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Differentially oriented populations of actin filaments generated in lamellipodia collaborate in pushing and pausing at the cell front

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Eukaryotic cells advance in phases of protrusion, pause and withdrawal¹. Protrusion occurs in lamellipodia composed of diagonal networks of actin filaments and withdrawal terminates with the formation of actin bundles parallel to the cell edge. Using correlated live cell imaging and electron microscopy we show that actin filaments in protruding lamellipodia subtend angles from 15-90 deg to the front and that transitions from protrusion to pause are associated with a proportional increase in filaments oriented more parallel to the cell edge. Microspike bundles of actin filaments also show a wide angular distribution and correspondingly variable bilateral polymerisation rates along the cell front. We propose that the angular shift of filaments in lamellipodia serves in adapting to slower protrusion rates while maintaining the filament densities required for structural support; further, that single filaments and microspike bundles contribute to the construction of the lamella behind and to the formation of the cell edge when protrusion ceases. Our findings suggest an explanation for the variable turnover dynamics of actin filaments in lamellipodia observed by fluorescence speckle microscopy² and are inconsistent with a current model of lamellipodia structure that features actin filaments branching at 70deg in a dendritic array³.

Migrating cells exploit two properties of actin filaments to move: the property to polymerise and push, to effect protrusion and the ability to slide with myosin II, to drive retraction. Protrusion is effected by lamellipodia^{1,4}, thin sheets of cytoplasm composed of networks of actin filaments that have their fast growing, plus ends abutting the leading membrane⁵. Current ideas of how protruding lamellipodia are organized have come mainly from electron microscopy of cells that show constant motility, in particular the epidermal keratocyte^{3,6}. And from images obtained using a critical point drying procedure for specimen preparation, a model of lamellipodium organization has been proposed that features a dendritic network of actin filaments with the Arp2/3 complex situated at 70degree branch points^{3,7,8}.

In migrating cells lamellipodia not only protrude, they undergo phases of protrusion, pause and withdrawal, the latter often associated with ruffling¹. Filopodia and related bundles embedded in the lamellipodia mesh, also referred to as microspikes⁴, contribute to these activities. To gain insight into the structural basis of changes in protrusive activity we have developed procedures for correlating the local movements of lamellipodia, monitored by live cell imaging, with their organization after negative stain electron microscopy. Results using this approach reveal filament arrangements and rearrangements in lamellipodia that are difficult to reconcile with the dendritic model. At the same time they show how filament remodeling in lamellipodia can contribute to construction of stationary cell edges and to the assembly of the cytoskeleton of the lamella region⁹ behind the lamellipodium.

Fig.1 shows the final frames of a video sequence (supplementary video, S1) of a fast and steadily protruding B16 melanoma cell expressing GFP-actin and mCherry-VASP, before and after fixation on the light microscope (Fig. 1a,b) and an overview of the cell in the electron microscope (Fig. 1d). VASP is a useful indicator of protrusion, since the intensity of lamellipodia tip labeling is proportional to the protrusion rate¹⁰. The time between the final video frame and the fixation event was around 3sec. Scans of the GFP-intensity across the lamellipodium (Fig. 1b, inset) in the main protruding zone (box in Fig. 1b) demonstrated that the gradient of actin fluorescence in the lamellipodium of the living cell was preserved by the fixation process. Frames of the video sequence in the boxed area in Fig. 1b are shown in Fig. 1c (upper panels) together with the velocity profile in this position (Fig. 1c, bottom panel). The mean protrusion rate over the terminal 60 sec was 3.5 $\mu\text{m}/\text{min}$. Electron micrographs of the same region close to and 5 μm behind the lamellipodium front (small, boxed regions in Fig. 1d) are shown in Figs. 2a and b (for overview, see supplementary Fig. S2). The number of filaments crossing a 1 μm line drawn 0.1 μm behind the cell front in this advancing lamellipodium averaged 90 (+/-10). Particularly noteworthy was the wide angular distribution of filaments, from 15-90degrees, with respect to the cell edge (left inset, Fig. 2a). A region 5 μm behind the front of the same lamellipodium is shown in Fig. 2b (small box inset, Fig. 1d and Supplementary Fig. S2); here the filament density was lower (59 +/-15 fils/ μm). In addition, there was an increase in the proportion of filaments at low angles to the cell front (inset Fig. 2b). The gradient of filament density across the lamellipodium back to 5 μm from the front correlated with the gradient of actin-GFP intensity in the same position of the living and fixed cell (Fig. 2a, right inset) and revealed a drop in filament number of 11% over the first micron.

Fig. 3 (supplementary video S3) shows a cell for which three regions of different protrusive activity are highlighted (Fig. 3b), with enlarged video frames in Figs. 3c-e. At position c, the protrusion rate (around 2 $\mu\text{m}/\text{min}$) and VASP intensity were more or less constant over the terminal two minutes (middle panel, Fig. 3c). Fig. 3f shows the organization of actin filaments at the front of the lamellipodium in the

same region of the cell. The histogram (lower panel, Fig 3c) shows the angular distribution of filaments in a region $0.2\mu\text{m}$ from the cell front; note again the wide distribution of angles down to 15° as well as the presence in Fig. 3f of a small population of filaments subtending angles below 20° to the cell edge, that also extend up to the tip of the lamellipodium (arrow). The cell in Fig 3 moved throughout the video sequence without a significant change in overall shape. As a result, the radial protrusion rate steadily decreased from the advancing shoulders out to the lateral flanks. In position d, the rate of protrusion just prior to fixation was $1\mu\text{m}/\text{min}$ and the VASP label steadily declined over the terminal two minutes (middle panel, Fig. 3d). In this position a large proportion of filaments were oriented at lower angles to the cell edge (Fig. 4a, see also histogram Fig.3d) and could be seen to originate from foci at the lamellipodium front. The same reorganization of filaments from the peak towards the flank was seen in equivalent positions of the lamellipodium on the opposite half of the cell (not shown). At the extreme lateral flank of the same cell (region e, Fig. 3,b, e) there was no net protrusion, but small fluctuations of the cell edge that ended in a minor retraction and complete loss of VASP label (Fig. 3e). Electron microscopy (Fig. 4b) showed that the lamellipodium at this position was around $0.5\mu\text{m}$ wide and contained a major component of filaments oriented parallel to the cell edge, superimposed on a narrow band ($0.2\mu\text{m}$ wide) of divergent filament arrays characteristic of early protrusions.

Analysis of MTLn3 carcinoma cells that migrate actively in vitro and in vivo¹¹ revealed essentially the same changes in filament organization between regions of protrusion and pause; namely, a shift in orientation of filaments to lower angles (some to below 15°) and in addition, to the appearance in pausing zones of a noticeable proportion of filaments with curved trajectories (supplementary Fig. S4). Slowing and pause was associated with a reduction in filament numbers at the front, albeit to variable extents; in position c and d of the B 16 melanoma cell in Fig 3 by 30% (inset, Fig.3f) and in the MtlN3 cell (supplementary Fig. S4), by 12%.

According to their varied orientation to the cell front, actin filament tips must move laterally along the cell edge as they grow¹². This lateral flow was reflected in the movement of microspike bundles, commonly embedded in protruding lamellipodia of B16 melanoma cells (supplementary Fig. S5a-g). The angle that such bundles subtended with the cell front was variable, ranging from 90° to below 10° (Fig. S5h), in the same range observed for individual filaments. In addition, the proportion of microspikes at lower angles was higher in slowing and pausing lamellipodia as compared to continuously protruding ones (Fig. S5h). A direct consequence of this angular distribution was a varied velocity of lateral movement of microspike tips along the cell edge and a variation in length of the bundles. Using photoactivatable GFP (Fig. S5e) we could show that the polymerization rate in microspikes increased up to $15\mu\text{m}/\text{min}$ for those oriented at around 15° to the cell edge (Fig. S5i). Microspike bundles moved laterally in opposite directions and could be observed to cross each other (Fig S5b) or to fuse. One consequence of these bi-lateral movements was the generation of antiparallel arrays of actin filaments that formed bundles at the base of the lamellipodium, from where they entered the lamella and accumulated myosin (Fig. S5f). Other microspikes contributed directly to the formation of retracting edges (Fig. S5a). While the lateral flow of microspikes could lead to their integration into the lamella with myosin, the lateral flow was not dependent on myosin II, as shown by its persistence in blebbistatin (Fig. S5g). In the short term (within 2-10 mins), blebbistatin ($50\text{-}100\mu\text{M}$) induced an increase in the rate of lamellipodia protrusion by a factor of 1.5 to 3 and an

increase in lamellipodium width (Figs. S5j and k), analogous to observations in immobilized *Aplysia* growth cones¹³.

The negative staining method, as employed here and in earlier studies (reviewed in 14) has the advantage that the linearity of filaments within the delicate meshwork of the lamellipodium is maintained and the dorsal and ventral arrays of actin are included in the electron microscope images. For the first time, we are thus able to relate the angular distributions of filaments and filament densities to the history of protrusion. Our direct measurements indicate that around 100 filaments per micron are utilized during protrusion. This value is in the order of half that estimated indirectly by comparison of the fluorescence intensity of phalloidin labeled single actin filaments with phalloidin labeled lamellipodia of fixed 3T3 fibroblasts¹⁵. From the present and earlier studies it is clear that the filament density in lamellipodia does not determine the protrusion rate, since it differs little between keratocytes that can move at 15 $\mu\text{m}/\text{min}$ ⁶ and B16 melanoma and MTLn3 adenocarcinoma cells protruding 5 times slower. Correspondingly, we found that slowing of the cell edge was not accompanied by a significant decrease in filament density. Instead, there was a consistent increase in the number of filaments oriented at a low angle to the cell edge. The wide angular distribution of filaments, all with their plus ends forward, indicate that there is a spread of polymerization rates, with filaments at low angles growing the fastest, to keep up with the front. The factors determining this variation in rate are unknown, but may be influenced by a type of force dependent feedback mechanism¹⁶, due to progressively lower resistance to filament growth at lower angles. We suggest that the transition from protrusion to pause is signaled by the net, local down-regulation of actin plus end polymerization complexes, but with some polymerization complexes being down regulated more than others. In this scenario, actin filaments polymerizing slowest act as a brake on protrusion through tethering with the leading membrane, analogous to the tethering of actin filaments to beads in mimetic models of cell motility^{17,18}. Other, faster growing filaments must reorient their trajectory relative to the front edge to grow, resulting in an increase in the population of filaments at lower angles (supplementary video S6). If pause persists, more filaments become parallel to the cell front; some may also detach and move backwards with retrograde flow to the base of the lamellipodium, as is in fact observed with microspike bundles (Fig. S5c and ref. 19).

The advance of the lamellipodium depends on a balance between the rate of actin polymerization and the rate of retrograde flow. Recent findings on immobilized *Aplysia* growth cones indicate that a component of retrograde flow is driven by myosin II activity in the lamella¹³, consistent with our own observation of an increase in lamellipodia protrusion rate in B16 cells in blebbistatin. Pausing in lamellipodia may therefore be explained by a reduction in net forward polymerization rate at filament plus ends to the rate component of retrograde flow contributed by myosin-actin interactions at the base of the lamellipodium. An analogous situation apparently pertains in the fish keratocyte lamellipodium, for which protrusion is maximal at the front and reduces to zero at the flanks. At the front there is minimal retrograde flow²⁰ whereas at the flanks retrograde flow is maximal, and explained as the combined contributions of actin polymerization at the membrane and the myosin-dependent withdrawal of filaments into the cell body²⁰.

According to the present results we propose a simple model in which different ramifications of filament reorganizations in the lamellipodium lead to the contribution of anti-parallel filament arrays to the lamella, including arcs^{9,12,21} during protrusion

(Fig. 5a) and to the cell edge during retraction (Fig 5b); in both cases filament arrays are produced to allow the formation of contractile assemblies with myosin. In this scheme, there is a gradation of filament lengths in lamellipodia according to angular distribution and the drop in actin filament density away from the front. From elementary geometric considerations, filaments oriented at 15 deg to the cell edge that extend to the rear of the lamellipodium will be 3 times longer than those oriented at 55deg. For a lamellipodium of 3 μm in width, this would correspond to maximum filament lengths of 11.6 μm and 3.7 μm respectively (disregarding those that extend further into the lamella). During persistent pause, depolymerization from filament minus ends will eliminate shorter filaments at higher angles to the cell edge first, leaving the longer filaments shorter, but still long enough to form a parallel bundle. We assume that the association of these latter filaments with tropomyosin protect them from further depolymerization²² and promote their interaction with myosin to consolidate the cell edge. Rather than being static, filaments that enter the lamella continue to turnover (Fig. S5e; ref.2), so that their lamellipodia precursors can be viewed as seeds of the lamella cytoskeleton.

The currently popular dendritic network model of lamellipodium protrusion^{7,8,23} features 70deg Y junctions within 20-50nm of each other in the actin network³ and branched filament segments behind the cell front that are capped at their plus ends. The model derives from analysis of electron micrographs of cytoskeletons prepared by the critical point drying method³ and extrapolation of data showing the ability of the Arp2/3-complex to induce the branching of actin filaments in vitro^{24, 25}. Definitive proof of whether or not branches exist at all in lamellipodia will need to come from cryo-electron tomography, to waylay any arguments about artifacts induced by specimen preparation²⁶ and three-dimensional organization. Medalia and colleagues²⁷ have already shown the feasibility of visualizing actin filaments in vitreously frozen *Dictyostelium* amoebae and the challenge now is to combine this methodology with information about the motile activity of the imaged regions. Nevertheless, we already show that there is no regular angle of 70deg between filaments at the front of a protruding cell edge. We also find no evidence of branches of actin filaments, using a stronger primary fixation than employed by Svitkina and Borisy³.

From analysis of actin filament turnover by fluorescence speckle microscopy^{2, 28} and lamellipodia spreading dynamics²⁹ it has been concluded that the lamellipodium surfs on top of the lamella underneath. Our results indicate however that all filaments in the lamellipodium originate from nucleation centres at the tip, with no superposition of the lamellipodium network on another array beneath; the lamellipodium and lamella are spatially and structurally distinct, although coupled at their boundaries⁹ where myosin engagement begins. At the same time, we provide an alternative explanation for the different populations of actin speckles observed in lamellipodia². The longest-lived speckles can be explained as belonging to the long filaments that extend to the base of the lamellipodium and the short-lived speckles to those that terminate within the lamellipodium network. The possibility exists that a variable rate of treadmilling of individual filaments and bundles, at different angles, also contributes to the variability in retrograde flow rate. This idea could be tested by electron microscopy in conjunction with speckle microscopy² or spatiotemporal image correlation microscopy³⁰.

In conclusion, correlated live cell imaging and electron microscopy has revealed a shift in the angular distribution of filaments in lamellipodia according to protrusive activity. These findings shed new light on the way actin is utilized to drive cell motility and also prompts a re-evaluation of current ideas about actin filament organization in lamellipodia.

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Competing financial interests

The authors declare no competing financial interests.

Materials and Methods

1. Correlated light and electron microscopy

The technique used for correlated light and electron microscopy is described in detail elsewhere¹. In brief, the procedure was as follows. Formvar films cast on a glass slide were floated onto a water surface and coverslips (22x30mm) placed onto the film, retrieved on parafilm and dried. A grid pattern was then embossed on the film by evaporation of gold through tailor made masks placed onto the coverslips. The transfected cells were plated onto the coverslips following coating with laminin (B16) or fibronectin (MTLn3). After video microscopy and fixation, the coverslips were transferred to a 9cm Petri dish filled with cytoskeleton buffer and the film gently peeled off the coverslip with forceps. At this stage the film was inverted and brought to the buffer surface to spread out under surface tension. Under a dissecting microscope, the film was floated onto a stainless steel ring platform and liquid removed until the film was immobilized, with the grid pattern centred. An EM grid (50 mesh hexagonal, copper) was then placed onto the film with the central hole over the region containing the cell of interest. For this manipulation the grid was mounted in forceps held in a modified Leica dual pipette holder, to allow controlled release, with the pipette holder mounted on a Narashige micromanipulator. The film was then floated off the stand by addition of buffer, recovered with a piece of parafilm, rinsed with negative stain solution and dried on the cell side. The negative stain was composed of a mixture of sodium silicotungstate (2%; Agar Scientific) and aurothioglucose (1%; Wako Chemicals, Germany), pH 7.0. Cells were observed and imaged immediately in the electron microscope (FEI Morgagni).

2. Cells, constructs and transfection

B16-F1 mouse melanoma cells were maintained as described² and transiently transfected using Fugene 6 (Roche) according to manufacturers' instructions with pEGFP- β -actin (BD Biosciences), actin fused to Ruby, a monomeric RFP variant³, a mixture of pEGFP-actin and mCherry-VASP, or a mixture of mCherry-actin and photoactivatable GFP-actin. mCherry-VASP was generated by exchanging EGFP in EGFP-VASP⁴ for mCherry⁵, an improved version of mRFP, kindly provided by Roger Tsien. Photoactivatable GFP (PA-GFP) - actin was made by exchanging EGFP in pEGFP- β -actin (Clontech) for PA-GFP, kindly provided by George Patterson and Jennifer Lippincott-Schwartz. mCherry-actin was kindly provided by Malgorzata Szczodrak. The myosin light chain GFP construct and Ruby construct were kindly provided by Rex Chisholm and Annette Muller-Taubenberger, respectively. Carcinoma MTLn3 cells were kindly provided by Jeff Segall (New York) and Bob van de Water (Leiden) and maintained in alpha-MEM containing ribonucleosides and deoxyribonucleosides, 5% FBS (Sigma) and penicillin/streptomycin. Transfection was performed using Fugene HD (Roche).

3. Live cell imaging and fixation

For light microscopy, the film-coverslip combination carrying the cells was mounted in a plexiglass, home made, flow through chamber that fitted on a temperature controlled heating platform (Harvard Instruments). The chamber (40x20mm x 8mm) featured two syringe needles glued into each end that connected to a central channel 0.5 mm deep between the filmed coverslip on the base and an upper, round coverslip glued to a central depression in the chamber.

Imaging was performed on a Zeiss Axiovert 200M inverted microscope equipped with a rear illuminated, cooled CCD camera (Micromax or Cascade, Roper) together with a filter wheel and shutters controlled with Metamorph software. Halogen lamps were used for imaging in both the phase contrast and fluorescence channels. Images were collected in one or two fluorescent channels and in phase contrast, with a time between frames between 8 and 15s (see legends).

Cells were fixed at the end of a selected video sequence by sucking the fixative/detergent mixture through the chamber. For B16 cells, the composition of the mixture was: 0.5% Triton, 0.25% glutaraldehyde in a cytoskeleton buffer (CB: 10mM MES, 150mM NaCl, 5mM EGTA, 5mM glucose, 5mM MgCl₂; pH 6.1), with added 1 μ g/ml phalloidin. For MTLn3 cells the procedure was the same but with a different ratio of Triton and glutaraldehyde: namely 0.25% Triton and 1% glutaraldehyde. An initial fixation of 2 mins in this mixture was followed by a post fixation in 2% glutaraldehyde (in CB plus 1 μ g/ml phalloidin) for 5-10 mins. Final fluorescence images of the selected cell were recorded during the initial fixation period. The coverslip was removed from the chamber and stored in cytoskeleton buffer containing 2% glutaraldehyde and 10 μ g/ml phalloidin at 4degC until processing for electron microscopy.

For acquisition of the images displayed in Fig. S5a-d, cells were maintained in an open heating chamber (Warner Instruments) at 37°C on an inverted microscope (Axiovert 100TV, Zeiss) equipped with a rear illuminated CCD camera (TE/CCD-1000 TKB, Princeton Instruments) driven by IPLab software (Scanalytics Inc.). Images were collected with a time between frames of 12.5 sec (a) or 18 sec (b-d).

Experiments with photoactivation of fluorescence were performed on cells co-transfected with photoactivatable-GFP-actin and mCherry-actin using a Zeiss LSM510 laser scanning confocal microscope equipped with Argon and Helium-Neon lasers for fluorescence observation and a diode-UV laser for photoactivation.

4. Image analysis and processing

Electron micrographs were processed in ImageJ using a bandpass filter to equalize the staining density and enhance filament contrast. For filament counts and angle measurements a line of 1 μ m or 0.5 μ m (MTLn3 cells) was drawn parallel to the cell edge using Adobe Photoshop: filaments crossing this line were overlaid with short lines and the angles between these lines and the cell edge measured in Photoshop.

Fluorescence intensities were measured with the linescan tool of Metamorph (Molecular Devices). For velocity measurements sequential GFP-actin images were processed with the detect edges filter in Metamorph. The position of the peak value of a line scan across the lamellipodium was used to define the position of the cell edge and the velocity was calculated from one frame to the next. Regression curves were drawn with Microsoft Excel.

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Figures:

Fig.1. Arrest of a steadily protruding lamellipodium and preservation of actin gradient. **a.** Final frame (GFP channel) of a video sequence of a B16 melanoma cell expressing GFP-actin and mCherry-VASP. **b.** Actin-GFP image of cell fixed within 3sec after last video frame (a); inset in b shows intensity scans [I (Actin)] across the lamellipodium (region boxed in b) before (green line) and after fixation (red line). **c.** Upper panels: video sequence leading to fixation of region boxed in b (green, GFP-actin; red, mCherry-VASP). Lower panel: protrusion rate over the terminal period (v , $\mu\text{m}/\text{min}$; blue) and the relative mCherry-VASP intensity at the front edge of the lamellipodium [I(VASP); red]. **d.** Overview electron micrograph of cell after negative staining. See also supplementary video S1. Bars: a,b,d, 10 μm ; c, 3 μm .

Fig. 2. Actin filaments are variably orientated in protruding lamellipodia. **a.** Electron micrograph of front region of the lamellipodium shown in Fig. 1b-d (peripheral zone boxed in Fig. 1d and supplementary figure S2). Histogram shows angular distribution of filaments crossing a 1 μm long line drawn parallel to and 100nm behind the front edge of the lamellipodium (average of 5 adjacent regions; total of 451 filaments). Filaments diverge at variable angles from foci at the cell front. Arrows indicate some examples of filaments at low angles. **b.** region in same lamellipodium, 5 μm behind the cell edge (inner region boxed in Fig. 1d and supplementary figure, S2); histogram shows angular distribution of filaments crossing a 1 μm line parallel to the cell front (average of 4 adjacent regions 5 μm from front edge). Note decrease in filament density and increase in number of filaments at lower angles to cell edge. Inset right in **a**: comparison of the fluorescence signal across the lamellipodium (Fig. 1b), with the actin filament density (per 1 μm of lamellipodium width) determined from electron micrographs taken from the region including Figs. 2a and b. The filament counts at 200nm from the front were normalized to the peak of the fluorescence scan of the fixed cell (red). The fluorescence traces correspond to the signal between the dotted lines shown in the inset. Bars, 200 nm.

Fig. 3. Actin filaments reorganize during the transition from protrusion to retraction.

a, b. Overview electron micrograph (a) and terminal video frame (b) of a B16 melanoma cell transfected with GFP-actin and mCherry-VASP (see supplementary video S3). Inset in **a**: intensity scans of GFP-actin fluorescence across the lamellipodium in a protruding region before (green) and after fixation (red) showing retention of actin density gradient. **c, d, e:** Upper panels, terminal video sequences of boxed regions c, d and e marked in b (green, GFP-actin; red, mCherry-VASP); Middle panels, protrusion rate (v , $\mu\text{m}/\text{min}$, blue) and relative VASP intensity at lamellipodium front [I (VASP), red] as a function of time (t , sec); Lower panels, Histograms of the angular distribution of filaments relative to the cell front for the corresponding electron micrographs (Fig 3f and Fig. 4,b) measured at 200nm from cell edge across a 1 μm line. “n” corresponds to the number of filaments measured. **f.** Electron micrograph of front region of protruding lamellipodium in boxed

region c. Note partial grouping of filaments oriented at high angles to the front (arrowhead), as well as a subpopulation of individual filaments at shallow angles (arrow; see also histogram in c). Inset: comparison of filament counts back to 1 μm in this part of the lamellipodium (blue) with the pausing region shown in Fig. 4a (green). Bars: a, 5 μm ; c-e, 2 μm ; f, 200 nm.

Fig. 4. Slowing and pause is associated with an increase in the number of filaments at shallow angles to the lamellipodium front.

a. Electron micrograph of region d in Fig. 3, where the protrusion rate had slowed down to 1 $\mu\text{m}/\text{min}$ at the point of fixation (middle panel, Fig. 3d). Note prevalence of filaments at low angles to the cell edge (see also histogram: Fig. 3d, lower panel) and that these filaments originate from foci at the lamellipodium front. **b.** Electron micrograph of region e in Fig. 3, on the lateral flank of the cell where protrusion had ceased (middle panel, Fig. 3e). Note dominance of filaments parallel to the cell edge (see also histogram, bottom panel, Fig. 3e). Bars, 200 nm.

Fig. 5. Filament reorganizations during protrusion, pause and retraction.

a. Proposed scenarios of actin filament and microspike reorganizations within the lamellipodium and their contribution to the lamella. Lp, lamellipodium, containing actin (A) and associated proteins: La, lamella, containing actin, associated proteins and myosin (A+M). Myosin filaments are depicted by orange-filled bars. The variable orientation of individual filaments (black lines) and filament bundles (microspikes; thick, grey lines) results in a variable, bi-lateral component of polymerization of filament plus ends along the front edge of the lamellipodium. Scenarios: 1, marks the two ends of a long microspike bundle that contributes actin filaments directly to the lamella; 2, a microspike translating to the right is arrested by a more radial microspike-it dissociates from the cell edge and moves with retrograde flow into the lamella; 3, a microspike bundle can lead a ruffle that propagates laterally along the lamellipodium; 4, a ruffle folding rearwards from the front can generate bundles at the base of the lamellipodium. Myosin engages with the subpopulation of anti-parallel actin filaments generated through bi-lateral flow, that survives the depolymerization activity of cofilin and other factors present in the lamellipodium. This filament population contributes to the construction of the lamella.

b. Scheme of transitions from protrusion to retraction. During persistent protrusion, the orientation of filaments is distributed preferably around higher angles to the cell front, with predominantly linear filaments. Slowing and pause is associated with a variable local reduction in actin polymerization rate at filament plus ends and with depolymerization from the rear. The slowest growing filaments retard protrusion and faster growing filaments turn more laterally, adopting curved trajectories. As pause persists, the longer filaments at low angles increase in proportion due to the preferential loss of the shorter filaments at high angles by depolymerization. Retraction is associated with the recruitment of myosin into the peripheral actin bundle and through linkage of actin filaments into contractile assemblies of the lamella network. The contractile bundle is anchored at both ends into adhesion foci that developed

during retraction (not shown). Microspikes also contribute filaments directly to retracting edges (Fig. S5a).

Supplementary figures

S1. Video Fig.1, including actin-GFP image after fixation. Time between frames was 8s.

S2. Electron micrograph overview of the region corresponding to Fig1c, with the positions of Figs 2a and b boxed.

S3. Video Fig.3. Time between frames was 8s.

S4. Transition from protrusion to pause in MTLn3 cell lamellipodia.

a, video frame, prior to fixation, of an MTLn3 cell transfected with EGFP-actin. **b**, overview of the fixed and stained cell in the electron microscope. **c**, region of the cell boxed in **a**, with the last video frame before fixation (red) overlaid with the frame 30sec before (green) to show the relative degrees of protrusion along the lamellipodium segment. **d** and **e** show the velocity traces of the cell edge for the corresponding positions marked in “c” by the white rectangles. **f** and **g**, electron micrographs of the positions shown in the overview **b** and corresponding to the cell edge positions **d** and **e** in **c**. Histograms in **f** and **g** show the angular distribution of filaments measured at 200nm from the cell edge. Additional inset in **f** shows the filament density as a function of the distance from the cell front for positions corresponding to **f** (dark blue) and **g** (light blue). For position **f**, counts were made along 0.5 μ m lines drawn parallel to the cell edge, to avoid overlap with adjacent zones, and the value doubled. For position **g**, counts were made along 1 μ m lines at the given distances from the front. The anomalous increase in filament number behind the protruding front in **f** was attributed to a contribution of filaments from an adjacent microspike bundle. Bars, **a,b**, 5 μ m; **c**, 3 μ m; **f,g**, 200nm.

S5. Bi-lateral flow of microspike bundles along the lamellipodium results in the contribution of bipolar arrays of actin filaments to the lamella.

a. Video sequence of a lamellipodium segment of a B16 melanoma cell transfected with actin-GFP showing contribution of a laterally translating microspike to the construction of a retracting edge that forms in the last frame (arrow). This and the following sequences (**b-d**) are shown in negative contrast. All times are in secs. Bar, 4 μ m.

b-d, selected frames of video sequences of the same region of a B16 melanoma cell expressing Ruby-actin. **b** shows two pairs of microspike bundles (numbered 1-4) crossing during the sequence (at 18 and 36sec). **c**, the tip of a laterally translating, long microspike (arrow) is stopped by a small radial microspike at 36sec (arrowhead), leading to dissociation of the long microspike from the lamellipodium tip and retrograde flow of the remaining bundle to the base. **d**, generation of an actin bundle parallel to the base of the lamellipodium (144sec) from a ruffle (**v**) that folds rearwards from the front (36-108sec), without a microspike. Bars, 2 μ m.

e. Lamellipodium segment of a B16 melanoma cell transfected with mCherry-actin and photoactivatable GFP-actin. The PA-GFP was photoactivated at 4s. Note contribution of microspike bundles to the lamella as well as the continued turnover of actin. The time between frames was 8sec. Bar, 3 μ m.

f. Engagement of myosin at base of lamellipodium. Selected video frames of B16 melanoma cell expressing mCherry-actin and GFP-myosin regulatory light chain. Note again bi-lateral translation of microspike bundles and their

contribution to bundles at the base of the lamellipodium, where myosin is recruited. Bar, 3 μm .

g. Myosin is not required for the lateral translation of microspikes. Frames of a video sequence of a B16 melanoma cell expressing mCherry-actin that was treated with 50 μM blebbistatin. Numbers indicate translating microspike; time in seconds; bar, 3 μm .

h,i. Angular distribution and growth rate of microspikes in B16 melanoma cells. **h**, comparison of microspike angles (to the cell edge) in pausing or slowing cells (purple bars) as compared to fast moving cells (blue bars). 14 microspikes each measured in 5 slow cells and 3 fast cells. **i**, distribution of actin polymerization rates in microspikes measured using photoactivatable GFP-actin in cells co-transfected with mCherry-actin (as for e). The rate was determined from the extension of microspikes from the point of photoactivation.

j,k. Data from 4 cells on the effect of 50-100 μM blebbistatin (BS) on lamellipodium width and protrusion rate in B16 melanoma cells. Within 2-10mins of blebbistatin treatment, the extended width of the lamellipodium increased by 15-100% (**j**) and the protrusion rate by at least two fold (**a**)

S6. Simulation of slowing, pause and retraction in a lamellipodium. During slowing to pause, filaments show a varied reduction in polymerisation rate (red and yellow plus ends), resulting in the faster filaments (red) adopting curved trajectories, culminating in a subpopulation of filaments parallel to the cell edge. Slower polymerising filaments (yellow to green) act as tethers and restrain protrusion. Shorter filaments at high angles depolymerise first, leaving longer filaments in antiparallel arrays to recruit myosin (orange bars) for retraction. Microspike bundles also contribute filaments to retracting edges (not depicted).

Supplementary references

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