Live-cell imaging of G-actin dynamics using sequential FDAP

Tai Kiuchi,* Tomoaki Nagai, Kazumasa Ohashi, Naoki Watanabe and Kensaku Mizuno*

Department of Biomolecular Sciences; Graduate School of Life Sciences; Tohoku University; Sendai, Japan

Correspondence to: Tai Kiuchi
and Kensaku Mizuno;
Email: tai.kiuchi@m.tohoku.ac.jp
and kmizuno@biology.tohoku.ac.jp

Various microscopic techniques have been developed to understand the mechanisms that spatiotemporally control actin filament dynamics in live cells. Kinetic data on the processes of actin assembly and disassembly on F-actin have been accumulated. However, the kinetics of cytoplasmic G-actin, a key determinant for actin polymerization, has remained unclear because of a lack of appropriate methods to measure the G-actin concentration quantitatively. We have developed two new microscopic techniques based on the fluorescence decay after photoactivation (FDAP) time-lapse imaging of photoswitch-able Dronpa-labeled actin. These techniques, sequential FDAP (s-FDAP) and multipoint FDAP, were used to measure the time-dependent changes in and spatial distribution of the G-actin concentration in live cells. Use of s-FDAP provided data on changes in the G-actin concentration with high temporal resolution; these data were useful for the model analysis of actin assembly processes in live cells. The s-FDAP analysis also provided evidence that the cytoplasmic G-actin concentration substantially decreases after cell stimulation and that the extent of stimulus-induced actin assembly and cell size extension are linearly correlated with the G-actin concentration before cell stimulation. The advantages of using s-FDAP and multipoint FDAP to measure spatiotemporal G-actin dynamics and the roles of G-actin concentration and ADF/cofilin in stimulus-induced actin assembly and lamellipodium extension in live cells are discussed.

Actin filament dynamics and architecture in cells are highly organized in space and time. Cells integrate intracellular and extracellular signals to regulate the activities of various actin regulatory proteins and actin filament reorganization, which leads to cell migration, morphogenesis and division. To understand the mechanisms that spatiotemporally regulate actin filament dynamics and architecture in cells, various microscopic techniques have been developed. These techniques have provided quantitative data on the kinetics of actin filament dynamics that are useful for mathematical model analysis.[1,2] In particular, the technique of fluorescence single-molecule speckle imaging, which can be used to observe directly the behavior of single fluorescence-labeled molecules attached to actin filaments, has provided extensive kinetic data on the processes of actin filament assembly and disassembly in live cells.[2]

During migration, cells extend F-actin-rich lamellipodia at their leading edge. In lamellipodia, dendritic arrays of actin filaments are constructed with fast-growing barbed ends directed to the plasma membrane. Actin polymerization at the barbed end generates a physical force to push the plasma membrane forward, resulting in lamellipodium extension. Next, new focal adhesions are formed in the lamellipodium, and the actomyosin contraction force pulls the cell body forward. In principle, cells migrate by cycling through these steps. Actin polymerization-based lamellipodium protrusion is the first and most important step in cell migration. Lamellipodia sense environmental cues around the cell and determine the potency
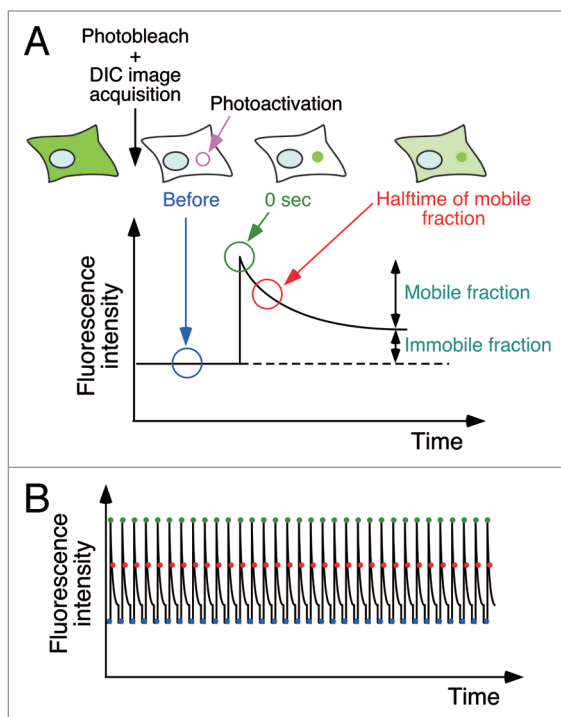
Figure 1. Outline of s-FDAP analysis.[9] (A) Time course of Dp-actin fluorescence decay after single photoactivation (FDAP). Dp-actin expressed in the cell was prebleached to the background level, and a DIC image was acquired. After photoactivation, time-lapse images were acquired. The fluorescence decay was fitted to calculate the mobile and immobile fractions and diffusion coefficient of Dp-actin.[9] (B) s-FDAP analysis. Fluorescence images were acquired before (blue) and at 0 (green) and 40 ms (red) after photoactivation. Each set of procedures was repeated 84 times at 10 sec intervals. FDAP values are expressed as the difference between the fluorescence intensities at 0 and 40 ms after photoactivation.

and directionality of cell migration by dynamically changing their morphology in response to these cues.[3,4]

Fluorescence single-molecule speckle imaging has provided many kinetic parameters of actin and actin-binding proteins on F-actin, which underlie the dynamic behavior of lamellipodia, including the rate of mDia (FH1-FH2)-mediated F-actin elongation (2 μm s^{-1}), the rates of dissociation of Arp2/3 (0.048 sec^{-1}) and capping protein (0.58 sec^{-1}) from F-actin, and the rate of F-actin disassembly (0.03 sec^{-1}) in lamellipodia.[5-7] Thus, substantial kinetic data on actin and actin-binding proteins in the processes from actin assembly to disassembly on F-actin have been accumulated. In contrast, kinetic data on G-actin and its binding proteins in the processes after actin disassembly and before actin assembly (which occur primarily in the cytosol) have remained insufficiently characterized, owing to a lack of appropriate methods to measure the changes in G-actin concentration quantitatively in live cells.

In most cells, the cytoplasmic G-actin concentration (~100 μM) is much higher than the critical concentration at the barbed end of an actin filament (~0.1 μM).[8] The high concentration of cytoplasmic G-actin is maintained by several factors: (1) the supply of actin monomers via actin-depolymerizing factor (ADF)/cofilin-mediated F-actin disassembly; (2) the prevention of spontaneous actin nucleation by G-actin-sequestering proteins, profilin and thymosin-β4; and (3) the interruption of actin assembly by F-actin-capping proteins. To evaluate the contribution of each actin regulatory protein to the maintenance of high cytoplasmic G-actin, it is essential to quantify the cellular G-actin concentration. To assess the cytoplasmic G-actin concentration in live cells, time-lapse analysis of fluorescence decay after photoactivation (FDAP) of Dronpa (Dp)-labeled actin was used (**Fig. 1A**).[9,10]

Dp is a GFP-like photochromic protein whose fluorescence can be reversibly and repeatedly photoactivated and photobleached by irradiation with light.[11] After photobleaching the whole cell, Dp-actin was photoactivated in a small region of the cytoplasm, and the time-dependent change in fluorescence intensity in the photoactivated region was measured (**Fig. 1A**). Fluorescence intensity in the photoactivated region decreased quickly due to the diffusion of Dp-labeled G-actin. Because the rate of the fluorescence decay of Dp-actin was much faster than the rate of F-actin disassembly, the FDAP value primarily reflects the amount of G-actin (mobile fraction of Dp-actin) in the local area of the cytoplasm. The contribution of F-actin disassembly to the FDAP value is negligible at least in the initial phase after photoactivation.

The fluorescence recovery after photobleaching (FRAP) of GFP-actin was also utilized to assess the local amount of G-actin.[12] However, FDAP analysis has an advantage over FRAP analysis for quantifying the amount of G-actin with high signal-to-noise ratio: the fluorescence signal in the region of interest (ROI) is maximal at the beginning of FDAP measurements but minimal at the beginning of FRAP measurements. The FDAP analysis revealed that the cytoplasmic G-actin concentration was decreased by the inactivation or depletion of ADF/cofilin to less than half of the concentration in control cells.[10] This result provides clear evidence that ADF and cofilin play a central role in the production and maintenance of high concentration of cytoplasmic G-actin.[10] A similar approach can be used to evaluate the contributions of other actin regulatory proteins to the control of cytoplasmic G-actin concentration.

In the cytoplasm, the ability of G-actin to polymerize to F-actin is controlled by its state of association with G-actin-binding proteins. Major components of the cytoplasmic G-actin pool are free ATP-G-actin, profilin-bound and thymosin-β4-bound ATP-G-actin, and ADF/cofilin-bound ADP-G-actin. Free ATP-G-actin and profilin-bound ATP-G-actin are polymerizable, whereas thymosin-β4-bound ATP-G-actin and ADF/cofilin-bound ADP-G-actin are
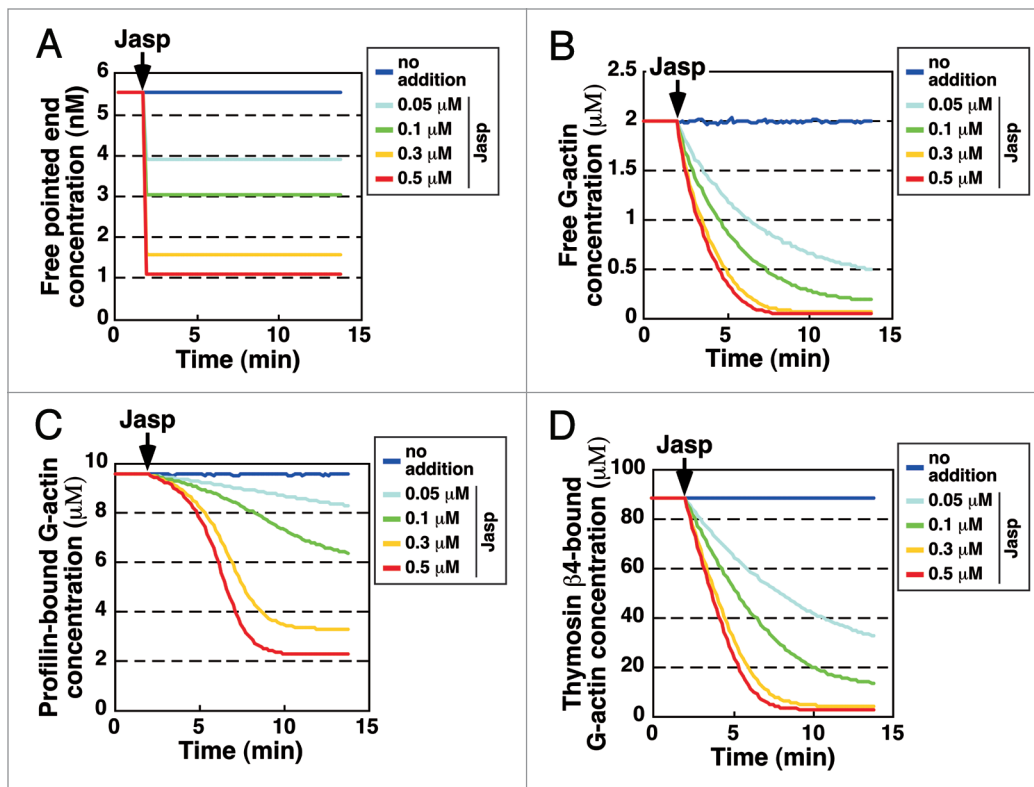
Figure 2. Model analysis of Jasp-induced changes in the concentrations of free pointed ends (A), free G-actin (B), profilin-bound G-actin (C) and thymosin-β4-bound G-actin (D). Temporal changes in FDAP values were measured after the treatment of MCF-7 cells with Jasp at various concentrations. Cellular concentrations of free G-actin, profilin-bound G-actin, thymosin-β4-bound G-actin, and the free barbed and pointed ends of F-actin were estimated by a differential equation model.[9] With these concentrations and the chemical reaction equations in reference 9, the time course of the concentration of each component was plotted by KINSIM. The free barbed end concentration is constant in the chemical reaction equations.

nonpolymerizable.[13-15] Although most cytoplasmic G-actin consists of nonpolymerizable thymosin-β4-bound ATP-G-actin, this complex is readily converted to free ATP-G-actin when the free G-actin concentration is reduced by an increase in the actin assembly rate or a decrease in the actin disassembly rate.[16] Thus, thymosin-β4-bound ATP-G-actin also contributes to actin assembly by acting as a reservoir of ATP-G-actin. Kinetic data on these biochemical reactions in actin dynamics have been extensively studied in the cell-free system.[14,16]

To investigate the mechanisms of intracellular reactions of G-actin dynamics, such as G-actin disassembly from F-actin, G-actin assembly to F-actin, and the interactions between G-actin and its regulating proteins, it is essential to measure time-dependent changes in cytoplasmic G-actin concentration in live cells quantitatively and with high temporal resolution. As described above, the amount of cytoplasmic G-actin can be assessed by

FDAP analysis of Dp-actin. Because Dp is a unique protein whose fluorescence can be reversibly and repeatedly switched on and off, Dp-actin is a useful probe for sequential measurements of FDAP. These characteristics allow the quantitative analysis of time-dependent changes in the G-actin concentration in live cells.[9]

Sequential FDAP (s-FDAP) analysis consists of repetitions of a set of procedures: differential interference contrast (DIC) image acquisition, photobleaching of the whole cell, and photoactivation of a small (1.8 μm diameter circle) region of the cytoplasm (**Fig. 1B**).[9] In each cycle, a set of three fluorescence images of the photoactivated region was acquired before and at 0 and 40 ms after photoactivation. The FDAP value, which reflects the G-actin concentration, was calculated as the difference between the fluorescence intensities at 0 and 40 ms after photoactivation. The time point for image acquisition (40 ms after photoactivation) was set on the basis of the half-life of the mobile

fraction of Dp-actin (41 ms) measured by a single FDAP analysis under similar conditions. The short-term measurement of FDAP enables s-FDAP analysis to measure temporal changes in the G-actin concentration with high temporal resolution. As described above, the effect of F-actin disassembly on s-FDAP analysis is negligible.[9,10]

Fluorescence correlation spectroscopy (FCS) has been developed to measure the cytoplasmic concentration of diffusive molecules.[17,18] FCS detects the number and diffusion time of fluorescence-labeled single molecules in the observed volume. Its autocorrelation function is calculated to determine the concentration and diffusion coefficient of the fluorescent molecule in the cytoplasm. However, FCS can only measure nanomolar concentrations of a fluorescent molecule; high concentrations of fluorescent molecules make it difficult to detect each fluorescent molecule separately in the observed volume.[18] Thus, FCS has an upper limit of the fluorescent signal

intensity and requires a relatively long measuring time (typically 10 sec x 5~10 times).[18] In addition, FCS is based on the premise that the concentration of fluorescent molecules does not change during the measuring time. Thus, FCS is unfavorable for measuring temporal changes in cytoplasmic G-actin concentration because the changes in G-actin concentration are very fast in cells and a high temporal resolution is required to measure them.[9]

The s-FDAP analysis was used to measure time-dependent changes in cytoplasmic G-actin concentration after the treatment of cells with an F-actin-stabilizing drug, jasplakinolide (Jasp).[9] The FDAP value gradually decreased after Jasp treatment in a time- and dose-dependent manner. The high temporal resolution of s-FDAP analysis was sufficient to use the data of Jasp-induced changes in G-actin concentration for model analysis. In the best-fit model, the cellular concentrations of free G-actin, profilin-bound and thymosin-β4-bound G-actin, and free barbed and pointed ends of actin filaments in the untreated cell were estimated to be 1.98 μM, 9.52 μM, 88.5 μM, 4.8 nM and 5.52 nM, respectively, assuming that the total concentrations of G-actin, profilin and thymosin-β4 were 100, 10 and 200 μM, respectively.[9] Based on these values, time courses of the concentrations of each component after Jasp treatment were estimated, as shown in **Figure 2**. Thus, the high temporal resolution and quantitative features of s-FDAP analysis make it useful for estimating the cellular concentrations of key elements involved in the regulation of actin dynamics.

The s-FDAP analysis was also applied to measure the temporal changes in G-actin concentration after cell stimulation with neuregulin (NRG), a growth factor that induces lamellipodium extension and cell migration.[9] Results from s-FDAP analysis demonstrated that the G-actin concentration decreased by ~40% at 2–4 min after NRG stimulation and then reached a plateau. This result indicates that ~40% of G-actin in the cytoplasm is polymerized to F-actin in response to NRG stimulation.[9]

The s-FDAP analysis can be used to measure temporal changes in cell morphology by DIC image acquisition, simultaneously with changes in G-actin concentration, in individual live cells. The DIC image analysis showed that the cell area increased about 1.6-fold at 6–8 min after NRG stimulation, with a lag time of 2–4 min after the decrease in cytoplasmic G-actin concentration. Data on the time-lapse fluorescence analyses of YFP-actin localization and measurements of free barbed-end formation in NRG-stimulated cells suggest that the cytoplasmic G-actin is used for F-actin assembly and Arp2/3 complex-mediated dendritic formation of barbed ends at the cell periphery in the initial phase after NRG stimulation, and thereafter the cell area begins to extend.[9]

Because the rate of actin polymerization is proportional to the G-actin concentration in cell-free assays,[19] it is reasonable to assume that the cytoplasmic G-actin concentration is a critical parameter for the intracellular rate of actin polymerization. However, some studies have paid little attention to the role of G-actin concentration in stimulus-induced actin assembly and lamellipodium extension, probably because the cytoplasmic G-actin is more than enough for actin assembly. Thus, it remains unclear to what extent the cytoplasmic G-actin concentration contributes to stimulus-induced actin assembly and cell extension.

To address this issue, the G-actin concentration in cells was artificially reduced by Jasp treatment, and its effects on NRG-induced actin assembly and cell size extension were analyzed by s-FDAP.[9] The extent of NRG-induced actin assembly (measured as the decrease in the cytoplasmic G-actin concentration) and cell size extension exhibited a linear correlation with the G-actin concentration in the prestimulated cell.[9] Strong correlations over a wide range of G-actin concentrations indicate that the extent of actin assembly and cell size extension after cell stimulation strongly depend on the cytoplasmic G-actin concentration before cell stimulation over a wide range of G-actin concentrations.

Inactivation or depletion of ADF/cofilin suppresses stimulus-induced actin assembly and lamellipodium extension, indicating that ADF/cofilin plays a critical role in these processes. In general, the rate of actin assembly depends on the concentrations of both G-actin and the free barbed ends of F-actin. Based on the potency of ADF/cofilin to increase both the G-actin concentration and free barbed ends, two models for the roles of ADF/cofilin have been proposed: ADF/cofilin contributes to stimulus-induced actin assembly and lamellipodium extension by (1) abundantly supplying G-actin to maintain a high concentration of cytoplasmic G-actin for polymerization,[9,10] or (2) severing F-actin to create free barbed ends that are used as nucleation sites for actin polymerization.[20,21] We showed that the (1) inactivation or depletion of ADF/cofilin remarkably decreases the cytoplasmic G-actin concentration, indicating that ADF/cofilin primarily contributes to the maintenance of high cytoplasmic concentrations of G-actin; (2) stimulus-induced actin assembly and cell extension strongly depend on the cytoplasmic G-actin concentration in the prestimulated cell; and (3) microinjection of actin monomers into ADF/cofilin-inactivated cells rescues actin assembly and cell extension.[9,10] These results suggest that ADF/cofilin contributes to stimulus-induced actin assembly and cell extension by supplying a large amount of G-actin into the cytoplasm, thereby maintaining a high concentration of cytoplasmic G-actin for polymerization. Although ADF/cofilin is required for stimulus-induced barbed-end formation at the cell periphery, it probably indirectly supports the creation of barbed ends by increasing the cytoplasmic G-actin concentration (which, in turn, accelerates the rate of actin polymerization and increases the Arp2/3 complex-mediated branching and barbed-end formation of actin filaments), rather than by directly creating barbed ends via their F-actin-severing activity.

The FDAP technique was also applied to measure the spatial distribution of G-actin concentration by sequentially performing single FDAP analyses at multiple points in a live cell.[9] Multipoint FDAP analysis of N1E-115 cells extending stable lamellipodia has shown that the G-actin concentration in lamellipodia is comparable to that in the cell body.[9] This finding indicates that the G-actin concentration in lamellipodia is sufficiently high to support F-actin polymerization at the leading edge. Time-lapse fluorescence imaging has

shown that G-actin disassembled from the rear of the lamellipodia rapidly diffuses into the cytoplasm, and that cytoplasmic G-actin is effectively incorporated into F-actin in the lamellipodia.[10] Thus, it is likely that the rapid diffusion of a large amount of cytoplasmic G-actin, which is much greater than the amount of G-actin generated by lamellipodial F-actin disassembly in a given period of time, equalizes the G-actin concentrations in the lamellipodia and cell body. It will be interesting to investigate whether or not the spatial distribution of G-actin concentration differs in various types of migrating or polarized cells.

In motile cells, the lamellipodium extends and retracts repeatedly.[22,23] In the spontaneous polarization of isotropically spreading fibroblasts and keratocytes, membrane retraction in the prospective rear of the cell was initiated prior to directional cell movement.[24,25] Actin assembly and disassembly are local biochemical reactions regulated by different reaction components. However, spatiotemporal linkage between these reactions suggests their coordination in the entire cell.

We have shown that the change in G-actin concentration in the cytoplasm is directly correlated with stimulus-induced actin assembly and cell extension.[9] Changes in the total G-actin concentration probably alter the biochemical reactions between G-actin and its regulatory proteins in the cytoplasm. In addition, the mDia1-mediated nucleation of actin filaments is presumably regulated by fluctuation in the G-actin concentration,[26] and the rate of G-actin incorporation at the tip of the lamellipodium is linearly correlated with the cytoplasmic G-actin concentration.[10] Thus, the quantitative analysis of the correlations between cytoplasmic G-actin concentration and kinetics of local actin assembly/disassembly will shed light on the molecular mechanisms by which motile cells coordinate local actin assembly and disassembly in the entire cell.

The s-FDAP and multipoint FDAP analyses are applicable to measure temporal and spatial changes in the concentrations of mobile components of various cytoskeletal and membrane-bound proteins in live cells. These techniques will serve to investigate the roles of these proteins in diverse cell processes, including cell migration, morphological changes and cytokinesis.

References

1. Dehmelt L, Bastiaens PI. Spatial organization of intracellular communication: insights from imaging. Nat Rev Mol Cell Biol 2010; 11:440-52; PMID:20485292; http://dx.doi.org/10.1038/nrm2903

2. Watanabe N. Inside view of cell locomotion through single-molecule: fast F-/G-actin cycle and G-actin regulation of polymer restoration. Proc Jpn Acad, Ser B, Phys Biol Sci 2010; 86:62-83; PMID:20075609; http://dx.doi.org/10.2183/pjab.86.62

3. Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. Cell 2003; 112:453-65; PMID:12600310; http://dx.doi.org/10.1016/S0092-8674(03)00120-X

4. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science 2003; 302:1704-9; PMID:14657486; http://dx.doi.org/10.1126/science.1092053

5. Higashida C, Miyoshi T, Fujita A, Oceguera-Yanez F, Monypenny J, Andou Y, et al. Actin polymerization-driven molecular movement of mDia1 in living cells. Science 2004; 303:2007-10; PMID:15044801; http://dx.doi.org/10.1126/science.1093923

6. Miyoshi T, Tsuji T, Higashida C, Hertzog M, Fujita A, Narumiya S, et al. Actin turnover-dependent fast dissociation of capping protein in the dendritic nucleation actin network: evidence of frequent filament severing. J Cell Biol 2006; 175:947-55; PMID:17178911; http://dx.doi.org/10.1083/jcb.200604176

7. Watanabe N, Mitchison TJ. Single-molecule speckle analysis of actin filament turnover in lamellipodia. Science 2002; 295:1083-6; PMID:11834838; http://dx.doi.org/10.1126/science.1067470

8. Pollard TD, Blanchoin L, Mullins RD. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu Rev Biophys Biomol Struct 2000; 29:545-76; PMID:10940259; http://dx.doi.org/10.1146/annurev.biophys.29.1.545

9. Kiuchi T, Nagai T, Ohashi K, Mizuno K. Measurements of spatiotemporal changes in G-actin concentration reveal its effect on stimulus-induced actin assembly and lamellipodium extension. J Cell Biol 2011; 193:365-80; PMID:21502360; http://dx.doi.org/10.1083/jcb.201101035

10. Kiuchi T, Ohashi K, Kurita S, Mizuno K. Cofilin promotes stimulus-induced lamellipodium formation by generating an abundant supply of actin monomers. J Cell Biol 2007; 177:465-76; PMID:17470633; http://dx.doi.org/10.1083/jcb.200610005

11. Ando R, Mizuno H, Miyawaki A. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. Science 2004; 306:1370-3; PMID:15550670; http://dx.doi.org/10.1126/science.1102506

12. McGrath JL, Tardy Y, Dewey CF, Meister JJ, Hartwig JH. Simultaneous measurements of actin filament turnover, filament fraction, and monomer diffusion in endothelial cells. Biophys J 1998; 75:2070-8; PMID:9746549; http://dx.doi.org/10.1016/S0006-3495(98)77649-0

13. Safer D, Nachmias VT. Beta thymosins as actin binding peptides. Bioessays 1994; 16:473-9; PMID:7945275; http://dx.doi.org/10.1002/bies.950160706

14. Kang F, Purich DL, Southwick FS. Profilin promotes barbed-end actin filament assembly without lowering the critical concentration. J Biol Chem 1999; 274:36963-72; PMID:10601251; http://dx.doi.org/10.1074/jbc.274.52.36963

15. Carlier MF, Pantaloni D. Control of actin assembly dynamics in cell motility. J Biol Chem 2007; 282:23005-9; PMID:17576764; http://dx.doi.org/10.1074/jbc.R700020200

16. Pantaloni D, Carlier MF. How profilin promotes actin filament assembly in the presence of thymosin-beta-4. Cell 1993; 75:1007-14; PMID:8252614; http://dx.doi.org/10.1016/0092-8674(93)90544-Z

17. Bacia K, Kim SA, Schwille P. Fluorescence cross-correlation spectroscopy in living cells. Nat Methods 2006; 3:83-9; PMID:16432516; http://dx.doi.org/10.1038/nmeth822

18. Kim SA, Heinze KG, Schwille P. Fluorescence correlation spectroscopy in living cells. Nat Methods 2007; 4:963-73; PMID:17971781; http://dx.doi.org/10.1038/nmeth1104

19. Pollard TD. Rate constants for the reactions of ATP-actin and ADP-actin with the ends of actin-filaments. J Cell Biol 1986; 103:2747-54; PMID:3793756; http://dx.doi.org/10.1083/jcb.103.6.2747

20. Chan AY, Bailly M, Zebda N, Segall JE, Condeelis JS. Role of cofilin in epidermal growth factor–stimulated actin polymerization and lamellipod protrusion. J Cell Biol 2000; 148:531-42; PMID:10662778; http://dx.doi.org/10.1083/jcb.148.3.531

21. Zebda N, Bernard O, Bailly M, Welti S, Lawrence DS, Condeelis JS. Phosphorylation of ADF/cofilin abolishes EGF-induced actin nucleation at the leading edge and subsequent lamellipod extension. J Cell Biol 2000; 151:1119-28; PMID:11086013; http://dx.doi.org/10.1083/jcb.151.5.1119

22. Giannone G, Dubin-Thaler BJ, Döbereiner HG, Kieffer N, Bresnick AR, Sheetz MP. Periodic lamellipodial contractions correlate with rearward actin waves. Cell 2004; 116:431-43; PMID:15016377; http://dx.doi.org/10.1016/S0092-8674(04)00058-3

23. Ohashi K, Fujiwara S, Watanabe T, Kondo H, Kiuchi T, Mizuno K. LIM-kinase has a dual role in regulating lamellipodium extension by decelerating the rate of actin retrograde flow and the rate of actin polymerization. J Biol Chem 2011; 286:36340-51; PMID:21868383; http://dx.doi.org/10.1074/jbc.M111.259135

24. Yam PT, Wilson CA, Ji L, Hebert B, Barnhart EL, Dye NA, et al. Actin-myosin network reorganization breaks symmetry at the cell rear to spontaneously initiate polarized cell motility. J Cell Biol 2007; 178:1207-21; PMID:17893245; http://dx.doi.org/10.1083/jcb.200706012

25. Mseka T, Bamburg JR, Cramer LP. ADF/cofilin family proteins control formation of oriented actin-filament bundles in the cell body to trigger fibroblast polarization. J Cell Sci 2007; 120:4332-44; PMID:18042624; http://dx.doi.org/10.1242/jcs.017640

26. Higashida C, Suetsugu S, Tsuji T, Monypenny J, Narumiya S, Watanabe N. G-actin regulates rapid induction of actin nucleation by mDia1 to restore cellular actin polymers. J Cell Sci 2008; 121:3403-12; PMID:18827014; http://dx.doi.org/10.1242/jcs.030940