as “LP”), STP (“TP”), and [(SdLP + SpLP) − STP] (“Δ”). (e, top) Cross-correlation coefficient for the example cell in d. Red horizontal lines represent the approximate upper and lower confidence bounds (95%), assuming that migration velocity and traction energy are completely uncorrelated. (e, bottom) Averaged cross-correlation coefficient for all 28 migrating cells examined.
Figure 3. **Temporal correlation between fluctuations in CC activity and soma translocation.** (a) Line scan of the stress along the typical GC axis (left) and soma translocation (right) with time for the same migrating GC. Green dot, location of the dominant CC; cyan line, soma center. Additionally, four more cases were shown in Fig. S3, and a total number of 28 migrating GCs were summarized in Fig. 2 e. (b) Soma displacements during the boxed period in a.
taxol at a concentration (50 nM) known to stabilize MTs (Xiao et al., 2006) produced no obvious effect in the stress (Fig. 4 f) and strain energy (Fig. 4 g). Interestingly, in the presence of myosin-II inhibitor, the elevated strain energy caused by nocodazole was totally abolished and reduced to a level below that found before the treatment (Fig. 4 g), which indicates that the enhanced traction force caused by MT depolymerization depends on myosin-II activity, which is consistent with that found in nonneuronal cells (Rape et al., 2011). Thus, MT dynamics could also regulate traction force generation in neurons.

The role of substrate adhesion in neuronal migration and force generation

The focal adhesion mechanically links the ECM with the termini of F-actin bundles (Patla et al., 2010) and is necessary for the generation of traction force against the substrate. Like most motile cells, neurons migrate in stereotyped steps: leading edge extension, nuclear translocation, and TP retraction. For fruitful forward migration, there must be net forward movement of the soma and TP. This suggests that the degree of substrate adhesion of the LP is larger than that of the soma and TP. We tested this notion by pushing the middle of the LP with a micropipette and determined which part of the neuron becomes detached from the substrate (Fig. 5 a). This assay showed that in 17/25 of migrating GCs, the soma was the first to be detached from the substrate (Fig. 5 b), demonstrating that substrate adhesion at the distal half of LP is stronger during migration. In contrast, in stationary neurons, the adhesion of soma and TP is stronger than the distal LP (14/16; Fig. 5 b). Thus, net forward migration appears to depend on an increased adhesion at the LP to a level above that of the soma and TP.

FAK is a crucial signaling component of the cell adhesion complex. It can be activated by numerous stimuli and serves as a biosensor or integrator to control cell motility (Mitra et al., 2005). We found that inhibition of FAK kinase activity by the specific drug Y11 (50–200 μM; Golubovskaya et al., 2012) enhanced the strain energy generated by cerebellar GCs (Fig. 5 d). This is consistent with that found in fibroblasts (Rape et al., 2011). The enhancing effect may be attributed to the stabilization of focal contacts via inhibition of their turnover by FAK inhibition (Webb et al., 2004). Alternatively, FAK inhibition can also enhance MT depolymerization (Kaverina et al., 1998; Palazzo et al., 2004) and elevate the RhoA activity (Mitra et al., 2005), which can facilitate myosin-II activation (Kimura et al., 1996), drive actin assembly (Burridge and Wittchen, 2013), and promote growth cone adhesion (Woo and Gomez, 2006). Further studies using total internal reflection fluorescence (TIRF) imaging of immunostained adhesion proteins FAK, paxillin, and vinculin (Fig. 5 c) showed that, unlike the typical clustered focal adhesions found in glial cells (Fig. S4 d), these proteins appeared to be more diffusely distributed in GCs (Fig. 5 c). Similarly, immunostaining of α-actinin and phalloidin-stained F-action showed that stress fibers are less prominent in GCs than in glial cells and fibroblasts (Fig. S4, e and f). Previous studies have also shown that most high-migratory cells lack stress fibers, and the presence of stress fibers is correlated more with strong adhesion than rapid migration (Burridge and Wittchen, 2013).

BDNF and Slit2 regulate traction force and guide neuron migration

In developing cerebellum, BDNF secreted by the Purkinje cell layer is necessary for GCs to migrate across the molecular layer (Borghesani et al., 2002; Poblete-Naredo et al., 2011). We found that local perfusion of BDNF (0.5 μg/ml) to the tips of processes steers the soma translocation in these cultured GCs toward the site of BDNF application. As shown in the example cell in Fig. 6 a, BDNF application enhanced forward soma translocation when applied at a dLP, but reversed soma translocation when applied at a TP. Measurements of the changes of soma translocation velocity, either positive (forward) or negative (rearward), showed that BDNF application at the longer process of the cell (12/21 leading and 9/21 trailing) resulted in an overall positive increase in the velocity (Fig. 6 a, right, n = 21). A previous study has shown that application of Slit2 at the dLP of these cultured GCs resulted in the reversal of soma translocation (Guan et al., 2007). Given these guidance effects of BDNF and Slit2 on soma translocation, we further inquired whether the action of the two guidance factors is mediated by regulating CC activities in these GCs.

When BDNF was bath applied to the culture medium, we found that force generation by the entire neuron was markedly enhanced (Fig. 6 b) and the total strain energy of the cell was increased (by up to fourfold; Fig. 6 c). As shown by the example cell in Fig. 6 d, we found that local perfusion of BDNF to dLP resulted in a local increase in the stress. A forward shift in the location of dominant CC (green line) was accompanied by a gradual forward soma translocation (cyan line). The summary of results from all nine GCs examined showed significant forward CC shifts after BDNF application at dLP (Fig. 6 d, right).

BDNF activates Cdc42 (Sieg et al., 2000; Yuan et al., 2003; Myers et al., 2012), a member of the Rho family of small GTPases, which further activates myosin-II via MRCK (Gomes et al., 2005; Wilkinson et al., 2005), promotes F-actin polymerization by the N-WASP–Arp2/3 pathway (Carlier et al., 1999), and enhances point–contacts dynamics (Myers and Gomez, 2011; Matsuda et al., 2012; Myers et al., 2012), all of which can enhance the contractile activity of the cell. We found that when BDNF was applied together with the Cdc42 inhibitor ML141 (10–20 μM), the enhancement of force generation induced by BDNF was markedly reduced (Fig. 6 c). Such a result supports the idea that the BDNF effect on force generation is mediated by Cdc42.

Previous studies have shown that frontal application of Slit2 to migrating GCs reversed the front-to-end distribution of RhoA and led to the reversal of neuronal migration in vitro (Guan et al., 2007). RhoA is a small GTPase and can facilitate myosin-II activation by inhibiting myosin phosphatase (Kimura et al., 1996); drives actin assembly by stimulating myosin activity, inhibition of cofilin-mediated F-actin severing, and ac-
Figure 4. The roles of myosin-II, actin filaments, and MTs in the contractile activity of GCs. (a, left) The stress field generated by a GC before and after treatment with the myosin-II inhibitor blebbistatin (Bleb, 50 µM, black line). (a, right) Total strain energy generated by GCs before and after Bleb treatment, with the red thick trace representing the average of 17 cells (individual traces in faint colors). Before averaging, data from each cell were normalized by...
tivation of mDia driving actin polymerization (Burrage and Wittchen, 2013); and promotes the formation and stabilization of growth cone point contacts (Woo and Gomez, 2006). In our study, we found that bath-applied Slit2 (0.5 µg/ml) caused the collapse and retraction of GC growth cones. The total strain energy of the cell was largely unchanged after retraction (Fig. 6c), indicating that Slit2 had not altered the overall level of contractility in GCs. When Slit2 (5 µg/ml) was applied locally to the LP growth cone, we found an initial increase of the stress in pLP followed by an increase in the TP (Fig. 6e). Concurrently, the position of dominant CC shifted from dLP to TP (Fig. 6e), accompanied by the reversal of soma translocation. Summary of results from all 10 cells analyzed showed significant rearward shifts of the dominant CC after exposure to Slit2 at dLP (Fig. 6e). Finally, inhibition of RhoA by Y27632 decreased the total strain energy by ~50% (Fig. 6c). These findings of RhoA’s contribution in force generation and LP-to-TP shift of the dominant CCs are consistent with the previous report (Guan et al., 2007) that frontal application of Slit2 reverses the distribution of RhoA toward TP. Thus, Slit2 reverses neuron migration by reversing the location of the dominant CC through a process mediated by RhoA.

Discussion

This study of spatiotemporal dynamics of traction forces in migrating GCs using TFM led to three major findings (Fig. 7). First, three distinct and independently fluctuating CCs exist in each GC, and the net action of CC activity drives soma translocation. Second, both F-actin and MTs contribute to the traction force generation, through myosin-II–dependent processes. Third, the attractive and repulsive guidance cues, BDNF and Slit2, respectively, exert their influence on GC migration by altering spatiotemporal dynamics of CC activities at LPs and TPs. To our knowledge, this was the first demonstration of coexistence of multiple CCs and their dynamic regulation during neuronal migration. These results resolved the previous controversy over the location of force generation that drives neuronal migration, and showed that CC-generated pulling rather than pushing is responsible for soma translocation.

The role of cytoskeletons in traction force generation

Although MTs represent the main cytoskeleton conferring the stiffness of the neuron (Spedden et al., 2012) and bearing the compressive load (Brangwynne et al., 2006), they also play an important regulatory role in cellular force generation. Consistent with the previous finding in migrating fibroblast (Rape et al., 2011), we found that in migrating neurons nocodazole-induced depolymerization of MTs enhanced the traction force, whereas inhibition of depolymerization by taxol had no detectable effect. It’s also consistent with our laboratory’s previous report that local perfusion of nocodazole in the LP can accelerate the neuron migration (He et al., 2010). Furthermore, the inhibition of myosin-II activity by blebbistatin abolished the traction force increase caused by nocodazole. The fact that MT depolymerization enhances traction force may be because its shortening can activate RhoA by releasing GEF-H1 (Waterman-Storer and Salmon, 1999; Kaverina and Straube, 2011; Akhshi et al., 2014) and help to stabilize cell adhesions (Akhshi et al., 2014). Via the MT binding proteins, there is a balance between the amounts of F-actin and MTs. Increasing F-actin is accompanied by decreasing MTs (Even-Ram et al., 2007), and MTs decreasing can increase the amounts of F-actin (Even-Ram et al., 2007). MTs can also promote changes at specific regions of the actomyosin cortex, and in turn the actomyosin cortex influences MT stability (Akhshi et al., 2014). Thus, although myosin-II–dependent actomyosin contractility provides the main source of traction force during neuronal migration, MT dynamics could also have a modulatory effect.

Other contractile activities affecting traction force generation

Besides the contraction of actomyosin, other factors such as the molecular clutch (Oakes and Gardel, 2014) and cortical actin (Salbreux et al., 2012) may also play important roles in generating the traction force. Stress generated within the cytoskeleton drives the retrograde flow of F-actin. Although engagement of focal adhesions results in a reduction of actin retrograde flow rates around adhesion sites and an increase in traction stresses (Gardel et al., 2008), the “clutch engagement” at a focal adhesion can increase the density and affinity of the binding between F-actin and ECM (Oakes and Gardel, 2014). As a cross-linked actin network beneath cell membrane, the cortical actin can generate tension by myosin activity and F-actin–cross-linking (Salbreux et al., 2012). When the cortical tension is not uniform throughout the cell, contractility gradients can result in local contraction, as exemplified by the cell body retraction during lamellipodial cell migration and leading edge protrusion in amoeboid motion (Salbreux et al., 2012).

The role of substrate adhesion in migration and force generation

Cell adhesion is necessary for the generation of the traction force against the substrate. Although cell adhesion does not actively generate forces, they mediate force transmission between the cytoskeleton and ECM. The asymmetry of adhesion strength found in our study (Fig. 5) may be caused by the asymmetric distribution of surface adhesion molecules or differential binding affinity between these molecules with their ECM ligands. Among various adhesion molecules, the integrin family is the best known and plays a prominent role in cell migration (Parsons et al., 2010). The binding affinity of integrins can be regulated by an inside-out signaling pathway that includes many intracellular activators (Shattil et al., 2010;
Kim et al., 2011) and inhibitors (Kim et al., 2011). Conversely, outside-in signaling initiated by extracellular integrin ligands can cause their conformational switch and clustering of integrins, which in turn enhances the adhesion strength (Shattil et al., 2010; Kim et al., 2011).

Cell adhesion can also link many structural and signaling molecules (Plotnikov et al., 2012). For example, as an integrator and biosensor localized in adhesion sites (Mitra et al., 2005), FAK mediates the modulation of Cdc42 by extracellular guidance cues (Matsuda et al., 2012; Myers et al., 2012) and regulates cytoskeletal fluidity, GTPase activity, membrane composition, and the dynamics of focal adhesion, actin, and MTs (Mitra et al., 2005). Adhesion formation and disassembly drives the migration cycle by activating Rho GTPases, which in turn regulate actin polymerization and myosin-II activity, and therefore adhesion dynamics (Parsons et al., 2010).

Physical and chemical components can influence each other reciprocally in an interaction network. Directed actin polymerization brings integrins together to form “nascent” adhesions (Oakes and Gardel, 2014) and increases the membrane tension (Houk et al., 2012). The membrane tension is primarily determined by the mechanical force balance between the cell membrane and traction forces (Lieber et al., 2013) and its increase can activate the myosin contraction (Keren, 2011), but a local increase can inhibit the actin assembly and Rac activation in the distal end (Houk et al., 2012). Myosin-II activity further promotes the polymerization of F-actin (Burridge and Wittchen, 2013; Courtemanche et al., 2013), and the mechanical force exerted on integrins drives focal adhesion growth, protein recruitment, adhesion stabilization, and signal activation (Guilluy et al., 2011; Kuo et al., 2011). In response to mechanical tension, the composition and signaling of substrate adhesion changes,

Figure 5. The role of substrate adhesion in neuronal migration and force generation. (a) Pulling assay. Pulling the LP of GCs at the middle point. Broken lines show the location of soma and LP growth cone before and after pulling. Bars, 10 µm. (b) Summary of results of the pulling experiments. Under migratory or stationary conditions, the number of GCs that showed the first detachment site at the soma or at the growth cone of LP after pulling is shown. (c) Immunostaining of markers of adhesion complexes (FAK, paxillin, vinculin). (d) FAK inhibition with Y11 (~50–200 µM) enhanced the strain energy of GCs (n = 7). Broken lines show the normalized strain energy (1 unit) before drug application.
Figure 6. **Guidance cues for neuronal migration regulate activities of CCs.** (a, left) Local perfusion with the attractive cue BDNF (50 ng/ml) guided neuron migration. Broken lines, perfusion pipette for BDNF delivery sequentially at the end of the LP and TP. Note the direction of soma translocation after BDNF perfusion at each location. (a, right) Summary of the BDNF effect on the velocity of soma translocation. BDNF was applied at the distal region of the long process, and the soma velocity (+ and −, toward and away from the long process) was monitored for 9–40 min before and after BDNF application. Note the increase in velocity toward the tip of the LP (P = 0.014, paired t test). (b) Line scan (along the red trace shown on the left) of the stress produced by a neuron before and after bath application of BDNF (50 ng/ml), showing overall increase in the stress. Red dots, location of dominant CC. (c) Changes in the total strain energy of the neuron after bath treatment with various agents: BDNF (50 ng/ml) + ML141 (15 µM), Slit2 (0.5 µg/ml), Y27632 (25 µM), ML141 (5 µM). (d, e) Similar experiments with Slit2.
leading either to increased anchorage or controlled disassembly of cell matrix adhesion complexes, both of which are critically involved in cell migration (Wehrle-Haller, 2012).

**Fluctuation of traction forces**

Fluctuation of traction forces is often observed at subcellular, cellular, and tissue levels, in many cell types and at different stages of developmental processes (Galbraith and Sheetz, 1997; Chan and Odde, 2008; Martin et al., 2009; Trepat et al., 2009; Plotnikov et al., 2012). Such fluctuation helps the cells to sense substrate stiffness (Chan and Odde, 2008; Plotnikov et al., 2012), to align along a special direction (De et al., 2007), and to reshape tissue morphology (Martin et al., 2009). We found that activities of multiple CCs of migratory GCs fluctuate independently during both migratory and stationary phases. The fluctuating CC activities generate apparently random net driving force on the soma, leading to fluctuating soma movement along neurite processes, with the net translocation along the direction of neuronal migration. In analogy to chemotactic migration of Escherichia coli (Macnab and Koshland, 1972) and Caenorhabditis elegans (Frady et al., 2012), the guidance cues such as BDNF and Slit2 bias the net soma translocation by biasing the apparently random soma translocation through modulation of the relative CC activities at LP and TP.

**Regulation of traction forces by BDNF and Slit**

Our studies showed that neuronal guidance factors such as BDNF and Slit2 induce GC migration by altering CC activities. Such a mechanism may be generalized to other types of migratory cells. For example, hepatocyte growth factor promotes cell motility and induces strong contractile activity of Madin-Darby canine kidney cells growing on substrates with micropillars (du Roure et al., 2005), and VEGF could enhance the traction force of human umbilical vein endothelial cells by promoting F-actin formation (Yang et al., 2011). The effect of BDNF on traction force may be mediated by cytoplasmic cAMP and the protein kinase A (PKA) activity (Cheng et al., 2011), which then modulate the local activity of Cdc42 (Yuan et al., 2003). However, global modulation of CC activities requires long-range signaling to distant regions of the migrating neuron. The existence of long-range signaling within neuronal cytoplasm is suggested by previous findings that local elevation of cAMP due to BDNF exposure in one neurite may result in cAMP reduction in other faraway regions of the migrating neuron. The existence of such global second messenger signaling is likely to play an important role in the spatiotemporal regulation of CC activities in migrating neurons.

Besides pulling the soma forward, physical forces generated by contact with the environment in the absence of diffusible molecular cues could also bias the direction of cell migration (Theveneau et al., 2010; Weber et al., 2012). The tension on the cell can regulate many signal pathways such as in recruiting myosin-II and regulating its activity (Fernandez-Gonzalez et al., 2009) and regulating gene expression and eventual cell fate (Hiramatsu et al., 2013). Thus, migrating neurons not only passively react to external forces, but also actively influence the mechanical environment for itself and other nearby cells by its active contractile activity and secreted factors. The use of TFM in mapping of traction force generated by migrating cells offers useful means in addressing the interplay between mechanical forces and cellular signaling.

**Summary**

We have examined the traction force due to neuronal contracility in the presence of cell–substrate adhesion by analyzing cell-induced distortion of the substrate, as revealed by embedded microbeads. While movements of some microbeads could be caused by the cell movement (especially those near the trailing ends of the cell), the cell migration as a whole must result from the action of the net traction force on the entire cell due to asymmetric substrate adhesion at two ends of the cell. This notion is supported by the result of our analysis of the temporal relationship between soma translocation and contractile activity, and by the finding that pharmacological treatments that up- and down-regulate the net traction force correspond in soma translocation. This study provides the first comprehensive analysis of the traction force generation by migratory neurons, and suggests an integrated model for the spatiotemporal control of contractile activities in neuronal cytoplasm that reconciles previous findings on force generation in migrating neurons.

**Materials and methods**

**Preparation of PAA gel substrate**

The protocol of PAA gel preparation followed a previous report (Wang and Pelham, 1998) with some modifications. In brief, the surface of a glass-bottom culture dish was processed by NaOH (0.1 M, 5 min), (3-Aminopropyl) trimethoxysilane (100%, 3 min), and glutaraldehyde (0.5%, 20 min) to facilitate the covalent attachment of PAA. The PAA gel was prepared by mixing ddH2O (258 µl), acrylamide (40%, 17 µl), bis-acrylamide (2%, 12 µl), ammonium persulfate (10%, 1.5 µl), and N,N,N’,N’-Tetramethylethylenediamine (100%, 0.45 µl), and 15 µl of the mixture was then mixed with 1.4 µl fluorescent microspheres (F8809, F8806; Invitrogen), dropped onto a previously prepared coverslip, and flattened with another coverslip. The dish was then put onto the surface of 24°C water under lamp light for polymerization. 20 min later, the coverslip over the gel was removed and the gel was exposed to UV for activating PAA after adding sulfo-SANPAH solution (1 mM; 22589; Thermo Fisher Scientific). The PAA gel was prepared by mixing ddH2O (258 µl), acrylamide (40%, 17 µl), bis-acrylamide (2%, 12 µl), ammonium persulfate (10%, 1.5 µl), and N,N,N’,N’-Tetramethylethylenediamine (100%, 0.45 µl), and 15 µl of the mixture was then mixed with 1.4 µl fluorescent microspheres (F8809, F8806; Invitrogen), dropped onto a previously prepared coverslip, and flattened with another coverslip. The dish was then put onto the surface of 24°C water under lamp light for polymerization. 20 min later, the coverslip over the gel was removed and the gel was exposed to UV for activating PAA after adding sulfo-SANPAH solution (1 mM; 22589; Thermo Fisher Scientific). The solution was removed 10 min later, and the gel was washed with Hepes (50 mM) on a shaker for 30 min, three times. The gel-coated dish was used immediately after preparation.

**Animals**

Newborn Sprague Dawley rats (P0–P2) used in the present study were provided by Shanghai SLAC Laboratory Animal, Inc. Experimental protocols were approved by the Bioethics Committee of the Institute.
Fluorescence images were recorded for 40–240 min at either objective lens (60×, NA 1.4, oil). In local perfusion experiments, the three CCs, resulting in preferential net traction toward a specific direction. Gradients of guidance cues from the environment bias the relative activities at the three CCs are weak and fluctuate independently, leading to the stationary state or apparently random fluctuation of soma location. Migratory neurons possess three potential CCs where activated myosin-II generates the traction force. In the absence of guidance cues, contraction activities at the three CCs are weak and fluctuate independently, leading to the stationary state or apparently random fluctuation of soma location. Gradients of guidance cues from the environment bias the relative activities at the three CCs, resulting in preferential net traction toward a specific direction.

of Neuroscience at the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Cultures of cerebellar GCs
Cultures of dissociated cerebellar GCs were prepared as described previously (Guan et al., 2007) with some modifications. In brief, the cerebellum cortex was dissected on ice from P0–P2 Sprague Dawley rats, digested with 0.125% trypsin (0.25%; Gibco) for 9–11 min at 37°C, and dissociated into single cells by gentle trituration. The PPA gel coverglass was prepared by coating with 100 µg/ml poly-d-lysine and 25 µg/ml laminin before cells were plated at a low density (200–300 cells/mm²) in Neurobasal medium (Invitrogen) supplemented with 10% FBS and 2% B27 (Invitrogen).

Time-lapse imaging and characterization of culture substrate
After 8–10 h of culturing, GCs were placed on a microscope stage for observation. The stage was equipped with a heated incubator to maintain the samples at 5% CO₂ and at 37°C, together with the objective lens (60x, NA 1.4, oil). In local perfusion experiments, the culture medium was changed to prewarmed L-15 (Gibco) without CO₂. Fluorescence images were recorded for 40–240 min at either 0.3 or ~10–21 s frame interval time using a confocal laser scanning microscope (FV1000; Olympus) with photomultiplier detectors, with 0.103 µm/pixel resolution in bidirectional mode. The acquisition software used was the FV10-ASW program and the pinhole was set to 200 µm to image the beads in the superficial layer of the PAA gel. The fluorochromes of the fluorescent microspheres includes orange (540/560) and crimson (625/645).

After image acquisition, all cells were removed by replacing the culture medium with prewarmed ddH₂O to lyse the cells and to obtain the original locations of the beads before cell attachment. Finally, we performed the stiffness measurement by placing glass balls (200 µm; Polysciences) onto the PAA gel and determining the depth of the pit caused by the glass balls by z-series imaging. The stiffness of the gel was calculated by the Hertzian equation (Chan and Odde, 2008):

\[
E = \frac{3(1 - \nu^2)}{4 \pi R^2} \delta \rho \cdot f,
\]

where \(E\) is Young’s modulus (Pa) of the PAA gel, \(\nu\) is the Poisson ration of the gel (\(\nu = 0.3\)), \(R\) is the radius of the glass ball, \(\delta\) is depth of the pit, and \(f\) is the buoyancy-corrected weight of the glass ball.

The stiffness used in the present study is within the range of 200–400 Pa.

Local perfusion
Local perfusion of pharmacological reagents was performed according to a previous study (Zheng et al., 1994), with some modifications. The drugs were injected into the culture medium by a microinjector (Ultra-MicroPump III; World Precision Instruments, Inc.) with a flow rate of 3–5 nl/min, which produced lower pressure and caused less disturbance to the PAA gel than the Picospritzer (Parker Inc.) used previously. The inner diameter of the micro pipette tip is ~1–2 µm.

Estimation of deformation, traction force, and strain energy
The microscopic images (12 bit) were transformed into TIF pictures (8 bit) using the Loci plugin (Linkert et al., 2010) for MATLAB, and then aligned and tracked using registration algorithms based on nonlinear optimization (Guizar-Sicairos et al., 2008). All beads were recognized by edge detection and their center locations were determined by the peak of 2D Gaussian fit (Chan and Odde, 2008). The displacement of the bead center was determined as the peak location for the cross correlation between the images before and after cell lysis (over a bead-centered square window of ~80 × 80 pixels; Butler et al., 2002). The bead displacement map was transformed into traction force map and the strain energy was calculated by Fourier transform traction cytometry (FTTC; Butler et al., 2002). Simply, the Boussinesq solution,

\[
u_i = \sum_{i=1}^{n} K (r_j) F_j,
\]

where \(K\) is green function, \(\nu\) is the strain of the gel, and \(F\) is the traction force in the gel) is Fourier transformed (\(\mathcal{F}\)) into a simple multiplication,

\[
\sigma(k) = \mathcal{K}(k) \mathcal{F}(k),
\]

where \(\sigma, \mathcal{K},\) and \(\mathcal{F}\) are the Fourier transform of each parameter; and \(k\) is the wave vectors. Then the stress can be calculated by

\[
F = \mathcal{F} T_{\mathcal{F}}^{-1} (\mathcal{K}^{-1} \mathbf{u}),
\]

where \(\mathcal{F} T_{\mathcal{F}}^{-1}\) is the two-dimensional inverse Fourier transform. The kernel \(\mathcal{K}\) is

\[
\mathcal{K}(k) = \frac{1 + \nu v_k}{E} \begin{bmatrix}
1 - v_k^2 & v_k k_j \\
v_k k_j & 1 - v_k^2
\end{bmatrix},
\]

where \(E\) is Young’s modulus of the PAA gel, \(k = \sqrt{k_x^2 + k_y^2}\), and \(v\) indicates the Poisson ratio of the gel (\(v = 0.3\)).

The total strain energy of a neuron was calculated by summing the energy density around the neuron.

2D interpolation
2D scattered displacement vectors were interpolated by the MATLAB scatteredInterpolant class. The vector components \(x\) and \(y\) were interpolated, respectively, and then joined together.

Determination of the location of dominant CC
We first determined the direction of traction forces at all pixels along the neuron axis and then identified the CC location as the region where...
near-neighbor pixels exhibited the maximal change in the direction of the traction force (Δangle).

Immunostaining
Neurons were fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature and then incubated with 0.25% Triton X-100 (in PBS) and 1% BSA (in PBS) for 1 h for permeabilization and blocking. Primary antibodies (FAK [pTyr397] antibody, 700255, Invitrogen; Paxillin [pY118] antibody, 44722G, Invitrogen; anti-vinculin antibody, V9264, Sigma-Aldrich; Phospho-Myosin Light Chain 2 [Ser19] Mouse mAb, 3657, Cell Signaling Technology; anti-green fluorescent protein, A11120, Molecular Probes) were added with corresponding blocking solution and incubated overnight at 4°C. Corresponding secondary antibodies and DAPI were added and incubated for 2 h at room temperature.

TIRF microscopy
After transfected with recombinant vector (GFP–α-actinin) and 8–10 h of culturing, GCs were immunostained with primary antibodies of FAK, Paxillin, vinculin, and GFP and then with secondary antibodies. The prepared samples were imaged with an inverted microscope (Ti-E; Nikon) at room temperature. The setup was equipped with an Apochromat TIRF oil-immersion objective lens (60×, NA 1.49), together with a camera (DU-897 X-4696; Andor) with a resolution of 0.27 µm/pix.

Online supplemental material
Fig. S1 shows that CCs can also be visualized by a 3D color map of stress magnitude and pinwheel color map of stress directions. Each process of the GC with multiple primary neurites can generate its own CC. Fig. S2 shows more cases of stress dynamics analysis by the line scan of the stress maps along GC axis and distribution of dominant CCs for 18 cells. Fig. S3 shows more cases of asymmetric stress maps during soma translocation. Fig. S4 shows that there are typical focal adhesions and stress fibers in glial cells but not in GCs, and that the traction force generated by glia cells is much larger than that of GCs. Table S1 shows the numbers of GCs with one, two, or three CCs in all the GCs we examined. Video 1 shows the spatiotemporal dynamics of the stress generated by a migrating GC during a 30-min period. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201410068/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201410068.dv.

Acknowledgements
We thank Dr. David J. Odde of University of Minnesota for kindly sharing the program codes for microbead recognition.

This work was supported by grants from Ministry of Science and Technology (973 Program, 2011CBA00400) and the Chinese Academy of Sciences (Strategic Priority Research Program, XDB02020001).

The authors declare no competing financial interests.

Submitted: 20 October 2014
Accepted: 27 April 2015

References


Borgbesian, P.R., J.M. Feyrin, R. Klein, J. Rubin, A.R. Carter, P.M. Schwartz, A. Lister, G. Corfas, and R.A. Segal. 2002. BDNF stimulates migration of cerebellar granule cells. Development. 129:1345–1442.


nals to promote cell migration. Nat. Cell Biol. 2:249–256. http://dx.doi.org/10.1038/35010517
Figure S1. **CC in neurons.** (a–c) A typical CC produced by the migrating GC, visualized by different graphing methods. (a) A typical CC is identified by stress vectors converging toward the dark area. The white circle labels the location of the CC. (b) 3D display of the magnitude of the traction field. The broken line and arrow indicate CC location. (c) Pinwheel direction map of stress around the CC. The stress directions were divided into six equal sectors, each labeled with different colors. (d–f) The CCs of a GC with three primary neurites. (d) Outline (gray) and axes (colored lines) of a GC with three primary neurites. The axes of the three neurites were labeled with three colored lines (green, red, and purple) and numbers. (e) Circles label the location of dominant CCs at three different times determined by the stress map. Note that coexistence of two CCs could be observed at some time points (i.e., t = 4 min). (f) Dynamic changes of the stress along the axes of the three neurites, as shown by line scans of the stress over a period of 40 min. The location of the CC is marked by green dots.
Figure S2. Distribution of dominant CCs relative to the soma center. The location of the dominant CC (marked by green dots) was determined as the site of maximal absolute value of the angular difference between adjacent stress vectors. The cyan line depicts the location of the soma center.
Figure S3. Migratory neurons generating asymmetric stress. (a, left) Soma translocation with time. The green asterisks label the time points that were chosen to show on the right. (a, right) Stress produced by the migratory neuron at five different time points were shown by hot maps and 3D graphs. (b–d) The asymmetrical stress maps of another three GCs. (b–d, left) Soma translocation with time. Two asterisks indicate the time interval shown on the right. (b–d, right) Stress maps at a continuous series of time points corresponding to the time interval marked by the arrow on the left. The cyan lines represent the cell outlines.
Figure S4. **The stress generation and focal adhesions of glial cells.** (a) The stress map of a glial cell (green line) and a neuron (red line) at nearby regions on the same substrate. The CC of the glial cell is labeled a white circle. (b) Strain energy around the glial cell and neuron were analyzed during the period of ~40 min. The total strain energy of the glial cell was ~10-fold larger than that of the neuron. A total number of 34 GCs and 19 glial cells were summarized in c. (c) Distribution and normal fitting of the total strain energies of GCs (purple, n = 34) and glial cells (green, n = 19). The mean strain energy of GC and glia were 2^{-6.5} and 2^{-2.2} pJ, respectively. (d) Focal adhesion sites in glial cells were indicated by clustered integrin and paxillin immunostaining. (e) F-actin labeled with phalloidin in the GC (left) and glia cell (right). (f) α-Actinin was expressed in live cells with transfection by YFP-α-actinin vectors (left, GC; right, 3T3 fibroblast). Bars, 10 µm.

<table>
<thead>
<tr>
<th>GC type</th>
<th>Three CCs</th>
<th>Two CCs</th>
<th>One CC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP, pLP, dLP</td>
<td>TP, pLP</td>
<td>pLP, dLP</td>
<td>TP, dLP</td>
</tr>
<tr>
<td>Migrating</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Stationary</td>
<td>3</td>
<td>6</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>10</td>
<td>20</td>
<td>2</td>
</tr>
</tbody>
</table>

Numbers of GCs with different CC locations, as detected by the absolute stress map. The GCs were divided into two groups by their migratory state. Note that the use of stress differential map yielded a higher fraction (72/72) of GCs with two or three CCs than that found by using the stress map (41/72).

<table>
<thead>
<tr>
<th>GC type</th>
<th>Three CCs</th>
<th>Two CCs</th>
<th>One CC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP, pLP, dLP</td>
<td>TP, pLP</td>
<td>pLP, dLP</td>
<td>TP, dLP</td>
</tr>
<tr>
<td>Migrating</td>
<td>17</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Stationary</td>
<td>34</td>
<td>2</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>3</td>
<td>18</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers of GCs with different CC locations, as detected by the stress differential map. The GCs were divided into two groups by their migratory state. Note that the use of stress differential map yielded a higher fraction (72/72) of GCs with two or three CCs than that found by using the stress map (41/72).
Video 1. **Spatiotemporal dynamics of the stress generated by a migrating GC in culture.** The deformation of the substrate was calculated by microbead displacements caused by GC contraction and then converted into the stress map as shown in Fig. 1. The magnitude of the local stress was color-coded and the direction was marked with discrete green arrows. Cyan line, outline of the GC. Purple arrow, the CC, where stress vectors converge. Frames were taken every 21 s for 30 min.