Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Neurobiology

Actin Assemblies in the Axon Shaft – some Open Questions

Pankaj Dubey[1], Kent Jorgenson[1,2] and Subhojit Roy[1,2]

The actin cytoskeleton in neurons plays critical roles in axonal growth and synaptic organization. Until recently, most studies on axonal actin were limited to terminal growth cones or synapses, whereas the organization of actin along the shaft of the axon was relatively ignored. However, experiments using super-resolution microscopy and live imaging have revealed previously unknown actin structures along the axonal shaft, such as periodic 'actin rings' circumferentially wrapping underneath the plasma membrane and dynamic actin pools deeper within the axon shaft (termed actin 'hotspots' and 'trails'). In this short review, we highlight some open questions that have surfaced as a direct result of these discoveries.

Addresses
[1] Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, 1111 Highland Avenue, Madison, WI 53705
[2] Department of Neuroscience, University of Wisconsin-Madison, 1111 Highland Avenue, Madison, WI 53705

Corresponding author: Roy, Subhojit (roy27@wisc.edu)

Current Opinion in Neurobiology 2018, **51**:163–167

This review comes from a themed issue on **Cellular Neuroscience**

Edited by **Juan Burrone** and **Erika Holzbaur**

doi:https://doi.org/10.1016/j.conb.2018.06.012

Introduction

Actin is one of the most conserved proteins known, exchanging between a monomeric and filamentous form. This cytoskeletal protein is highly abundant in neurons and relatively easy to visualize by routine staining with actin-filament binding probes such as phalloidin. Thus it was a surprise when super-resolution imaging revealed a remarkable, previously unknown actin-based lattice structure along the axon shaft. A major component of this lattice are 'actin rings' – periodic, circumferential rings of actin filaments wrapping underneath the plasma membrane, spaced at ~ 190 nm – just below the resolution limits of light microscopy (see Figure 1). Mainly (but not exclusively) found along the shaft of the axon, adjacent actin rings are spaced by tetramers of another cytoskeleton-associated protein called spectrin; thus generating alternating "bands" of actin and spectrin in axon-shafts, when visualized by super-resolution microscopy. In addition to these sub-plasmalemmal structures, axons also have deeper assemblies of actin that have been visualized by both super-resolution microscopy and live imaging. These deeper actin assemblies are quite dynamic, comprised of focal "hotspots" – where actin continuously polymerizes and depolymerizes – as well as linear actin filaments that longitudinally extend along the shaft of the axon (termed 'actin trails'; see Figure 1). For a more detailed description of these structures, we refer the reader to several recent reviews [1••,2,3]. While these observations exponentially advanced our understanding of the axonal cytoskeleton, they also unearthed a plethora of new questions. Here we discuss two such questions that have surfaced from these discoveries.

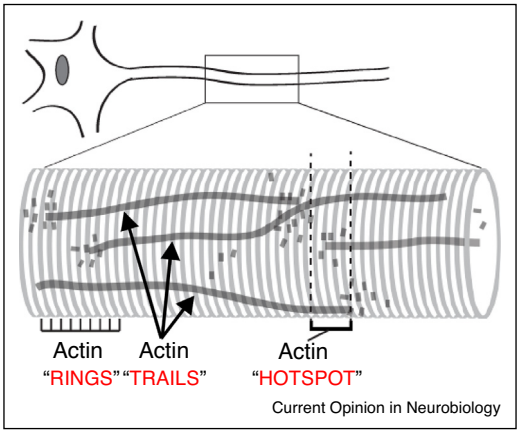
How do actin rings and trails arise in axons?

Actin Rings

In the axon shaft, circumferential axonal actin rings – spaced at ~ 190 nm – are interspersed by βII-spectrin tetramers that span across adjacent actin rings, thus forming a sub-plasmalemmal network of alternating actin and βII-spectrin assemblies, which we refer to here as the axonal 'ring-structure'. A key feature of neurons is their extreme polarity. As neurons differentiate, a single process breaks symmetry and dramatically elongates to form the axon [4]. Though axons are capable of limited protein synthesis, the vast majority of neuronal proteins are synthesized in the neuronal soma and transported into the axon, up to the tip. This axonal transport not only sustains elongation of the axon during development, but also maintains axonal and synaptic homeostasis throughout the life of the neuron [5,6]. Interestingly, most of the cytoskeletal and cytosolic proteins comprising the axonal ring-structure – including actin, spectrin, adducin, and ankyrin [7] – are known to be conveyed in a mysterious transport group known as slow axonal transport; as shown by classic pulse-chase type radiolabeling studies [8–10]. Thus the initial emergence of actin rings and related structures is likely to be an interplay between slow axonal transport of the ring-components and an orderly supramolecular organization of these transported elements into the ring-structure as the axon elongates.

Examining the βII-spectrin lattice structure in developing axons of cultured hippocampal neurons – an ideal model-system where axonal differentiation has been extensively studied – Xu et al. reported that at early stages when the axon has just broken symmetry (days in vitro-2 or DIV-2), the ring-structure is restricted to the proximal segment [11]. However, at a later stage when the axon is established (DIV-6), the βII-spectrin lattice is

Figure 1

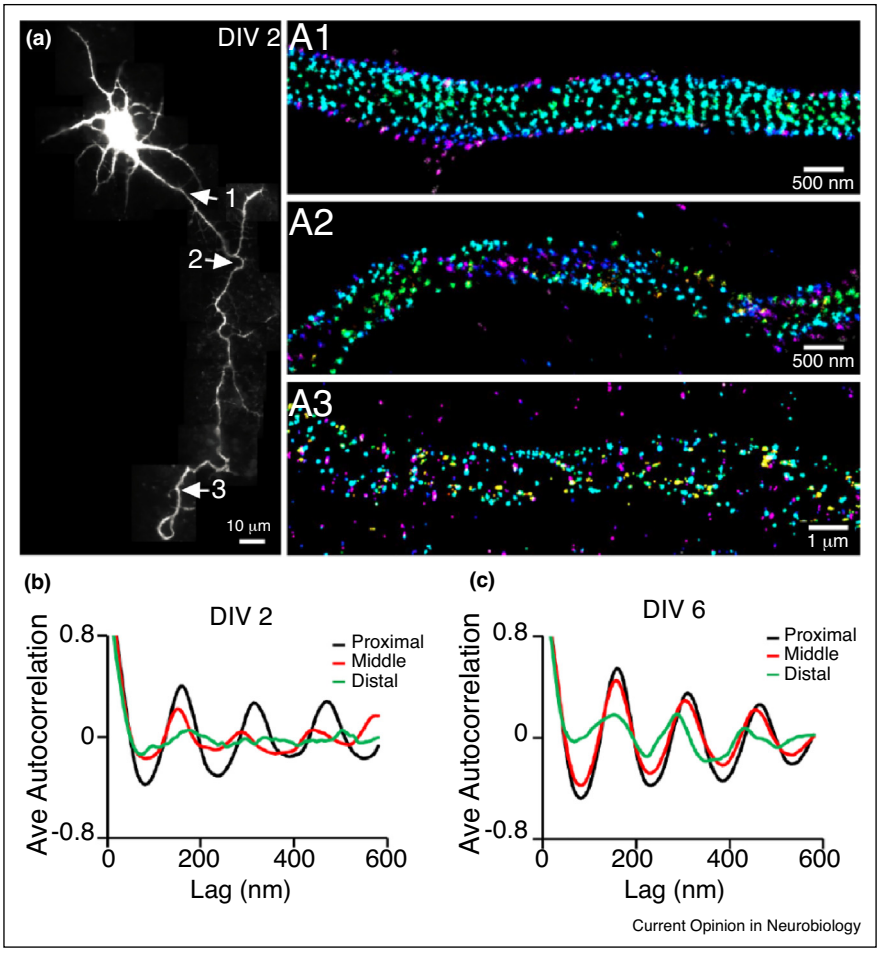


Schematic of an axon shaft with circumferential actin rings, focal hotspots of actin assembly, and long actin polymers extending bidirectionally.

more continuous along the length of the axon (Figure 2). Following these observations, they proposed a "propagation model" where the ring-structure first emerges at the base of the axon, and then spreads distally as the axon matures [11]. Though the propagation model is a reasonable approximation of STORM imaging, it is based on extrapolation from two static time-points in development, and other possible mechanisms – for instance, local assembly from pre-assembled intermediates along the length of the axon – can also explain the data. Importantly, the putative role of slow axonal transport of ring-components in this assembly process has not been explored yet, probably due to inherent difficulties in imaging slow transport [6].

A notable feature of the data from Xu et al. is that in the early stages where the ring-structure is established at the proximal part of the axon, the distal axon is not completely devoid of ring-components. Instead, the βII-spectrin in this region has a diffuse, speckled

Figure 2



3-D STORM of βII-spectrin in DIV2 axons of cultured hippocampal neurons. Note that the distribution of βII-spectrin is periodic in the proximal axonal segment (A1), while this periodicity is lost in the distal segment (A3). Quantified in b, c. Data from Zhong et al., [11], reproduced with permission.

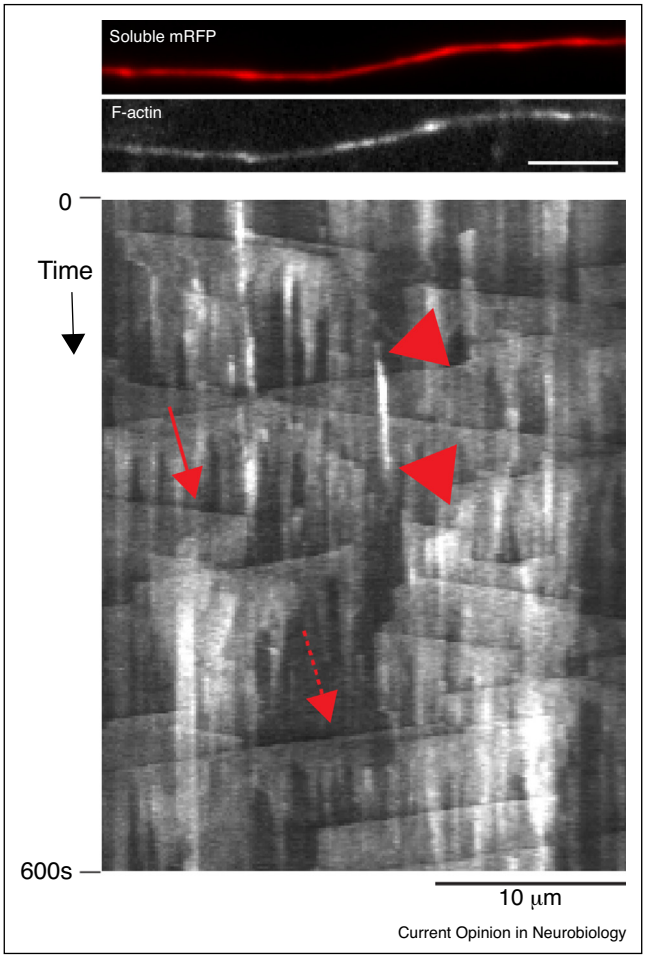
appearance, sometimes resembling poorly organized ring-like patterns (see Figure 2 A2 and A3). Interestingly, this speckled pattern of distribution is also seen with other slow component proteins, and is qualitatively different from the discrete, punctate distribution of vesicles and mitochondria in axons (that move in a distinct rate-class called fast axonal transport). For example, the endogenous distribution of synapsin and Hsc70 – two proteins that are also conveyed in slow axonal transport – is also speckled in axons. A closer view of these synapsin and Hsc70 particles by DNA-PAINT/STORM-imaging indicates that these proteins are organized into nano-scale "transport complexes" in axons [12•]. Thus it seems reasonable to surmise that at least a portion of the βII-spectrin particles seen in axons represent the transported fraction of ring-components (not the assembled fraction), and that this population ultimately contributes to the assembly of these proteins into an organized ring-structure. Relatedly, it is also possible that the distal punctate pattern represents "nascent ring-components" that will give rise to the organized pattern in older axons, a view that is supported by the STORM data in distal axons, where an indistinct sub-plasmalemmal organization is evident (see Fig. 2A2 for example). Future studies combining live imaging of ring-components and super-resolution microscopy may shed light on this issue. Questions regarding the stability and turnover of the ring-structure also remain. FRAP experiments indicate minimal recovery of GFP-tagged βII-spectrin in axons over ~ 30 minutes, suggesting a stable organization [11]. However, treatment with cytoskeletal depolymerizing drugs can disrupt the ring-structure [11,13,14], indicating a dynamic network. Future experiments looking at slow axonal transport, assembly, and turnover of individual ring-components may reveal some answers.

Actin Trails

Recently, actin was visualized in axons of cultured hippocampal neurons (DIV 7-10), using probes specific for the filamentous form of actin [15]. A combination of live imaging and STORM showed that axons have dynamic actin assemblies, distinct from the sub-plasmalemmal actin/βII-spectrin ring-structure [15]. Specifically, focal hotspots of actin were seen in axons, spaced at ~ 3-4 μm, along with longer actin filaments that were bidirectionally extending into the axon shaft (actin trails; see kymograph in Figure 3). The elongating filaments often emerged from (or very close to) where the focal hotspots were, supporting a model where the actin hotspots act as a nidus for generating the elongating actin filaments [15]. A recent study in mature touch receptors neurons (TRNs) in *C. elegans* also saw focal actin hotspots and trails, indicating that these assemblies are remarkably conserved, and can also be seen in a true in vivo setting [16••].

Mechanistically, these studies suggest that the assembly of actin at the hotspots might occur on the surface of stationary axonal endosomes [15], but definitive proof of this is still elusive. Pharmacologic studies suggest that the rapid elongation of actin trails is mediated by formins, and not Arp2/3 [15]. Formins are a family of proteins that bind to the barbed end of actin and facilitate rapid linear elongation [17]. Though the rate of elongation of actin trails (~ 1μm/s) is consistent with a formin-mediated phenomenon, the specific formin(s) involved are yet unknown. Also, though global formin-inhibition attenuated actin trails, interestingly, there was no effect on the actin hotspots [15]. In most formin-mediated cellular events, formins are responsible for both nucleation and elongation of actin; thus the selective effect of formin inhibition on axonal actin trails – but not hotspots – is surprising, and future studies into this may also lead to new insights about formin function in general. A more comprehensive itinerary of the players involved in actin hotspots and trails is also lacking.

Figure 3



Live imaging of actin in axons (using GFP-tagged to Utr-CH, see [31]. Images above show an axon co-transfected with soluble mCherry (top) and GFP:Utr-CH (bottom). Kymograph from this axon shows many hotspots (arrowheads marking one), and well as anterograde and retrograde trails (marked by filled and dashed arrowheads respectively). Reproduced from [15], with permission.

The initial appearance of actin trails in developing axons is also unclear. So far, actin trails have been reported in more mature (DIV 7-10) axons, where these assemblies can be readily seen, and it will be interesting to compare the developmental appearance of the rings and trails in axons. Since actin hotspots and trails are the only known dynamic actin structures along axon shafts, it seems reasonable to hypothesize that these assemblies are a visual correlate of actin transport. However, mechanistic details are still unclear and further studies looking at slow transport of actin may shed light on this issue. Finally, it is unclear if there is an anatomical or functional link between actin rings and trails, and understanding this issue might shed light on the function of these assemblies in the axon (see next).

What are the functions of actin rings and trails?

Actin Rings

The overall organization of the axonal ring-structure is reminiscent of a flexible but supportive scaffold – such as ribs inside a chest-wall. Similar scaffolds are found in other cell-types, offering clues into its potential function in axons. For example, an actin/βII-spectrin network is also seen in erythrocytes, where the scaffold is thought to give structure and flexibility to the red blood cell membrane [18]. Although the precise composition of the ring-structure differs in erythrocytes and neurons, the two assemblies might reflect evolutionary variations of the same basic theme, warranting comparisons. Interestingly, the spacing of actin/βII-spectrin network in erythrocytes is only ~80 nm, suggesting that the lattice network is relaxed in these cells, unlike axons, where it is may be under tensile stress [19••]. Indeed, several studies indicate that axons are under high tensile stress [20,21,22]; thus the rings might play a role in maintaining axonal tension. Axonal myosins may be involved in this process [23], but neither the repertoire of molecular players, nor the mechanistic details of this process is understood at this time. Other examples of actin-myosin networks include *Drosophila*, where non-muscle myosin II associates with rings of actin in the formation of tracheal tubes [24]. Non-muscle myosin II is also vital in the separation of daughter cells during cytokinesis, creating a functional contractile ring [25].

Axons invariably need to endure stretching and movement, and the stretched axonal βII-spectrin tetramers could provide overall rigidity and stability to the axonal shaft; thus protecting the elongated structure from damage. Indeed such a role is suggested by spectrin knockout experiments in *C. elegans*, where axons break as the organism starts to move [26]. However, the role of the ring-structure in maintaining axonal tension and overall integrity is not established yet. The axonal ring-structure might have other roles as well. For instance, it is unclear whether axon shafts can undergo endocytosis, and the ring-structure might provide a way to attenuate membrane internalization along the length of the axon. Indeed it seems advantageous to suppress recycling of transported vesicles in axons – for instance, retrograde vesicles carrying growth factors and signaling molecules to the soma – but these questions have not been rigorously explored yet. A pool of stationary vesicles and endosomes is also seen in axons – both in cell culture and in vivo [15,16••] – and it remains possible that these stalled vesicles are physically associated with the axonal ring-structure. Such a scenario might also link actin rings and trails, and future experiments may shed light on these hypothetical scenarios.

Actin Trails

The dynamic and abundant axonal actin trails appear to be a remarkable metabolic investment by the cell, but putative functions are still unclear. These structures resemble actin 'comets' in listeria and other organisms, where they are known to propel vesicles [27–30]. However, pharmacologic inhibition of actin trails does not seem to have a major effect on vesicle transport [15]. It remains possible however, that there are specific classes of vesicles or organelles that can be propelled by actin trails. As mentioned before, one possibility is that actin trails represent the axonal transport of actin and is the mechanism by which actin is delivered to presynaptic boutons. In support of this, studies found that attenuation of actin trails interrupted the delivery of actin to boutons, with physiologic consequences [15]. However, details related to the polarization of these assemblies and movement in slow axonal transport remains to be elucidated.

In summary, although recent discoveries have revealed intriguing cytoskeletal assemblies along the axon shaft, fundamental questions related to the organization and function of these structures remain. In a way, this is a reflection of how new knowledge invariably leads to more questions, and it seems that there are plenty of interesting questions to keep the cytoskeletal community engaged in the coming years.

Acknowledgements

This work was supported by an NIH grant to SR (R01NS075233). The authors thank Christophe Leterrier for helpful comments.

References

1. •• Leterrier C, Dubey P, Roy S: **The nano-architecture of the axonal cytoskeleton**. *Nat Rev Neurosci* 2017, **18**:713-726.
A recent, comprehensive review of the neuronal cytoskeletal field, including organization and nanostructure, axonal transport of cytoskeletal elements, and a historical perspective.

2. Leite SC, Sousa MM: **The neuronal and actin commitment: Why do neurons need rings?** *Cytoskeleton* 2016, **73**:424-434.

3. Roy S: **Waves, rings, and trails: The scenic landscape of axonal actin**. *J Cell Biol* 2016, **212**:131-134.

4. Banker G: **The Development of Neuronal Polarity: A Retrospective View**. *J Neurosci* 2018, **38**:1867-1873.

5. Nirschl JJ, Ghiretti AE, Holzbaur ELF: **The impact of cytoskeletal organization on the local regulation of neuronal transport**. *Nat Rev Neurosci* 2017, **18**:585.

6. Roy S: **Seeing the unseen: The hidden world of slow axonal transport**. *Neuroscientist* 2014, **20**:71-81.

7. Xu K, Zhong G, Zhuang X: **Actin, Spectrin, and Associated Proteins Form a Periodic Cytoskeletal Structure in Axons**. *Science (80-)* 2013, **339**:452-456.

8. Black MM, Lasek RJ: **Axonal transport of actin: Slow component b is the principal source of actin for the axon**. *Brain Res* 1979, **171**:401-413.

9. Garner Ja, Lasek RJ: **Cohesive axonal transport of the slow component b complex of polypeptides**. *J Neurosci* 1982, **2**:1824-1835.

10. Levine J, Willard M: **Fodrin: Axonally transported polypeptides associated with the internal periphery of many cells**. *J Cell Biol* 1981, **90**:631-643.

11. Zhong G, He J, Zhou R, Lorenzo D, Babcock HP, Bennett V, Zhuang X: **Developmental mechanism of the periodic membrane skeleton in axons**. *Elife* 2014, **3**:1-21.

12. • Ganguly A, Han X, Das U, Wang L, Loi J, Sun J, Gitler D, Caillol G, Leterrier C, Yates JR *et al.*: **Hsc70 chaperone activity is required for the cytosolic slow axonal transport of synapsin**. *J Cell Biol* 2017, **216**:2059-2074.
Live imaging and DNA-PAINT suggest that the cytosolic proteins synapsin and Hsc70 are present as nano-scale multi-protein complexes that are conveyed in slow axonal transport, and that Hsc70 chaperone activity regulates this process.

13. Qu Y, Hahn I, Webb SEDD, Pearce SP, Prokop A: **Periodic actin structures in neuronal axons are required to maintain microtubules**. *Mol Biol Cell* 2017, **28**:296-308.

14. Leterrier C, Potier J, Caillol G, Debarnot C, Rueda Boroni F, Dargent B: **Nanoscale Architecture of the Axon Initial Segment Reveals an Organized and Robust Scaffold**. *Cell Rep* 2015, **13**:2781-2793.

15. Ganguly A, Tang Y, Wang L, Ladt K, Loi J, Dargent B, Leterrier C, Roy S: **A dynamic formin-dependent deep F-actin network in axons**. *J Cell Biol* 2015, **210**:401-417.

16. •• Sood P, Murthy K, Kumar TV, Nonet ML, Menon GI, Koushika SP: **Cargo crowding at actin-rich regions along axons causes local traffic jams**. *Traffic* 2017 http://dx.doi.org/10.1111/tra.12544.
Live imaging of actin in*C-elegans* axons reveal hotspots and actin trails in vivo. The authors propose that actin hotspots help stall vesicles along axons, and that this trapped cargo acts as a functional reservoir of vesicles.

17. Goode BL, Eck MJ: **Mechanism and Function of Formins in the Control of Actin Assembly**. *Annu Rev Biochem* 2007, **76**:593-627.

18. Gokhin DS, Nowak RB, Khoory JA, Piedra Adl, Ghiran IC, Fowler VM: **Dynamic actin filaments control the mechanical behavior of the human red blood cell membrane**. *Mol Biol Cell* 2015, **26**:1699-1710.

19. •• Pan L, Yan R, Li W, Xu K: **Super-Resolution Microscopy Reveals the Native Ultrastructure of the Erythrocyte Cytoskeleton**. *Cell Rep* 2018, **22**:1151-1158.
STORM imaging of erythrocytes reveal an $\sim$ 80nm junction-to junction distance, which is in agreement with relaxed spectrin tetramers. This is in contrast to the $\sim$ 190nm spectrin-tetramer spacing seen in axons.

20. Anava S, Greenbaum A, Jacob E, Ben, Hanein Y, Ayali A: **The regulative role of neurite mechanical tension in network development**. *Biophys J* 2009, **96**:1661-1670.

21. Dennerll TJ, Lamoureux P, Buxbaum RE, Heidemann SR: **The cytomechanics of axonal elongation and retraction**. *J Cell Biol* 1989, **109** 3073 LP-3083.

22. Fan A, Stebbings K, Llano D, Saif T: **Stretch induced hyperexcitability of mice callosal pathway**. *Front Cell Neurosci* 2015, **9**:292.

23. Berger SL, Leo-Macias A, Yuen S, Khatri L, Pfennig S, Zhang Y, Agullo-Pascual E, Caillol G, Zhu MS, Rothenberg E *et al.*: **Localized Myosin II Activity Regulates Assembly and Plasticity of the Axon Initial Segment**. *Neuron* 2018 http://dx.doi.org/10.1016/j.neuron.2017.12.039.

24. Hannezo E, Dong B, Recho P, Joanny J-F, Hayashi S: **Cortical instability drives periodic supracellular actin pattern formation in epithelial tubes**. *Proc Natl Acad Sci* 2015, **112**:8620-8625.

25. Zumdieck A, Kruse K, Bringmann H, Hyman AA, Jülicher F: **Stress Generation and Filament Turnover during Actin Ring Constriction**. *PLoS One* 2007, **2**:e696.

26. Hammarlund M, Jorgensen EM, Bastiani MJ: **Axons break in animals lacking β-spectrin**. *J Cell Biol* 2007, **176**:269-275.

27. Taunton J, Rowning Ba, Coughlin ML, Wu M, Moon RT, Mitchison TJ, Larabell Ca: **Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP**. *J Cell Biol* 2000, **148**:519-530.

28. Merrifield CJ, Moss SE, Ballestrem C, a Imhof B, Giese G, Wunderlich I, Almers W: **Endocytic vesicles move at the tips of actin tails in cultured mast cells**. *Nat Cell Biol* 1999, **1**:72-74.

29. Rozelle AL, Machesky LM, Yamamoto M, Driessens MHE, Insall RH, Roth MG, Luby-Phelps K, Marriott G, Hall A, Yin HL: **Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3**. *Curr Biol* 2000, **10**:311-320.

30. Lambrechts A, Gevaert K, Cossart P, Vandekerckhove J, Van Troys M: **Listeria comet tails: the actin-based motility machinery at work**. *Trends Cell Biol* 2008, **18**:220-227.

31. Burkel BM, von Dassow G, Bement WM: **Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin**. *Cell Motil Cytoskeleton* 2007, **64**:822-832.