Persistent and polarized global actin flow is essential for
directionality during cell migration

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Abstract

Cell migration is hypothesized to involve a cycle of behaviors beginning with leading edge extension. However, recent evidence suggests that the leading edge may be dispensable for migration, raising the question of what actually controls cell directionality. Here we exploit the embryonic migration of Drosophila macrophages to bridge the different temporal scales of the behaviors controlling motility. This reveals that edge fluctuations during random motility are impersistent and weakly correlated with motion. In contrast, flow of the actin network behind the leading edge is highly persistent. Quantification of actin flow structure during migration reveals a stable organization and asymmetry in the cell-wide flowfield that strongly correlates with cell directionality. This organization is regulated by a gradient of actin network compression and disassembly, which is controlled by myosin contraction and cofilin-mediated disassembly. It is this stable actin-flow polarity, which integrates rapid fluctuations of the leading edge, that controls inherent cellular persistence.
Introduction

Cell migration is hypothesized to involve a step-wise cycle of behaviors: 1) actin-based protrusion of the leading edge, 2) formation of new adhesions at the cell front, 3) acto-myosin contraction of the cytoskeletal network, and 4) de-adhesion at the rear. At the same time the cell must maintain a polarity, which is hypothesized to be controlled by a combination of reaction-diffusion modules and membrane tension to maintain an asymmetry in these behaviors. The integration of the stages of the migratory cycle leads to coherent cell motion whereby cells have an inherent persistence in cell speed and direction.

Since the first postulation of the migratory cycle, we now understand many of its molecular components. Actin-protrusion of the leading edge is driven by Arp2/3-mediated actin polymerization and branching. Pushing of the actin filaments against the leading edge, along with myosin-II driven contraction in many cell-types, subsequently induces a retrograde motion of the crosslinked actin-network. When integrins are engaged, these points of friction with the extracellular matrix lead to a slowing of the retrograde flow allowing for transmission of the forces of the flowing actin network into extracellular traction stresses. Finally, asymmetric release of integrin adhesions at the rear allows for net cell translation.

This model of cell migration is predicated on the idea that it is a linear step-wise process that starts at the leading edge. Therefore, with regards to the control of cell directionality and persistence, a major focus has been on the actin polymerization machinery at the cell front. However, evidence has arisen to bring this leading-edge centric view of motility into question. Loss of the leading edge lamellipodia does not grossly inhibit chemotaxis and the presence of actin protrusions, rather than being essential for cell translocation, may actually destabilize migration and enhance...
exploratory behavior\textsuperscript{20, 21}. Additionally, recent data revealed that cell shape is a predictor of migratory dynamics\textsuperscript{22, 23} suggesting that global cellular processes, rather than simply local edge fluctuations, are also important in controlling cell motility. However, the idea that cell movement may not be directly controlled from extensions at the cell edge is still controversial\textsuperscript{24} as the stepwise view of migration has remained nearly unchanged for decades\textsuperscript{25}.

What is currently lacking is a holistic understanding of how the hypothesized steps of cell motility are integrated in space and time to give a cell its inherent persistence and directionality. The complexity in bridging the stages of cell migration is partly due to the different time and length scales of its behaviors\textsuperscript{26}. For instance, it is difficult to understand how rapid fluctuation of the leading edge, which oscillates on the order of seconds\textsuperscript{27-30}, controls overall cell persistence that decays on the order of minutes to hours\textsuperscript{6}. To resolve such questions requires cell migration to be imaged at sufficiently high spatiotemporal resolution for long enough time periods to correlate leading edge fluctuation, actin dynamics, and overall cell motion. However, correlating behaviors on such different timescales is both experimentally and quantitatively challenging.

In previous work, we exploited the embryonic migration of \textit{Drosophila} macrophages (hemocytes), which are highly amenable to live imaging during their developmental dispersal\textsuperscript{31}, to develop tools to image cell-wide actin flows during embryonic migration \textit{in vivo}\textsuperscript{32}. Here we use this unique system to quantify, and correlate in time, the various behaviors of cell motility during both random and directed migration. This reveals that edge fluctuation is a weak predictor of directionality during random cell motility, with a persistence during random migration that is less than the actual persistence of overall cell motion. In contrast, the retrograde flow of the actin
network behind the leading edge, which has recently been revealed to couple cell speed and persistence\(^3\), is highly organized and stable in time. Through the development of approaches to quantify global actin flow organization, along with a minimal fluid-mechanical model of acto-myosin induced emergence of stable flows, we reveal that cell migration involves network-wide coordination and an intrinsic asymmetry in the flowfield that highly correlates with cell directionality. This asymmetry is controlled by a stable gradient of actin network compression and disassembly towards the rear of the lamellae, which is driven by myosin contraction and cofilin-mediated destruction. It is this destruction/contraction gradient that leads to a stable cell-wide polarity within the flowing actin network, which likely integrates the rapid fluctuations of the leading edge to control overall cell persistence.

Results

**Leading edge fluctuations are a weak predictor of cell directionality**

*Drosophila* hemocytes spread throughout the embryo taking stereotypical migratory routes. One of these routes is along the ventral surface of the embryo in which hemocytes migrate in a confined space that is essentially two-dimensional, allowing for imaging of their motility at high spatiotemporal resolution\(^3\),\(^2\),\(^3\). These cells can be automatically and precisely tracked using the nucleus as a fiducial marker, which is more accurate than tracking cell centroid at high temporal resolution (Movie 1). Here we focus on tracking freely moving hemocytes that do not contact any neighboring cells as they undergo random patrolling behavior. Randomly migrating hemocytes have a noisy cell contour during their movement (Figure S1A). We first used the natural variation in the fluctuation of hemocyte contours to determine whether
there was a relationship between edge fluctuations and cell motion at high temporal
resolution (5 s/frame). Morphodynamic analysis revealed that the speed of cell edge
extensions was significantly faster than cell speed regardless of whether examining
all extensions, the maximum extension, or only the extensions in the direction of
motion (Figure 1A-C,F). In contrast, the net speed of the edge in the direction of motion
(average of extension and retraction vectors) was significantly less than cell speed
(Figure 1D,F). Interestingly, the net edge speed of all fluctuations was around zero
with low variability highlighting that on average edge extensions are balanced by edge
retractions over this short temporal scale with hemocytes tending to maintain a
constant area over time (Figure 1E,F). These data suggest that edge fluctuations are
largely disconnected from cell speed.

We next quantified the positions of edge extensions during random migration
with respect to instantaneous cell motion to determine how protrusions are correlated
with the direction of cell travel. Normalizing the position vectors of edge extensions to
hemocyte trajectory revealed that the leading edge is rapidly fluctuating and weakly
correlated with motion (Figure 1G-J, Movie 2). Additionally, correlating the direction
of cell motion to the direction of the maximum extension or retraction similarly revealed
a very weak and variable positive and negative correlation, respectively, further
highlighting a disconnect between protrusions and cell translocation (Figure S1B).
These data are surprising, considering the high persistence of hemocytes
(directionality ratio 0.7 ± 0.1 SEM, this work)\textsuperscript{35}. Interestingly, considering both direction
and speed of extensions (resultant velocity), revealed that taking into account all
extensions around the cell showed stronger correlation to motion than the maximum
extension alone (Figure S1C); this suggests that even minor extensions that
individually are not obviously correlated with cell trajectory are integrated to provide
directional information. Finally, comparing the direction autocorrelations (a measure of persistence) of cell trajectory and the maximum extension, revealed that the leading edge was less persistent than overall cell motion (Figure 1K). These data highlight that randomly patrolling hemocytes spend significant effort generating extensions that are independent of cell motion in a mode of motility that has been termed ‘inefficient’, suggesting that other behaviors must be involved to provide their high migratory persistence.

Actin flow is globally organized in migrating cells

Recent mathematical modeling has suggested that actin retrograde flow may help establish the inherent persistence of migrating cells. To examine cell-wide actin flows in hemocytes and address whether they are correlated with cell motion we performed Particle Image Velocimetry (PIV) of LifeAct-GFP expressing cells, which allows for analysis of global actin flow speed and direction during their migration.

Global PIV analysis suggested an overall organization to the actin flow with vectors showing a high degree of alignment and a gradient of high to low flow speed from the leading edge to the cell body (Figure 2A, Movie 3). As Lifeact-GFP binds actin indirectly, and is constantly binding and unbinding actin filaments, we wanted to confirm that PIV was actually highlighting internal motion of the network. We therefore labeled hemocyte actin directly with Actin-GFP, and photobleached spots in the network, which allowed us to directly examine network transit. Photobleached spots at the leading edge provided stable fiducial marks, which moved in a retrograde fashion toward the cell body and mimicked the flowfield of Lifeact-GFP expressing cells, suggesting the PIV analysis was actually tracking actin motion (Movie 4). In order to understand how the actin flow was structured we first calculated its divergence,
which is a local measure of the flux through each point in the flowfield highlighting
sources and sinks in the network. While there was little positive divergence (Figure
S2A), the network showed large regions of high negative divergence at the rear of the
flowing lamellar network immediately adjacent to the cell body of the hemocyte (Figure
2B, Movie 3). In contrast to the rapid fluctuation of the leading edge, these negatively
divergent regions were persistent on the order of 30-60 seconds (Figure S2B). Furthermore, the regions of negative divergence correlated with zones of actin fiber
deformation and bundling (Figure S2C). Indeed, the presence of the photobleached
Actin-GFP fiducial spots began to disappear in the vicinity of the negatively divergent
regions at the rear of the network suggesting that they represent regions of actin
remodeling and/or destruction (Movie 4).

In order to examine the global organization of actin flow within hemocytes we
seeded streamlines at each point along the edge of migrating cells. Streamlines are
the lines of continuous tangent to the flow velocity, and therefore highlight a flow path
through the network. The evolution of streamlines during migration showed an overall
organization in the actin flowfield with many streamlines ending within a region of the
lamella anterior to the cell body in the direction of cell travel (Figure 2C, Movie 3). We
calculated the strength of the confluence of streamlines by quantifying the number of
streamlines ending in any one location within the lamella, which revealed a
predominant streamline endpoint that was asymmetrically distributed within the cell
(Figure 2D, Movie 3). Interestingly, this primary streamline endpoint was highly
negatively divergent and tended to represent a region of low actin flow speed (Figure
2E, Movie 3), showing that it represents a large sink within the actin flowfield. Additionally, the number of sinks correlated with the number of hemocyte lamellae
suggesting that they may be involved in maintaining cell polarity (Figure S2D,E, Movie 5).

The primary streamline sink appeared to represent some transition in the actin flow as its location was strongly correlated with sharp transition from retrograde to anterograde actin motion (Figure 2F-H), suggesting that they represent a region that coordinates global actin flow. We hypothesize that these transition regions are analogous to the retrograde to anterograde transitions observed in the lamellae of various migrating cells in vitro\(^\text{19, 37-39}\) and the transition of actin network gripping to actin network slipping. When we calculated the actin flow speed in the retrograde region compared to the cell-wide average, this revealed that the actin flow was slower anterior to the primary sink (i.e. in the retrograde region, Figure S2F), suggesting that this is where friction with the extracellular environment is highest. While this is consistent with the actin-clutch hypothesis, we observed no obvious relationship between actin flow speed and cell speed at this high temporal resolution as has been hypothesized should occur\(^\text{33}\), suggesting that this linear relationship between cell speed and actin flow may not be valid on shorter timescales (Figure S2G). Taken together, our data suggest that global actin flow in hemocytes is highly organized with large network sinks that coordinate global actin flow.

In order to examine whether actin flow organization can be observed in other migrating cells we examined fish keratocytes, growth cones\(^\text{40}\), and retinal pigmented epithelial cells (RPE1) (Figure S3A, Movies 6-8). All cell-types showed similar global organization of actin flow with streamlines converging at a large network sink deep within the cell, suggesting that this is a conserved feature of migrating cells.
During random migration, the polarity of global actin flow is highly correlated with hemocyte directionality

We next examined how actin flow organization correlated with leading edge dynamics and cell directionality. In order to correlate leading edge extension and flow polarity with cell motion we calculated vectors from the nucleus to defined points within the cell: maximum extension, streamline sink, and the retrograde/anterograde transition region. We then correlated the direction of these vectors with the cell’s direction of motion over 5 s timesteps. This revealed that the primary streamline sink and the retrograde/anterograde transition region showed a statistically higher correlation with cell motion when compared with edge extension/retraction (Figure 3A,S4A, Movie 9). Furthermore, we calculated the direction autocorrelation of the vector to the primary sink to quantify the persistence of the flow polarity, which revealed that the persistence of the flowfield was higher than the persistence of the leading edge (Figure 3B). These data suggest that the persistent and coordinated flow of actin within the cell may be integrating leading edge activity to provide an inherent persistence to randomly migrating hemocytes.

We next tested if there was a temporal hierarchy of these various migratory behaviors, which would suggest a stepwise motility process. We examined the relationship of leading edge extension, actin flow polarity, and cell directionality by calculating the temporal cross correlation of the direction of each of these vectors. Interestingly, even at our rapid temporal resolution of 5 s/frame we observed a maximum correlation at 0-lag highlighting that edge extension, actin flow polarity, and cell directionality are precisely correlated in time (Figure 3C). This is despite the fact that these different phenomena are separated by relatively large distances within the cell (Figure S4B). Considering an actin flow speed of only 4 µm/min, this means that
new actin filaments, which are predominantly added at the cell edge\textsuperscript{41, 42}, are taking approximately 2 minutes to reach the primary sink and it is therefore unclear how there is seemingly instantaneous crosstalk between these behaviours. Nevertheless, these data highlight that the hypothesized ‘stages’ of migration in hemocytes are highly integrated in time without any obvious temporal hierarchy.

We next examined the correlation of leading edge activity and the streamline sink with cell directionality in RPE1 cells to determine whether a similar integration of migratory behaviors could be observed in another cell-type. Similar to hemocytes, edge speed in RPE1 cells was uncorrelated with cell speed (Figure S3B). Furthermore, both the direction of the maximum extension and the primary sink were correlated with cell motion (Figure S3C,D, Movie 10). Additionally, the direction of the velocities of the maximum extension, primary sink, and cell motion showed a maximum correlation at 0-lag, highlighting that, similar to hemocytes, these behaviors were strongly coordinated (Figure S3E). However, in contrast to hemocytes the maximum extension and primary sink were equivalently correlated with cell motion (Figure S3D) suggesting that RPE1 cells are more efficient with regards to the production of leading edge protrusions during motility.

\textit{During directed migration, leading edge persistence controls hemocyte directionality}

While the organization of global actin flow appeared more important than leading edge fluctuations in defining the inherent persistence of randomly patrolling hemocytes, we wondered whether the same would be true during their directed migration. Hemocytes can be rapidly induced to migrate to epithelial wounds possibly through hydrogen peroxide release\textsuperscript{43, 44}. When migration behaviors were examined
during a wound response (Figure S4C, Movies 11,12) there was an increase in the
correlation of the maximum extension and primary sink with cell motion (Figure
3D,S4D-F). Furthermore, there was an increase in leading edge persistence, which
unlike randomly moving cells, closely matched the persistence of other migratory
behaviors (Figure 3E,F,S4G). Interestingly, cells undergoing directed versus random
migration showed equivalent distributions of edge extensions around the cell
perimeter when normalized to the direction of motion (Figure 3G). However, the
resultant edge velocity of these extensions was more correlated to motion in
directionally migrating cells (Figure 3H), suggesting that the wound cue may be
increasing the speed of edge extensions in the direction of the wound site. These
changes in leading edge activity were also correlated with an increase in the
persistence of cells migrating to the wound site (Figure S4H). These data suggest that
the leading edge in hemocytes is more critical for driving migration during chemotactic
responses, which is similar to what has been reported for mammalian dendritic cells\textsuperscript{21}.

**Nonequilibrium fluid-mechanical model spontaneously breaks symmetry,**
**resulting in highly stable actin flows and persistent motion**

To gain mechanistic insight into the connections between actin flows, sinks,
and persistent cell motion, we built a minimal one-dimensional fluid-mechanical model
with active processes based on only four coupled partial differential equations (see SI
and related models for more details\textsuperscript{45, 46}). First, actin polymerizes at the cortex with
nonlinear auto-catalytic dynamics\textsuperscript{47}. Second, active stresses and pressure from actin
polymerization in the cortex induce actin and myosin cortical flows. Third, cytosolic
myosin diffuses and can reversibly bind filamentous cortical actin (F actin). Fourth,
cortical myosin dynamics mirror the processes of the third equation and advect with
the cortical flow. This minimal model leads to an emergent actin flow profile with a
gradient of myosin intensity and a sink at the rear (Figure 3I). Note in our model, that
retrograde actin flow leads to forward cell motion due to friction at the cell/environment
interface and force balancing\textsuperscript{45}, so that persistent flow translates into persistent cell
motion. Interestingly, this emergent flow profile demonstrated remarkable robustness.
The induction of stable flow was relatively insensitive to changes in actin
polymerization (as long as a threshold level was reached; Figure 3J) or
depolymerization (see SI) but sensitive to changes in myosin levels (Figure 3J).
Additionally, it was relatively difficult to reorient the flow by perturbation of cortical actin
density. When we simulated a single pulse of actin polymerization at a new region of
the cortex (e.g. as would be induced by an external attractant source) the angle of
actin flow – and hence cell motion – was hardly deflected (Figure 3K). However,
increasing the strength of the perturbation had an increased capacity to reorient the
flow (Figure 3L), suggesting that an external cue could steer cell motion by increasing
the strength of actin polymerization. Indeed, this mechanism of cell steering may be
occurring in hemocytes migrating towards wounds as their increase in resultant edge
velocity in the direction of motion is likely driven by increased actin polymerization
(Figure 3G,H). Nevertheless, this minimal model suggests that actin flow organization
is inherently stable and strongly dependent on myosin advection.

Negatively divergent regions of the actin flowfield represent regions of actin
network strain and disassembly

Due to the presence of stable, negatively divergent regions within the actin
flowfield, we hypothesized that global actin flow may be coordinated by these points
deep within the network. There are two, non-mutually exclusive mechanisms
hypothesized to contribute to actin flows: motor driven contraction and actin network
disassembly. We therefore examined how the negatively divergent regions of the
network correlated with measures of compression and disassembly. From the PIV
analysis of actin dynamics we first calculated the principal component of the strain
rate, which is quantified from the spatial changes in the actin velocity field; this analysis
of network deformation allowed us to infer regions of the actin network undergoing
tensile or compressive strains and highlighted that the negatively divergent regions
were actually correlated with high rates of compression (Figure 4, Movie 13). We also
modeled the assembly/disassembly within the network by taking into account the actin
intensity and flow information as previously described\textsuperscript{11}. This revealed that the
negatively divergent regions were also correlated with regions of disassembly (Figure
4, Movie 13). These data suggest that the negatively divergent regions of the network
are controlled by a combination of both contraction and disassembly of actin filaments.

\textit{Myosin-II driven contraction and Cofilin-mediated disassembly are essential for
actin flow}

We next examined zygotic mutations in \textit{non-muscle myosin-II} and the actin-
severing gene, \textit{cofilin}, which have both been hypothesized to regulate actin flow
through contraction and severing, respectively\textsuperscript{11, 48-50}. Indeed, homozygous mutation
of either \textit{myosin-II} or \textit{cofilin} led to defects in hemocyte dispersal with fewer cells
migrating to reach the ventral surface of the embryo (Figure 5A,B, Movie 14).
Furthermore, while hemocytes were still capable of extending lamellae, both mutations
showed a reduction in cell speed, and similar to what has been observed in cultured
cells \textit{in vitro}, a reduction in actin flow velocity\textsuperscript{11, 48, 49, 51, 52} (Figure 5C-E, Movie 15).
We subsequently examined how the organization of the actin flow was affected by the absence of either Cofilin or Myosin-II. As mechanical gradients across the cytoplasm are hypothesized to be a property of polarized motility, we first examined the gradient of negative divergence by quantifying linescans of the divergence maps from the cell body to the lamella boundary. Wild-type cells showed a gradient of network divergence starting a few microns from the cell edge, which increased until peaking just before reaching the cell body (Figure 6A,B). In contrast, in both myosin-II and cofilin mutant cells, the overall divergence values increased and there was no obvious gradient from front to rear (Figure 6A,B, Movie 16). Furthermore, the primary streamline sink, which was negatively divergent in wild-type cells, showed a significant increase in divergence values in the mutants (Figure 6C) suggesting that Myosin-II and Cofilin are both playing some role in generating sinks within the actin network.

While the divergence profiles appeared similar in myosin-II and cofilin mutants, they showed other phenotypes suggestive of unique roles in the regulation of actin flow when we compared the rates of assembly/disassembly. Linescans of wild-type cells revealed a gradient of disassembly that peaked at the rear of the network in a region similar in location to the peak in negative divergence (Figure 6B, D,E). In cofilin and myosin-II mutant cells, overall disassembly was reduced suggesting that they both play a role in network destruction (Figure 6D,E, Movie 16), however, the profile of the disassembly rates was not identical. In the absence of Myosin-II (i.e. only Cofilin-mediated destruction is present) the net disassembly of the network was relatively flat until reaching the rear of the network. In contrast, in the absence of Cofilin (i.e. only myosin-mediated destruction is present) there was a similar profile of net disassembly to wild-type cells, with a peak at the rear of the network that failed to reach levels
observed in controls (Figure 6 D,E). These data suggest that both Cofilin-mediated
severing and Myosin-II contraction are essential to regulate disassembly, however,
Cofilin is setting a baseline level of actin depolymerization across the network while
Myosin-II is controlling its graded destruction.

Streamline analysis also revealed that the myosin-II mutant cells showed a
much more disorganized actin flow. Quantifying the strength of the streamline sink
revealed that the maximum streamline endpoint in myosin-II mutants accumulated far
fewer streamlines than either wild-type or cofilin mutants (Figure 6F,G, Movie 16).
Furthermore, quantifying a local alignment parameter of the flowfield revealed that
myosin-II mutants specifically had a more disorganized actin flow profile (Figure S5A-
C), suggesting that Cofilin on its own is playing a minor role in the global organization
of actin flow. These data suggest that while both contraction and severing are
controlling the strength of actin network sinks, myosin-II is more important in
controlling the long-range coordination of actin flow.

We also examined how changing polymerization dynamics may alter global
actin flow organization. As loss of actin polymerization factors (e.g. Scar and Arp2/3)
lead to a severe and near complete loss of lamellae55,56 it was not possible to analyze
their role in controlling actin flow. However, Drosophila Ena/Vasp, which antagonizes
capping of actin filaments and enhances leading edge dynamics, has no obvious role
in hemocyte lamellipodia formation, thus allowing us to examine how changing leading
divergence values (Figure S5D-G, Movie 17). However, we observed no obvious
change in actin flow organization (Figure S5H). Therefore, as predicted by the
modeling, the emergence of a stable flow profile is likely insensitive to changes in actin polymerization.

**A gradient of Myosin-II indirectly leads to actin network contraction**

As *myosin-II* mutant hemocytes showed a more perturbed organization in actin flow we hypothesized that a graded distribution of myosin-II may be driving long-range coordination of the network. Expression of GFP-tagged Myosin-II in hemocytes revealed Myosin-II puncta flowing within the actin network (Figure 7A, Movie 18). Maximum intensity projections of Myosin-II dynamics and linescan analysis revealed that Myosin-II puncta are indeed present in a gradient from front to rear within hemocytes (Figure 7A,B), which was similar to what has been observed in various cells *in vitro* \(^{11,53}\).

We next hypothesized that Myosin-II may be directly controlling the buildup in contractile forces causing the network compression that we observed within the negatively divergent regions of the actin network. We therefore simultaneously analyzed actin and Myosin-II flows while also calculating the divergence within the actin flowfield to examine whether Myosin-II would localize to the negatively divergent regions of the network. To our surprise, we observed little to no correlation of Myosin-II puncta with divergent hotspots (Figure 7C, S6A). Indeed, dynamic analysis of the divergence revealed that the negatively divergent hotspots often developed adjacent to Myosin-II puncta and in between dense actin fibers within the network (Figure 7D). Furthermore, while the negatively divergent regions appeared to be fixed points within the network, Myosin-II puncta flowed through these regions suggesting that these stable regions of actin network compression and disassembly are helping drive the
flowfield (Movie 19). Consistent with this, we observed that myosin puncta, while moving in the same direction as the overall actin flow, showed a statistically lower speed, and concomitantly a distinct divergence profile (Figure S6B-E, Movie 20), which is similar to what was observed in migrating fish keratocytes\textsuperscript{52}. These data suggest that Myosin-II is not directly responsible for generating local contractile stresses within the actin network sinks; instead, the points of actin network divergence are likely an emergent behavior driven by a stable gradient of actin network tension and disassembly of the network.

Discussion

Here, we have taken advantage of the embryonic migration of hemocytes to examine stereotypical behaviors hypothesized to control cell motility and determine how these processes correlate with cell directionality. Contrary to the previous front-driven, lamellipodial-centric model of cell motility, the leading edge in hemocytes is poorly correlated with instantaneous cell motion with a persistence that is less than the overall persistence of the cell. Hemocytes spend a significant amount of energy using cellular extensions to explore their environment rather than directly inducing motion. While this mode of motility has been termed ‘inefficient’\textsuperscript{28, 36}, this does not mean that these seemingly extraneous edge fluctuations are non-functional; indeed, hemocytes are necessary to engulf apoptotic debris\textsuperscript{58} and evenly deposit extracellular matrix within the embryo\textsuperscript{34}, and the decoupling of edge extensions from cell motion may be necessary for hemocytes to efficiently explore their environment to carry out these critical tasks. Nevertheless, the term ‘leading edge’ is a misnomer as it is not obviously playing a leading role during random migration of these cells; however, nor
is it completely uncorrelated with motion. The velocities of edge fluctuations in hemocytes – despite their inherent noise – are still correlated to motion and therefore cells must have an intrinsic capacity to integrate this activity in order to ‘decide’ on a direction of travel, and we speculate that this occurs through the flow of actin within the cell. Furthermore, edge extensions, actin flow, and cell motion are highly integrated behaviors with no obvious temporal hierarchy. The migratory process is not obviously step-wise and in the future only a holistic approach to understanding cell motility may explain how these migratory behaviors are coordinated in such a precise fashion to control coherent cell motion.

Our global view of actin flow in hemocytes revealed a highly structured flowfield that is coordinated across the entire cell. The sinks within the hemocyte actin network are also similar to what we observed in a number of other cell-types. Indeed, this is consistent with what was reported in one of the first publications using speckle microscopy of actin flow by Valloton et al.,\textsuperscript{37}; it is also interesting to note that the authors of this work presciently noted that organization of the flow, in contrast to the leading edge, “is time persistent over minutes”, and we hypothesize that this stable organization of the actin flowfield may be a consistent feature of cell motility. The network sink within hemocytes also represents a sudden transition from retrograde to anterograde actin flow. While actin flows within migrating cells are often generally termed ‘retrograde flow’ (likely due to the focus on the leading edge), there is significant anterograde motion of the actin network observed from the rear of numerous cell-types\textsuperscript{19, 37-39}. Furthermore, consistent with what we observed in hemocytes, modeling has predicted that the retrograde flow at the front of a migrating cell will transition to anterograde flow as the adhesions switch from gripping (within the retrograde region) to slipping (within the anterograde region)\textsuperscript{59, 60}. 
One outstanding question is, what is controlling the formation and stability of these network sinks during motility? As the motion of the leading edge and the sinks are correlated, it is possible that there is information being transmitted between these two sites. Recent work has suggested that actin flow mediates a coupling between cell speed and persistence through the advection of polarity cues from the leading edge and it is possible that these cues may converge on the network sink to regulate its stability. Another possibility is that the flowing actin network is inherently stable and flow patterns may develop spontaneously in the absence of any direct regulation by signaling cues. Indeed, our minimal fluid-mechanical model of actin flow, in which flows emerge primarily through myosin-II contraction, leads to a stable 'sink' at the rear and is seemingly insensitive to a number of perturbations.

The destruction of the actin network, which is likely occurring at these network sinks may also be directly providing forces for locomotion. Disassembly of cytoskeletal networks can generate force in the absence of motors through entropic contraction and network remodeling. Due to the absence of any obvious time lag between the direction of nuclear movement and the primary streamline sink we hypothesize that the sink may provide the force for motion of the trailing cell body. The major sink represents a unique region of the network where there is a sharp retrograde/anterograde flow transition. The actin network on the retrograde side of this sink (i.e. toward the cell front) is experiencing high friction while the anterograde side (i.e. toward the rear) is slipping, which may be expected as the integrin adhesions at the rear of migrating cells have also been shown to slip. This would imply that the retrograde region is anchored to the substrate, allowing the forces generated by the reorganization of the actin network at the sink to drive unidirectional retraction of the rear of the cell. This mechanism is also consistent with the network-contraction model
that has been hypothesized to drive rear retraction in other cell-types and may therefore be a more ubiquitous mechanism of cell locomotion.

The organization of actin flow we have observed may have wide-ranging implications for how cells interpret and respond to migratory cues. Due to the extreme stability of the global actin flows, it is possible that in some cells a complete loss of polarity may be required to reset the flow direction and redirect cell motion (e.g. during run and tumble modes of migration). The stable actin flow may also be providing a stable polarity to the cell that enhances the discrimination of guidance cues. While internal amplification mechanisms through reaction-diffusion signaling modules have been hypothesized to be required to accurately chemotax towards low concentrations of guidance cues, this may be unnecessary. The stable flow of the actin network itself may be sufficient to provide the directional memory that allows the leading edge to rapidly sample and read external cues with subtle biases in the noise of edge fluctuations stabilized and integrated by the actin flow. Indeed, directional memory can make chemotaxis more efficient and discriminatory, and while reaction-diffusion modules have been hypothesized to control this memory, recent work suggests that it may also come from the cytoskeleton itself; our work suggests that it may originate from the highly coordinated and stable flow of the actin network.
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Figure Legends

Figure 1. Leading edge fluctuations are a weak predictor of cell directionality

(A-E) Morphodynamic analysis of edge fluctuation in hemocytes by quantifying the speed of: (A) all edge extensions, (B) the maximum edge extension (longest contiguous extension of perimeter), (C) extensions in the direction of cell motion (30° cone), (D) edge extensions and retractions in the direction of motion, and (E) all edge extensions and retractions (green = extension; magenta = retraction; for display purposes ‘A-E’ only show unit vectors; scale bar = 10 µm.).

(F) Comparison of cell speed with the speed of edge fluctuations as measured in ‘A-E’ reveals that protrusion speed is significantly different to instantaneous cell speed. ***P < 0.001, Kruskal-Wallis test and Dunn’s multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for all samples).

(G) A representative snapshot of a randomly migrating hemocyte with edge extensions automatically segmented. Vectors are drawn from the nucleus to each individual extension (blue arrows) and correlated with the direction of cell motion (magenta arrow) in panel ‘H’. Scale bar = 10 µm.

(H) A rose plot showing the direction of all extension vectors normalized to the direction of cell motion.

(I) A representative snapshot of a randomly migrating hemocyte with the maximum edge extension (by area) automatically segmented. Vectors are drawn from the
nucleus to the centroid of the maximum extension (green arrow) and correlated with
the direction of cell motion (magenta arrow) in ‘J’.

(J) A rose plot showing the direction of the maximum extension vectors normalized to
the direction of cell motion.

(K) Direction autocorrelation comparing the persistence of cell motion and maximum
edge extension showing that the maximum edge extension is less persistent than
overall cell motion. Dotted lines are real data and solid lines represent fitted decay
curves.

Figure 2. Actin retrograde flow is globally organized in migrating hemocytes

(A) Particle Image Velocimetry (PIV) analysis performed on a LifeAct-GFP expressing
cell to highlight the direction and magnitude of global actin flow. The region of the
flowfield without vectors represents the soma of the hemocyte, which has no
observable actin flow, and this cell body information was removed for all subsequent
quantification (scale bar = 10 µm).

(B) Divergence calculated from the actin flowfield to highlight sinks within the actin
network. In this image only negatively divergent regions are highlighted.

(C) Streamlines calculated from the actin flowfield in which streamlines were seeded
along the entire boundary of the cell, which reveals the overall organization through
the flowing network.

(D) The confluence of streamlines quantified by calculating the number of streamlines
ending in any one particular location within the cell. In this image, the size of the spot
is normalized to the number of streamline endpoints at each location.

(E) The actin flow divergence calculated at the primary sink and compared to the
divergence values averaged across the entire cell, which revealed that the sink is more
negatively divergent. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows
medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as
whiskers (n = 443 for all samples).

(F) A correlation map in which the direction of cell motion was correlated to the
direction of every actin flow vector within the cell. Note that a positive correlation
highlights anterograde flow while a negative correlation denotes retrograde flow. The
dashed circle indicates the location of the primary streamline sink in this frame of the
time-lapse movie.
(G) Quantification of the gradient of the correlation map in ‘F’ reveals sharp transition regions within the flowfield. The dashed circle indicates the location of the primary streamline sink in this frame of the time-lapse movie.

(H) Quantification of the gradient of the retrograde/anterograde flow correlation at the primary streamline sinks compared to the gradient values averaged across the entire cell, which reveals that the sink represents a region of steep retrograde/anterograde flow transition. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for all samples).

Figure 3. The polarity of global actin flow is highly stable and correlated to hemocyte motion during random and directed migration

(A) The left panel shows a schematic depicting the regions of the cell that are correlated to cell motion in the adjacent boxplot. These regions include: the primary streamline sink; the position of the peak retrograde/anterograde gradient; and the maximum edge extension. Vectors are drawn from the nucleus to each of these regions of the cell (the position vector to the maximum extension is shown as an example) and correlated to the direction of cell motion. The boxplot shows that the primary sink and the peak gradient are strongly correlated with the direction of motion compared to the maximum extension. ***P < 0.001, Kruskal-Wallis test and Dunn’s multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for all samples).

(B) Direction autocorrelation comparing the persistence of cell motion, maximum edge extension, and the primary streamline sink during random migration. Dotted lines are real data and solid lines represent fitted decay curves. Note that the primary streamline sink is more persistent than the maximum extension (autocorrelations for cell motion and maximum extension are from Figure 1K).

(C) Temporal cross correlation comparing the direction of cell motion, maximum edge extension, and the primary streamline sink, which reveals a peak correlation at 0-lag showing no obvious temporal hierarchy in these migratory parameters.

(D) Visualization of all hemocyte edge extensions (green) during random and directed migration.
(E) A schematic of a cell undergoing directed migration depicting the correlation of migratory parameters as in ‘A’.

(F) Direction autocorrelation comparing the persistence of cell motion, maximum edge extension, and the primary streamline sink during directed migration. Dotted lines are real data and solid lines represent fitted decay curves. Note the slower decay in these parameters during directed migration compared to random migration in ‘B’.

(G) Correlation of the average edge extension velocities (unit vectors, i.e. direction only) to the direction of cell motion in cells undergoing random versus directed motion. Note that there is no difference in the distribution of edge extensions around the cell perimeter. Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25\(^{th}\) and 75\(^{th}\) percentiles as box limits, 10\(^{th}\) and 90\(^{th}\) as whiskers (n = 33617 for random, n = 19486 for directed).

(H) Correlation of the resultant edge extension velocities (i.e. direction and magnitude) to the direction of motion, showing an increased correlation in cells undergoing directed migration. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25\(^{th}\) and 75\(^{th}\) percentiles as box limits, 10\(^{th}\) and 90\(^{th}\) as whiskers (n = 443 for random, n = 272 for directed).

(I) A minimal one-dimensional fluid-mechanical model (see SI for mathematical details) shows that myosin contraction can spontaneously lead to acto-myosin cortical flows with a sink at the cell rear corresponding with a peak in myosin concentration (θ = 0).

(J) Linear stability analysis around an initially homogeneous steady state of actin and myosin shows (1) that fluctuations grow into patterned flows once threshold values for myosin levels (μ\(_{0}\)) and actin polymerization rate (k\(_{p}\)) are reached (blue region is denotes decay of fluctuations while red denotes growth), and (2) that the emergence of flows is sensitive to myosin levels (steep dependence) and insensitive to rate changes (flat dependence).

(K,L) Actin flows are stable with respect to perturbations in actin ‘polymerization’ (e.g. due to external stimulus). (K) Angular location of myosin peak and hence direction of cell migration, θ\(_{\text{motion}}\), (red solid line) as a function of the angle of actin perturbation, θ\(_{\text{perturb}}\), (black dashed line). Note that the angle at which the perturbation occurs has little effect on the direction of actin flow.
Angular location of myosin peak and cell motion as a function of the strength of actin perturbation, $s_{\text{perturb}}$. In this case the angle of perturbation (black dashed line) was kept constant while varying its strength. Similar to panel 'K', the actual direction of motion (red solid line) was relatively stable with respect to the perturbation. Note that a small reorientation of the direction of motion is possible with an increase in the strength of the perturbation.

**Figure 4. Negatively divergent regions of the actin flowfield represent regions of actin network strain and disassembly**

(A-C) Heatmaps comparing the quantification of divergence (A), network compression (B), and actin network disassembly (C) in an individual hemocyte. Bottom panels are high magnification images of the boxes outlined in the upper panels. Note the partial overlap of these parameters.

(D) Scatter plot comparing a random sample of points in the actin flowfield for divergence and principal strain. Note the positive relationship between negative divergence and the negative values of the principal strain (i.e. compression).

(E) Scatter plot comparing a random sample of points in the actin flowfield for divergence and amount of assembly/disassembly within the actin network. Note the positive relationship between negative divergence and the amount of disassembly.

**Figure 5. Loss of Myosin-II and Cofilin lead to reduced actin flow and cell speed**

(A) Images of hemocytes on the ventral surface of *Drosophila* embryos in wild-type, myosin-II, and cofilin mutant embryos. LifeAct-GFP is shown in green, nuclei are labeled in magenta. Scale bar = 30 µm.

(B) Temporal average projection of wild-type, myosin-II, and cofilin mutant embryos, highlighting domains occupied by migrating hemocytes. Note that the mutant embryos display a less homogenous domain distribution. Scale bar = 30 µm.

(C) PIV analysis of actin flow in wild-type, myosin-II, and cofilin mutant cells.

(D) Quantification of mean cell speed in wild-type and mutant cells showing that both myosin-II and cofilin mutant hemocytes are slower than wild-type cells. ***P < 0.001, ordinary one-way ANOVA test and Holm-Sidak's multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).
(E) Quantification of mean actin flow speed in wild-type and mutant cells. Both myosin-II and cofilin mutant hemocytes are slower than wild-type cells. ***P < 0.001, ordinary one-way ANOVA test and Holm-Sidak’s multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).

Figure 6. A gradient of Myosin-II driven contraction is essential for global organization of actin flow

(A) Heatmaps comparing the quantification of divergence in wild-type, myosin-II, and cofilin mutant hemocytes.

(B) Quantification of the mean divergence values in wild-type and mutant cells calculated by drawing linescans from the cell body to the edge. Error bars = SEM.

(C) Quantification of the mean divergence at primary sink in wild-type and mutant cells, which reveals that mutants have an increase in divergence values highlighting a reduction in network compression. ***P < 0.001, **P < 0.01, Kruskal-Wallis test and the Dunn’s multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).

(D) Heatmaps comparing the quantification of actin disassembly in wild-type and mutant cells.

(E) Quantification of the mean assembly/disassembly values in wild-type and mutant cells calculated by drawing linescans from the cell body to the edge as in ‘B’. Error bars = SEM.

(F) Images highlighting an analysis of streamlines through the actin flowfield in wild-type and mutant cells. Note the disorganized streamlines in myosin-II mutants.

(G) Quantification of the percentage of streamlines that end at the primary sink in wild-type and mutant cells. Note that myosin-II mutants show a significant reduction in their streamline confluence compared to wild-type or cofilin mutant cells. **P < 0.01, Kruskal-Wallis test and Dunn’s multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).

Figure 7. A gradient of Myosin-II indirectly leads to actin network contraction
(A) Temporal maximum projection of a Myosin-II-GFP expressing hemocyte highlighting that Myosin-II puncta within the lamellae are predominantly toward the rear of the network surrounding the cell body. Scale bar = 10 µm.

(B) Linescan analysis of Actin and Myosin-II localization within hemocytes. The plot profile of fluorescence intensity was performed on randomly chosen lines within the lamellae from the cell body towards the edge (see insert). Note that the average intensity of Myosin-II is high toward the cell body and decreases in a gradient approaching the edge, whereas Actin intensity remains constant. Error bars = SEM.

(C) Comparison of Actin and Myosin-II localization with actin flow divergence. Bottom panels are high magnification images of the boxes outlined in upper panels. Asterisks highlight regions of strong negative divergence, which show no obvious colocalization with Myosin-II. Scale bar = 10 µm.

(D) Time-lapse series comparing Actin and Myosin-II localization with actin flow divergence. Circles highlight example regions of strong negative divergence. Note that the negatively divergent regions are adjacent to Myosin-II puncta.

Supplementary Figure Legends

Supplementary Figure 1. Related to Figure 1.

(A) Three examples of cell contour analysis during hemocyte migration revealing highly dynamic edge activity.

(B) Left panel reveals a representative snapshot of a randomly migrating hemocyte with the maximum edge extension (green) and retraction (magenta) automatically tracked and compared to the direction of cell motion (white). Right panel shows that the maximum extension and retraction are positively and negatively correlated to motion. Note the high variance in the distribution. ***P < 0.001, Mann-Whitney two-tailed test. The graph shows mean and SD as bars; each datapoint is displayed as a dot (n = 443 for all samples).

(C) Left panel shows a representative snapshot of all extension vectors around the cell perimeter (green) and maximum extension vectors based on the longest contiguous extension (blue). White arrow shows the direction of cell motion. Right panel shows the correlation of the resultant velocity of extension vectors to the
direction of motion, showing that the resultant of all extensions is better correlated than maximum extension alone. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for all samples).

**Supplementary Figure 2. Related to Figure 2.**
(A) Probability density function (PDF) examining the distribution of divergence values within the actin flowfield. Note that most of the measured divergence within the actin flowfield is negative in value.
(B) Time-lapse series of divergence measurements within the actin flowfield during hemocyte migration. Dashed circle highlights a region of strong negative divergence that is persistent in time within the network.
(C) Time-lapse series of a LifeAct-GFP labeled hemocyte in which the direction of the actin flow and divergence of the flowfield were quantified. The bottom panel is a high magnification image of the boxes in the upper panel showing the direction of the actin flowfield (arrows) color coded for the strength of the negative divergence. Note that in the center of the flowfield is a region of actin network deformation which correlates with strong negative divergence.
(D) Comparison of streamlines with the speed and divergence of global actin flow. The dashed circle highlights the streamline sink in this frame of the time-lapse movie positioned within a region of low flow speed and high negative divergence.
(E) Example image of a hemocyte with sustained bipolar protrusions and opposing streamline sinks. Note the strong negative divergence within both streamline sinks. Scale bar = 10 µm.
(F) Quantification of the mean cell-wide actin flow speed versus retrograde actin flow speed. Note that the actin flow speed is significantly reduced in the retrograde region. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for all samples).
(G) Comparison of instantaneous cell speed with average global actin flow speed (left), flow speed within the retrograde region only (middle), and flow speed in the direction of motion (right). Note that there is no significant correlation in any of these comparisons.
Supplementary Figure 3. Related to Figures 2 and 3.

(A) PIV, divergence, streamline analysis, and quantification of streamline sinks of cultured cells containing labeled actin. Representative snapshots are displayed for a fish keratocyte (scale bar = 10 µm), a neural growth cone (scale bar = 5 µm), and a Retinal Pigment Epithelium (RPE1) cell (scale bar = 10 µm).

(B) Comparison of cell speed with the speed of the maximum edge extension in RPE1 cells reveals that protrusion speed is significantly higher than instantaneous cell speed. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 247 for all samples).

(C) Example cell track of an RPE1 cell in which the unit vectors of the maximum edge extension or the primary streamline sink were superimposed.

(D) Temporal cross correlation comparing the direction of cell motion, maximum edge extension, and the maximum streamline sink in RPE1 cells, which reveals a peak correlation at 0-lag showing no obvious temporal hierarchy in these migratory parameters.

(E) Correlation of the primary streamline sink and the maximum edge extension vectors to the direction of cell motion in RPE1 cells. Note that that both are strongly correlated with the direction of cell motion. Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 247 for all samples).

Supplementary Figure 4. Related to Figure 3.

(A) Example cell track of a randomly migrating hemocyte in which the unit vectors of the maximum edge extension or the primary streamline sink are superimposed. Note that the direction of the primary sink appears better correlated with the direction of cell motion.

(B) Probability density function (PDF) comparing the distribution of distances from the nucleus to the maximum edge extension and the nucleus to the primary streamline sink.

(C) Time-lapse series of hemocytes migrating directionally to a laser wound (asterisk) in the embryo. LifeAct-GFP is shown in green, nuclei are labeled in magenta. Scale bar = 30 µm.
(D) Rose plot showing the direction of maximum extensions normalized to the direction of motion comparing random migration (black outline) to directed motion (green).

(E) Rose plot showing the direction of the primary streamline sink normalized to the direction of cell motion comparing random migration (black outline) to directed motion (blue).

(F) Correlation of the direction of the maximum extension and primary streamline sink to the direction of cell motion. Note that both parameters are more highly correlated in cells undergoing directed migration. *P < 0.05, Mann-Whitney two-tailed tests. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for random, n = 272 for directed).

(G) Comparison of the direction autocorrelations of cell motion (left), the primary streamline sink (middle), and the maximum edge extension (right) during random and directed migration. Note the slower decay in the autocorrelations of all parameters during directed migration suggesting increased persistence.

(H) Quantification of the directionality ratio shows higher persistence in cells undergoing directed migration (walking average over 60 s intervals). *P < 0.05, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for random, n = 5 for directed).

Supplementary Figure 5. Related to Figure 6.

(A) Schematic showing an example of the alignment analysis of actin flow. The average cosine similarity between a reference velocity vector (F(r)) and its 8 nearest neighbours (F(n)) is calculated. This local analysis of alignment is calculated for every vector in the field and averaged to produce the mean alignment for each genotype, which reflects the organization of the actin flows.

(B) Color coded heatmap of the degree of alignment within the flowfield in wild-type and mutant cells with a value of 1 meaning perfect alignment.

(C) Boxplot representing average alignment of the actin flowfield. Note that the flow in myosin-II mutant cells is more disorganized than either wild-type or cofilin mutant cells. **P < 0.01, Kruskal-Wallis test and Dunn’s multiple comparison test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).
(D) PIV, divergence, streamline, and streamline sink analysis of LifeAct-GFP expressing wild-type and ena mutant cells. Scale bar = 10 µm.

(E) Comparison of the speed of edge extensions in wild-type and ena mutant cells reveals that edge speed is significantly lower in mutants. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, 10th and 90th as whiskers (n = 443 for wild-type, n = 509 for ena mutants).

(F) Quantification of mean actin flow speed in wild-type and ena mutant cells reveals that actin flow is slower in mutants. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).

(G) The primary streamline sink is more strongly negatively divergent and stronger in the wild-type cells. ***P < 0.001, Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).

(H) Quantification of the percentage of streamlines that end at the primary sink in wild-type and ena mutant cells. Note that ena mutants show a similar level of streamline confluence to wild-type cells. Mann-Whitney two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers; each datapoint is displayed as a dot (n = 9 for all samples).

**Supplementary Figure 6. Related to Figure 7.**

(A) Scatter plot of Myosin-II intensity and actin divergence for each point in the lamella of a hemocyte reveals no relationship between Myosin levels and strength of divergence.

(B) PIV analysis of actin and Myosin-II flow performed simultaneously in a migrating hemocyte.

(C) Correlation of the direction of actin and Myosin-II flow from simultaneous PIV analysis reveals that their direction of motion is nearly identical. The boxplot shows median as central lines, 25th and 75th percentiles as box limits, 10th and 90th percentiles as whiskers (n = 147).
(D) Comparison of actin and Myosin-II flow speed from simultaneous PIV analysis reveals that Myosin-II motion is significantly slower. ***P < 0.001, Wilcoxon matched-pairs signed rank two-tailed test. The boxplot shows medians as central lines, 25th and 75th percentiles as box limits, minimum and maximum values as whiskers (n = 142 for all samples).

(E) Comparison of actin and Myosin-II divergence from simultaneous PIV analysis reveals that they have distinct profiles.

Supplementary Movie Legends

**Supplementary Movie 1. Related to Figure 1**
Automatic tracking of a hemocyte comparing tracking of the cell centroid (magenta) or the nucleus (green). Note that in hemocytes at this temporal resolution (5 s/frame), tracking the cell centroid reflects overall shape changes more than cell motion. In contrast, the nucleus represents a fixed fiducial marker within the cell that more accurately reflects cell movement.

**Supplementary Movie 2. Related to Figure 1**
Time-lapse movie of a randomly migrating hemocyte in which edge extensions were automatically segmented. Vectors (green arrows) were drawn from the nucleus to either each individual extension or the maximum extension (extension of the largest contiguous area), while simultaneously tracking the cell direction of travel (magenta arrow).

**Supplementary Movie 3. Related to Figure 2.**
Time-lapse movie of a randomly migrating hemocyte in which analysis of the actin flowfield was conducted using PIV, divergence, streamlines, and streamline sinks (the size of the spots are normalized to the number of streamlines ending within a defined region).

**Supplementary Movie 4. Related to Figure 2.**
Time-lapse movie of a hemocyte expressing GFP-actin (left panel) to directly label the actin network. A region within the network was photobleached (highlighted by the circle) and subsequently tracked as it transited through the lamella. Note that the bleached spot moves through the lamella in a direction predicted by the PIV (middle panel) and begins to disappear at the rear of the network, which shows an increase in negative divergence (right panel).

**Supplementary Movie 5. Related to Supplementary Figure 2.**

Time-lapse movies of a randomly migrating bipolar hemocyte in which the sinks colocalize with the negative divergent regions. Note that two independent sinks develop at the time when the cell develops a bipolar shape.

**Supplementary Movie 6. Related to Supplementary Figure 3.**

Time-lapse movie of a fish keratocyte in which analysis of the actin flowfield was conducted using PIV, divergence, streamlines, and streamline sinks.

**Supplementary Movie 7. Related to Supplementary Figure 3.**

Time-lapse movie of a neural growth cone in which analysis of the actin flowfield was conducted using PIV, divergence, streamlines, and streamline sinks.

**Supplementary Movie 8. Related to Supplementary Figure 3.**

Time-lapse movie of an RPE1 cell in which analysis of the actin flowfield was conducted using PIV, divergence, streamlines, and streamline sinks.

**Supplementary Movie 9. Related to Figure 3.**

Time-lapse movie of a randomly migrating hemocyte comparing the direction of motion (white arrow) to either the direction to the maximum extension (green arrow) or the primary sink (magenta arrow).

**Supplementary Movie 10. Related to Supplementary Figure 3.**

Time-lapse movie of an RPE1 cell in which cell motion, edge extensions, and streamline sink were automatically tracked. Vectors were drawn from the nucleus to
the maximum extension (green arrow), and the primary sink (magenta arrow), while simultaneously showing cell trajectory (white arrow).

**Supplementary Movie 11. Related to Figure 3.**
Time-lapse movie of hemocytes migrating towards a laser wound (asterisk). LifeAct GFP is shown in green, nuclei are labelled in magenta.

**Supplementary Movie 12. Related to Figure 3.**
Time-lapse movie of a randomly and a directionally migrating hemocyte highlighting the vector to their maximum edge extension (green) along with their direction of travel (white arrow). Asterisk denotes the wound site.

**Supplementary Movie 13. Related to Figure 4.**
Time-lapse movie of a randomly migrating hemocyte comparing negative divergence, compression, and actin disassembly.

**Supplementary Movie 14. Related to Figure 5.**
Time-lapse movies of wild-type, myosin-II, and coflin mutant hemocytes undergoing developmental dispersal (LifeAct GFP is shown in green, nuclei are labelled in magenta).

**Supplementary Movie 15. Related to Figure 5.**
Time-lapse movies of PIV analysis of actin flow on wild-type, myosin-II, and coflin mutant hemocytes expressing LifeAct-GFP.

**Supplementary Movie 16. Related to Figure 6.**
Time-lapse movies wild-type, myosin-II, and coflin mutant cells analyzed for divergence, disassembly, and streamlines of the actin flow.

**Supplementary Movie 17. Related to Supplementary Figure 6.**
Time-lapse movie of an ena mutant cell in which analysis of the actin flowfield was conducted using PIV, divergence, streamlines, and streamline sinks.
Supplementary Movie 18. Related to Figure 7.
Time-lapse movie of a hemocyte containing fluorescently labelled Actin and Myosin-II.

Supplementary Movie 19. Related to Figure 7.
Time-lapse movie of a hemocyte showing divergence of the actin flow and the location of Actin and Myosin-II. Crosses highlight transient regions of strong negative divergence. Note that the Myosin-II puncta do not accumulate at regions of negative divergence and instead continue to flow through.

Supplementary Movie 20. Related to Figure 7.
Time-lapse movie of PIV analysis and divergence of actin and Myosin-II flow performed simultaneously in a migrating hemocyte.
Materials and methods

Fly genetics

The following fly stocks were used in this study: w1118 strain as wild-type (Bloomington Drosophila Stock Center (BDSC), BL3605); myosin-II mutant (BDSC, BL4199); cofilin mutant (BDSC, BL9107). Hemocytes were labeled using the promoters, Srp-Gal473 or Sn-Gal474. The following fluorescent probes were used to label: nuclei [UAS-RedStinger, (BDSC, BL8546 and 8547]; actin [UAS-LifeAct-GFP74 or UAS-Moesin-Cherry75]; Myosin-II heavy chain [(UAS-Zip-GFP)76]. Flies were left to lay eggs on grape juice/agar plates overnight at 25°C. Embryos were dechorionated in bleach and the appropriate genotype was identified based on the presence of fluorescent markers.

Cell lines

Microscopy

Embryos were mounted as previously described4 and time-lapse images were acquired every 5 s with a PerkinElmer Ultraview spinning disk microscope equipped with a 63x NA 1.4 Plan-Apochromat oil objective during developmental dispersal (stages 15–16). Whole embryo snapshots were taken using the LSM 880 confocal microscope (Carl Zeiss) equipped with a 40x NA 1.3 Plan-Apochromat oil objective.

Data analysis

For the characterization of control hemocyte migratory parameters (i.e. direction autocorrelation, retrograde flow speed, streamline analysis, divergence, principal strain, assembly/disassembly, and flow alignment) data were gathered on a per/frame basis from 9 individual cells each imaged over approximately 4-5 minutes at intervals of 5 s/frame, representing an n number of 443 time-points. When comparing control and mutant genotypes, statistical tests were performed on a per cell basis due to the partial penetrance of the various mutant phenotypes. Here, comparisons were made between the 9 control cells, 9 myosin-II mutants, and 9 cofilin mutants. The specific statistical test, as well as the thresholds for significance are noted in the respective figure legends. The raw data that support the findings of this study are available from
the corresponding author upon reasonable request [BS]. The computational analysis was performed in MATLAB (Mathworks®) using custom code, which can be obtained from the corresponding author upon reasonable request [BS].

**Wounding**

Laser wounding was performed using an ablation laser (MicroPoint; Andor Technology) as previously described and imaged using a Perkin Elmer spinning disk microscope.

**Quantification of Moesin-Cherry and Myosin-GFP fluorescence**

In order to quantify the spatial distribution of Actin and Myosin-II within hemocytes, cells expressing Moesin-Cherry and Myosin-II-GFP were sampled by linescan analysis to measure average fluorescence intensity. Fiji line and profile functions were used to draw and record 2 μm wide lines from the cell body boundary to the cell edge in 12 different cells.

**Cell tracking**

Hemocytes containing labeled nuclei were first thresholded in Fiji. Tracking was then performed in MATLAB by calculating the positions of the centroid of the nucleus through time.

**Particle Image Velocimetry**

Time-lapse images of freely moving hemocytes were acquired at 5 s/frame. Actin was labeled with LifeAct-GFP for all figures with the exception of Figure 7 and Supplementary Figure 6, which used Moesin-Cherry in conjunction with Myosin-II-GFP (*Drosophila* non-muscle myosin heavy chain). Cells were then manually segmented prior to PIV analysis.

There is no grossly observable actin flow behavior within the cell body of the hemocytes, therefore information from the cell body was removed by manually segmenting the cell body region and using this as a mask to remove PIV values. The actin flow PIV information is therefore entirely from within the lamellae. To observe Myosin-II flow in the lamellae, the signal from the cell body was oversaturated. For this reason, no PIV information could be obtained from the cell body region and it was...
excluded from the myosin flow PIV analysis and its actin flow counterpart (Figure S6D-G).

A 2D cross-correlation algorithm adapted from classical PIV was implemented\(^\text{32}\). In brief, this method compares a region of interest in an image (source image) with a larger region of a subsequent image (search image). The sizes of the source and search regions are determined on the basis of the feature size to be tracked and the area of their expected displacement (i.e. actin bundles). For this analysis, source and search images encompassing areas of 1.2 µm\(^2\) and 2 µm\(^2\) were used. A cross-correlation map was computed by analyzing the cross-correlation coefficient between the source image and the search image, by shifting the source across the search one pixel at a time. Network displacement was measured by finding the maximum coefficient within the resulting cross-correlation map. To filter anomalous tracking data, only displacements that had a cross-correlation coefficient above a certain threshold, \(c_0\), were kept. For the present work, the threshold was set at \(c_0 = 0.5\). Finally, a spatial convolution with a Gaussian kernel (size of 5 µm, sigma of 1 µm), and temporal convolution with temporal kernel of 20 s (sigma 10 s) were used to interpolate the measured displacements to cover all the pixels within the cell outline. The complete algorithm for this analysis was implemented in MATLAB.

**Defining retrograde and anterograde flow regions**

Retrograde and anterograde flow were defined with respect to their respective alignment to cell motion. The direction of the actin flow at every point within the lamellae was correlated with the instantaneous direction of cell motion using the cosine of the angle between these velocity vectors. Retrograde flow was defined as a negative correlation while anterograde flow was a positive correlation to cell motion.

**Streamlines**

Streamlines were used to assess the global organization of the actin flowfield. Here each line is drawn tangent to a local velocity vector and describes a path that a massless particle would take if entering the actin flowfield at that point (MATLAB **stream2** function). The seed points for the streamlines were placed at every pixel along the cell boundary. For visualization purposes streamlines were represented at regular intervals (MATLAB **streamslice** function). Line Integral Convolution was employed to represent global streamline activity (Figure S2D) utilizing an open source vector field...
visualization toolkit (http://sccn.ucsd.edu/~nima/). Streamline sinks were defined by quantifying the frequency of streamline termini within non-overlapping 2.5 μm² regions of the cell image and the coordinates of these endpoints were set at the center of the boxes. For position vector analysis of these endpoints, vectors were constructed from the centroid of the nucleus to the endpoint coordinates.

**Principal strain**

Local deformation of the actin network can be quantified by evaluation of the principal strains which are derived from local velocity changes obtained by PIV. The relative positional changes of points within a deforming body are described with a velocity tensor, which is computed based on a central difference estimation over 2.5 μm in both spatial dimensions.

\[ V = \begin{pmatrix} \frac{\partial V_x}{\partial x} & \frac{\partial V_x}{\partial y} \\ \frac{\partial V_y}{\partial x} & \frac{\partial V_y}{\partial y} \end{pmatrix} \]

Decomposition of the velocity gradient provides a symmetric and an antisymmetric component, with the symmetric part being the strain rate tensor. This strain rate tensor is defined as \( S = \frac{1}{2}(V + V^T) \). Decomposition of \( S \) yields the eigenvalues and eigenvectors of the deformation, where eigenvectors denote the principle axes of the deformation and eigenvalues the principle components of the strain rate tensor. The eigenvalues sign accounts for compressive (negative) or tensile (positive) strain. We observed very little tensile strain inside the network along the major axis. Therefore, for visualization purposes, only the principal strain denoting compression was shown. For visual representation of control cells the principal strain field was normalized between -1 and 0. For comparing the principal strain field between genotypes, no normalization was performed because of the reduction of the strain values in the mutant conditions, however the colormap scaling was fixed between genotypes.

**Divergence and network turnover analysis**

For quantification of divergence a central difference scheme was implemented to compute the spatial derivatives of the actin flow velocities \((\nabla \cdot V)\). This method of
calculating divergence was also utilized in the computation of network turnover, to
determine the spatial distribution of network assembly and disassembly which was
calculated using the equation below.

$$\text{turnover} = \frac{\partial I}{\partial t} + (I \cdot (\nabla \cdot V)) + (V \cdot (\nabla \cdot I))$$

The temporal derivative of the fluorescence intensity $\frac{\partial I}{\partial t}$ was computed using

a forward difference scheme between two consecutive frame of the time-lapse. As

with the spatial gradients of flow velocity $\nabla \cdot V$ the fluorescence intensity $\nabla \cdot I$ was

computed using a central difference scheme. As there was not much assembly

information inside the lamellae of hemocytes, only the disassembly data were visually

represented and normalized to the maximum value in the field. However, both

assembly and disassembly was included in the quantification.

For visualization purposes in control cells, normalized disassembly or negative
divergence maps were shown normalized to the maximum value in the field, providing

values between -1 and 0. For comparing the disassembly or negative divergence

between genotypes, no normalization was performed because of the reduction of

these values in the mutant conditions, however the colormap scaling was fixed

between genotypes.

Linescans were used to show the contractile and destructive gradients of the

flowing actin network (MATLAB improfile function) by drawing three random lines of 1

pixel width per frame. Lines originate from the centroid of the nucleus and extend

through the lamella to the cell edge. Data points within the cell body were discarded.

Flow alignment

For determining the average alignment of actin flows, the cosine similarity between all

velocity vectors and their 8 nearest neighbors was computed using $\cos \theta = \frac{v_1 \cdot v_2}{|v_1||v_2|}$, and subsequently averaged to give flowfield alignment.

Extension/retraction analysis

For the extension/retraction analysis, segmented time-lapse images of freely moving

hemocytes were subtracted from the subsequent frames in the time-lapse series to

highlight regions of extension or retraction. The MATLAB regionprops function was

used to filter extensions and retractions by their respective area, and to attain their
centroid for the purpose of tracking these regions with respect to the position of the nucleus. Maximum extensions and retraits were defined as the regions for each frame with the largest area.

**Edge velocity analysis**

To evaluate edge dynamics, segmented time-lapse images of hemocytes were analyzed using a Segmentation and Windowing package 27, calculating edge extensions and retractions at each pixel along the cell boundary. Custom scripts implemented in MATLAB were used to calculate extension speed globally, and locally within specific regions of the cell boundary. To calculate the edge velocity in the direction of cell motion, the edge was segmented within a region bounded by a 30° cone centered on the direction of motion. To calculate the edge velocity within the maximum extension, the longest uninterrupted region along the perimeter of the cell edge was segmented. To quantify the average net edge activity, positive and negative sign was assigned to velocity vectors depending on whether they were classified as extension or retractions.

**Temporal cross-correlation**

Temporal cross-correlation was employed to evaluate whether there was any temporal hierarchy governing the dynamics of the considered migratory parameters (i.e. cell motion, primary sink, maximum edge extension). This analysis involves the directional correlation of two vectors at all potential time lags. The temporal cross-correlation function is described as $DC = \langle v_i(t) \cdot v_j(t + \tau) \rangle$, where $DC$ is the time averaged cosine similarity between vector $i \ (v_i)$ and parameter $j \ (v_j)$ at time $t$ and lagged time intervals $(t + \tau)$. 


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Figure 1

A. All extensions
B. Max extension
C. Extensions (direction of motion)
D. Net extensions (direction of motion)
E. All net extensions
F. Box plot showing speed (m/min) across different conditions
G. Diagram showing all extensions and direction of motion
H. Circular graph showing all extensions
I. Diagram showing max extension and direction of motion
J. Circular graph showing max extensions
K. Graph showing cell motion and max extension over time
Figure 2:

A. PIV
B. Divergence
C. Streamlines
D. Streamline sinks
E. Divergence (a.u.)
F. Retro/anterograde flow
G. Gradient
H. Gradient (a.u.)

0 μm/min

0 a.u.

0.0

-0.3

0.8

0.6

0.4

0.2

0.0

-1 cos(\(\theta\))

-1

-0.1

-0.2

-0.3

-0.2

-0.1

-0.0

0.0

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

primary sink cell-wide

primary sink cell-wide

***
Figure 3:

A

Max extension
Nucleus
Primary sink/peak gradient
Motion
Random

B

Direction autocorrelation

Cell motion
Primary sink
Max extension

C

Temporal cross correlation

Lags

D

Random
Extensions

Directed
Extensions

0 secs
100 secs

E

Primary sink
Max extension
Nucleus
Motion
Directed

F

Direction autocorrelation

Cell motion
Primary sink
Max extension

G

Random all
Directed all

H

Random all
Directed all

I

$\theta = 0$
front
$\theta = \pi$
rear
$
\rho$(actin)
$
\mu$(myosin)

$10^5$

J

growth rate of $l=1$ perturbation

myosin $\mu_0$
actin polymerisation $k_p$

K

angle of motion $\theta_{motion}$

angle of perturbation $\theta_{perturb}$

L

angle of motion $\theta_{motion}$

strength of perturbation $s_{perturb}$
Figure 4:

(A) Divergence

(B) Compression

(C) Disassembly

(D) Principal strain vs. Divergence (a.u.)

(E) Disassembly vs. Divergence (a.u.)

\[ R^2 = 0.43 \]

\[ R^2 = 0.42 \]
Figure 5:

(A) Wild-type, myosin-II mut, and cofillin mut cells showing actin filament localization.

(B) Wild-type, myosin-II mut, and cofillin mut cells showing cell body morphology.

(C) PIV analysis of wild-type, myosin-II mut, and cofillin mut cells indicating mean cell speed (µm/min). 

(D) Box plot showing mean cell speed (µm/min) for wild-type (wt), myosin-II mut, and cofillin mut cells.

(E) Box plot showing mean flow speed (µm/min) for wild-type (wt), myosin-II mut, and cofillin mut cells.
Figure 6:

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>myosin-II^{mut}</th>
<th>cofillin^{mut}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divergence</td>
<td><img src="image1.png" alt="Image A" /></td>
<td><img src="image2.png" alt="Image B" /></td>
<td><img src="image3.png" alt="Image C" /></td>
</tr>
<tr>
<td>Disassembly</td>
<td><img src="image4.png" alt="Image D" /></td>
<td><img src="image5.png" alt="Image E" /></td>
<td></td>
</tr>
<tr>
<td>Streamlines</td>
<td><img src="image6.png" alt="Image F" /></td>
<td><img src="image7.png" alt="Image G" /></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7:

A) Myosin-II

B) Graph showing the average fluorescence intensity of Actin and Myosin over time. The x-axis represents the distance from the cell body in μm, and the y-axis represents the fluorescence intensity in arbitrary units (a.u.).

C) Images showing the distribution of Actin and Myosin-II with a divergence map.

D) Time-lapse images of Actin and Myosin-II fluorescence intensity changes over 0 s, 25 s, and 50 s.
Supplementary Figure 1:

A  Cell contours

B  Direction of motion
- Max Extension
- Max Retraction

C  Direction of motion
- All extensions
- Max extension

Graphs showing the direction of motion and the cosine of theta for max extension and max retraction.
Supplementary Figure 2:

A) PDF of Divergence (a.u.)

B) LifeActGFP labelled hemocyte at different time points (0 secs, 25 secs, 50 secs, 75 secs)

C) LifeActGFP labelled hemocyte over time (0 secs, 5 secs, 10 secs, 15 secs)

D) Flow speed and Divergence maps

E) Sinks and Divergence representation

F) Box plot of Flow speed in direction of motion

G) Scatter plots showing R^2 values for average flow speed, retrograde flow speed, and flow speed in direction of motion vs. cell speed

**Legend:**

- PDF: Probability Density Function
- Flow speed: µm/min
- Divergence: a.u.
- Sinks: Depressions or regions where flow is reduced
- Retrograde flow: Flow moving against the main direction
- R^2: Coefficient of determination
Supplementary Figure 3:

A. PIV, Divergence, Streamlines, and Streamline sinks for Fish keratocyte, Growth cone, and RPE1 cells.

B. Box plot showing the comparison of speed (µm/min) for Cell motion vs Max extension with statistical significance indicated by "***".

C. Line graph showing temporal cross correlation with lag times ranging from -100 to 100.

D. Line graph showing the comparison of temporal cross correlation between Cell motion vs Primary sink, Cell motion vs Max extension, and Primary sink vs Max extension.

E. Box plot showing comparison of correlation coefficients between Primary sink and Max extension, with "ns" indicating no significant difference.
Supplementary Figure 5:

A. Local Alignment (cos(\theta))

B. Local Alignment

D. PIV

G. Divergence at primary sink (a.u.)

E. All extensions speed (\mu m/min)

F. Mean Flow speed (\mu m/min)

H. Percentage streamlines at primary sink (%)

---

F(3) = F(i,j+1)
F(1) = F(i+1,j)
F(2) = F(i+1,j+1)
F(7) = F(i,j-1)
F(6) = F(i-1,j-1)
F(5) = F(i-1,j)
F(4) = F(i-1,j+1)
F(r) = F(i,j)

---

[Graphs and diagrams showing comparisons between wild-type, myosin-II\textsuperscript{mut}, and cofillin\textsuperscript{mut} in a local alignment context.]
**Supplementary Figure 6:**

A. Scatter plot showing the relationship between actin negative divergence and myosin intensity. The correlation coefficient $R^2 = 0.07$.

B. PIV images of actin and myosin-II, with color gradients indicating flow speed in µm/min.

C. Graph showing the distribution of cosine of the angle ($\cos(\theta)$) between actin/myosin-II.

D. Box plot comparing flow speeds between actin and myosin-II, with statistical significance indicated by ***.

E. Images of actin and myosin-II divergence, highlighting the areas of negative and positive divergence.