Journal of Cell Science

# Shifting views on the leading role of the lamellipodium in cell migration: speckle tracking revisited

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/12/1955/DC1

*Journal of Cell Science 122, 1955-1958*
*Published by The Company of Biologists 2009*
*doi:10.1242/jcs.042036*

## Introduction

Migrating cells advance by first protruding a lamellipodium, a thin sheet of cytoplasm that consists mostly of filamentous actin (F-actin). The region immediately behind the lamellipodium is also relatively flat and has been termed the lamella (Heath and Holifield, 1991). Adhesion to the matrix, as required for traction, is initiated under the lamellipodium and at the boundary with the lamella (Kaverina et al., 2002). Recent interest has focused on understanding the turnover of actin at the cell front and the interplay between the lamellipodium and lamella in the process of migration (Small and Resch, 2005).

By introducing extremely low concentrations of fluorescently tagged G-actin monomers in live cells, it is possible to generate single-molecule fluorescent markers in the F-actin cytoskeleton and to track them (Watanabe and Mitchison, 2002). Even at slightly higher concentrations, statistical variations in the local density of polymerised fluorescent F-actin give rise to distinct features, termed speckles, which can, in principle, be tracked (Fig. 1; Box 1). This task can easily be mastered by a human operator, but there is considerable interest in automating this very time-consuming exercise.

A striking feature of lamellipodium dynamics, as revealed by fluorescence recovery after photobleaching (FRAP) (Lai et al., 2008; Wang, 1985) and fluorescent speckle microscopy (FSM), is retrograde flow, which is powered by actin polymerisation at the leading edge. Retrograde flow is also observed in the lamella, as revealed first by particle tracking (Harris, 1973; Heath and Holifield, 1991) and more recently by FSM (Vallotton et al., 2003). It is fast in the lamellipodium and slower in the lamella, with an interface between these two regions where, by virtue of conservation of matter, significant depolymerisation takes place (Vallotton et al., 2004).

A provocative diversion from the conventional idea of what happens at the cell front was stimulated by what we call here the 'lamella hypothesis' – the suggestion that the lamella extends to the very leading edge of motile cells and that the lamellipodium surfs on it (Ponti et al., 2004) (see also Box 1). Almost simultaneously, Gupton et al. proposed that the lamellipodium was not necessary for cell motion, and that the lamella was the organelle responsible for cell motility (Gupton et al., 2005). A feature that distinguishes the lamella from the lamellipodium is the presence of myosin II in the lamella and its absence in the lamellipodium (Fig. 2); the conclusion that a contractile actomyosin array in the lamella could push, therefore, when pushing is most effectively achieved by polarised actin arrays in lamellipodia and filopodia, was surprising.

In this Update, we take a second look at the evidence supporting the lamella hypothesis of Ponti et al. (Ponti et al., 2004) – a set of FSM movies. The hypothesis is based on the application of automated particle-tracking methods to follow speckles in the lamellipodium, a notoriously difficult type of analysis. Using their new tracking tools, Ponti et al. found that a large proportion (33%) of speckles were long-lived and moved slowly in the lamellipodium. Such speckles are usually observed only in the lamella, and their presence would indeed represent good reason to formulate the lamella hypothesis.

Following this lead, and using the same published image datasets, we recently sought to learn more about the properties of these slow, long-lived speckles – but failed to find them. We tracked speckles in the lamellipodium both manually and using an automated tracking tool, we used kymograph analysis, and we created close-up movies of the lamellipodium area, hoping to sight the elusive speckles more directly. We were also able to conjure the slow, long-lived speckles in question by intentionally using a poor methodology to track the speckles. From this analysis, presented in more detail below, we contend that the lamella should be understood as functionally, kinematically, kinetically and spatially distinct from the lamellipodium, as it was prior to 2004. These findings highlight the need for a more rigorous interpretation of FSM data.

## Object tracking

In order to extract quantitative information from time-lapse data, it is usual to identify discrete objects in every image of the sequence and establish a correspondence across successive frames positing their physical identity. When performed over all frames of the sequence, this process delivers trajectories for each object (Box 1). A long-lived speckle, then, is one that is identified as the same speckle through a large number of consecutive frames. The average velocity of a speckle is calculated as the total distance travelled, divided by its lifetime. Crucially, the numerical values that are obtained for these quantities depend on one's ability to correctly track objects.

In algorithmic terms, automated object tracking poses significant difficulties. Objects can appear or disappear either genuinely, by division, fusion, diffusion, polymerisation or depolymerisation, or apparently, by moving into or out of focus. Object dynamics can also be very different for different objects and might change over time. Finally, the actual amplitude of object displacements might be so large that determining the true object motion in the presence of many other targets becomes virtually impossible, even manually. We found that mathematical graphs were very useful to deal with such problems. Extensive tests were conducted using simulated data sets (Vallotton et al., 2003) to ensure that automated tracking results were consistent with those of manual tracking.

Object tracking is not the only method for quantifying dynamics, however. For example, kymograph analysis was in use long before automated object tracking, and it represents one of the gold standards against which such methods are evaluated.

### Kymograph analysis

To conduct a kymograph analysis, the user defines a line segment in the first frame of an image sequence. This segment is then cut out digitally from each subsequent image and they are mounted side by side to form a montage image. Oblique streaks indicate the presence of features moving along the kymograph axis, with a slope that reveals the velocity of these features (Fig. 1B).

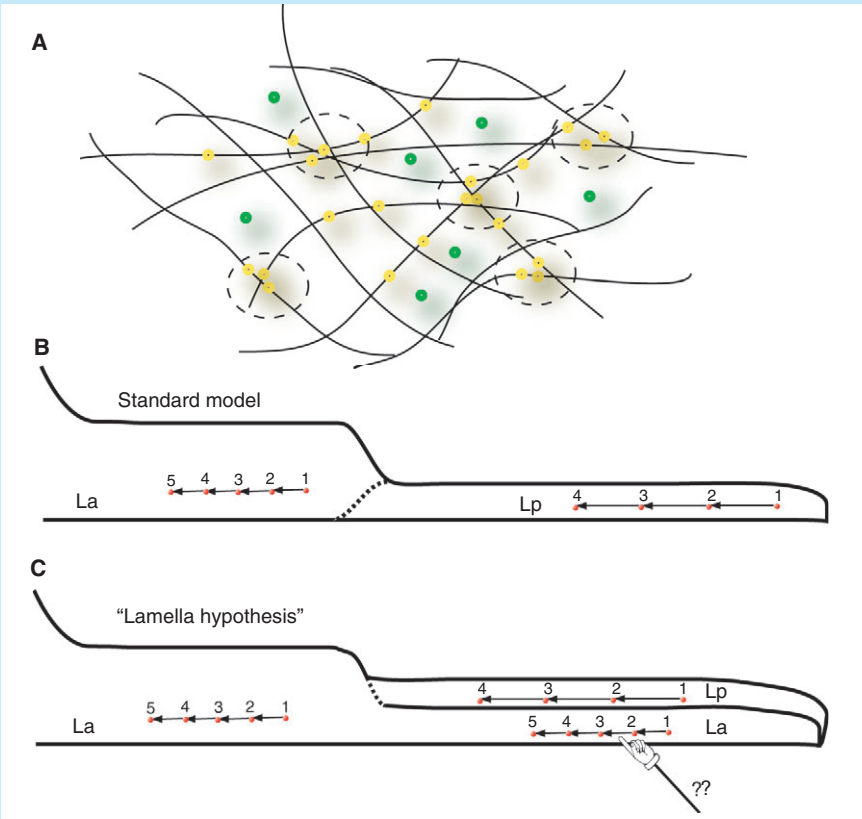
We have produced kymographs over the lamellipodium area, based either on the original images or on the images Gaussian-filtered for improved visibility. Many kymographs similar to the ones shown in Fig. 1B, using different axis directions and multiple positions along the leading edge, were produced (supplementary material Fig. S1). Long, oblique streaks at low incidence within the lamellipodium could not be observed. None of the kymographs that we found in the literature indicated the presence of such events either. Thus, it appears that speckles that display a lamella-like signature are typically not observed in the lamellipodium using traditional kymograph analysis. This is not a limitation of the methodology itself – lamella speckles give rise to the expected streaks in the lamellar region (Fig. 1B).

### Manual tracking

Automated tracking algorithms are used to save time, but invariably make tracking errors. Our sample results from manual tracking are shown in Fig. 1D, in which the tracks are overlaid on the last frame of the image sequence. Using MTrackJ (an ImageJ plug-in created by Erik Meijering, Erasmus MC, Rotterdam, NL), it took about 30 minutes to manually produce these trajectories. We could not identify stationary speckles in the lamellipodium using MTrackJ. The process of manual tracking is illustrated in supplementary material Fig. S2.

Visual attention can sometimes catch elusive events that the best computational frameworks or supporting tools may miss. Thus, we have produced and scrutinised many close-up movies of the lamellipodium region (supplementary material Movies 1, 2 and 3); slow, long-lived speckles could not be identified by this process, either.

### Box 1. Fluorescent speckle microscopy, tracking and the lamella hypothesis




Fluorescent speckles in F-actin meshworks arise in the presence of very low concentrations of fluorescently labelled G-actin monomers as a result of statistical variations in the number of labels that clustered locally. In part A of the figure, dashed circles highlight regions where several fluorophores (shown in yellow) polymerised within a diffraction-limited area to produce speckles. Speckles are very useful – being intimately associated with the meshwork, they move with it and also report on its assembly and disassembly as they appear and disappear. Note that the free G-actin monomers (shown in green) do not give rise to speckles because diffusive motion mostly averages out their contribution.

Tracking fluorescent speckles is not different from tracking any other target – in part B of the figure, a particular speckle is seen at a particular position at time-point 1. It is then seen at another position at time-point 2 as the speckle drifts rearward, entrained by the lamellipodium retrograde flow. By marking and linking the positions occupied in successive images by the same speckle, speckle trajectories can be generated and analysed. In particular, in the standard model the speed along the trajectories in the lamellipodium (Lp) are much greater than the speed in the lamella (La), as illustrated by the longer displacement vectors in the figure (see also Fig. 1E).

In the 'lamella hypothesis' (part C of the figure), the lamella is proposed to colocalise with the lamellipodium, an idea originally put forward on the basis of indirect evidence for slow speckles in the lamellipodium area.

### Automated tracking

Early tracking methods tended to produce jagged speckle trajectories in the lamellipodium (Ponti et al., 2004). This does not marry well with the impression of mostly linear tracks that comes from watching the movies (supplementary material Movies 1, 2 and 3). Early flow maps also tended to significantly underestimate flow speed in the lamellipodium. The reason for this discrepancy is that, under normal imaging conditions, speckles move so quickly in the lamellipodium (jumps of up to 10 pixels are not exceptional) that it is difficult to track them using computer vision. This difficulty is compounded by considerable speckle appearance at the cell front. Thus,

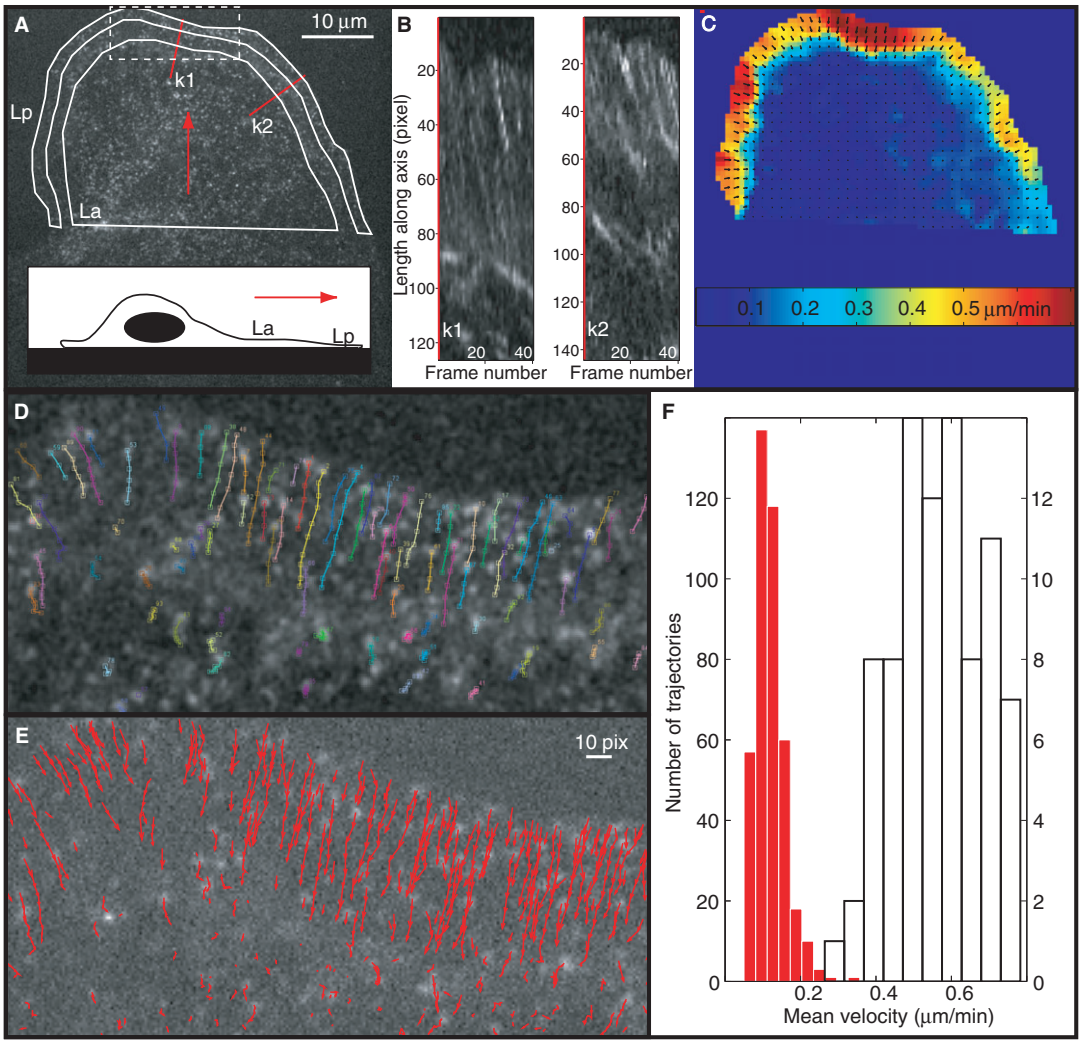



**Fig. 1.** Speckle tracking in motile cells. (A) First frame of a newt lung migrating-cell sequence viewed under FSM (Vallotton et al., 2004). The lamellipodium is marked Lp and the lamella La. The red arrow indicates the direction of cell motion. (B) Kymograph analysis conducted over the leading-edge region, with kymograph axes k1 and k2 drawn in A. Streaks display a typical slope in the lamella that is different from the slope in the lamellipodium. No streak can be generated in the lamellipodium that would feature a slope typical of those found in the lamella. (C) Flow map obtained on the basis of our automated tracking results. Velocity in the lamellipodium is up to three times that in the lamella. (D) Manual tracking results generated using MTrackJ, overlaid on the last frame of the sequence (the region of interest is indicated by a dashed frame in A). Note that MTrackJ only displays tracks that remain until the last frame. (E) Sample of our automated tracking results for speckles at the leading edge, overlaid on the first frame of the sequence (pixel size: 67 nm). (F) Distribution of average velocities (in red) for tracks in the lamella area (La in A), lasting more than five frames, together with distribution of average velocities (in white) for tracks in the lamellipodium area (Lp in A) lasting more than five frames. The two distributions have virtually no overlap.

instead of obtaining correct matches that show collective, coherent behaviour, tracking mistakes produce randomly oriented matches that bias the flow speed downwards.

By introducing a preference for coherent motion among neighbouring speckles directly in the structure of the tracking graph, it is possible to overcome these difficulties and obtain trajectories that are consistent with those obtained by manual tracking. We show a sample of our new automated tracking results at the leading edge in Fig. 1E. The corresponding time-lapse sequence can be downloaded from the supplementary information section, either in raw form (supplementary material Fig. S3), or in Gaussian-filtered form (supplementary material Fig. S4).

To isolate speckles that might display the behaviour reported by Ponti et al. (Ponti et al., 2004), we screened all of our tracks using the automated trajectory selection tools available with the Diatrack automated tracking software (version 2.3; Semasopht, Switzerland) and retained only those speckles that lasted at least five frames and had their origin within the lamellipodium. The distribution of average speed along these tracks is displayed in white in Fig. 1F, where it appears that none of the tracks are characterised by a speed of less than 0.2 µm/min. The behaviours of speckles in the lamella and in the lamellipodium, therefore, are fundamentally different, and automated tracking, by itself, does not suggest that the lamella extends to the very leading edge.

In any event, the presence of slow and long-lived speckles would not be convincing enough – the speckles would also need to display the systematic retrograde motion that is typical of lamella speckles.

## Simulations

For deriving the lamella hypothesis, Ponti et al. independently developed a

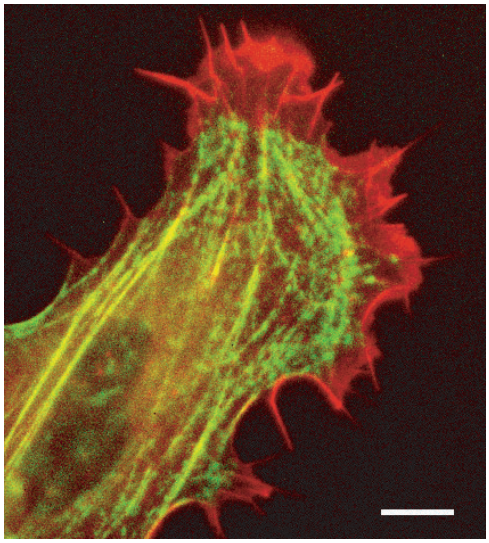


**Fig. 2.** Myosin II is localised in the lamella and is mainly excluded from the lamellipodium. Video-frame composite of a living fish fibroblast co-transfected with mCherry-actin and GFP-myosin light chain [conditions as described by Nemethova et al. (Nemethova et al., 2008)], showing segregation of myosin in the lamella behind the lamellipodium-filopodium boundary. Scale bar: 10 μm.

new algorithm based on neural networks [(Ponti et al., 2004), pages 4 and 5 of their supplementary information document 1], but a validation of this code on simulated data or manually tracked scenes was not offered. As a test, we used a value of only 5 pixels in Diatrack for the maximum speckle jump amplitude of any speckle in the sequence. It is important to note that this methodology is bound to trigger many mistakes because the longest speckle jumps are, in fact, as large as 10 pixels.

We then analysed all tracks lasting more than five frames in the lamellipodium and observed that a few tracks now appeared to be consistent with lamella behaviour, reproducing to some extent the results of Ponti et al. (Ponti et al., 2004). Viewing the tracks frame by frame, however, confirmed that the speckles had not been tracked correctly. In particular, the tracks looked mostly random. Generally, the poorer the quality of FSM movies in terms of speckle contrast and sampling rate, the more tracking mistakes are made. In fact, except for a few FSM movies of exceptional quality, it is very difficult to perform a reliable analysis (e.g. supplementary material Fig. S5 shows a sequence for which we would argue that speckle tracking is not feasible).

## Conclusions and perspectives

The presence of two different networks at the cell front would, one expects, have consequences at the level of their microscopic organisation. However, the actin meshworks in lamellipodia that have been observed by Koestler et al. (Koestler et al., 2008) lack any underlying array that could be equated with a component of the lamella. From experiments in which tropomyosin was injected into living cells, Gupton et al. concluded that lamellipodia were abolished, without compromising cell migration (Gupton et al., 2005). Closer inspection of their phase-contrast videos and phalloidin-labelling data, however, indicates that lamellipodia were reduced but not eliminated by tropomyosin injection.

A considerable body of literature using quantitative FSM has followed the exploratory work of Ponti et al. Biological conclusions reached using that technique should now be carefully re-examined – an undertaking too ambitious for the space available here.

Object-tracking methods are becoming increasingly useful in quantitative biology as they open up new ways to interrogate and gather useful information from biological systems. Graph-based algorithms have been shown to be particularly effective in this context, at least for FSM data, and we anticipate that considerable developments will take place in the near future. However, FSM should preferably be used alongside complementary optical approaches and correlated electron microscopy. By applying such methods, we are learning a great deal about how cells move; however, in the light of current evidence, the lamella hypothesis is now very thin – lamellipodia and filopodia are still up front, leading the way.

We are grateful to Maria Nemethova (IMBA) for providing Fig. 2. Pascal Vallotton is founder of Semasopht and developed the Diatrack software. Funding from CSIRO and the Austrian Academia of Science is acknowledged.

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