Forces from the rear: deformed microtubules in neuronal growth cones influence retrograde flow and advancement

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Abstract. The directed motility of growth cones at the tip of neuronal processes is a key function in neuronal path-finding and relies on a complex system of interacting cytoskeletal components. Despite intensive research in this field, many aspects of the mechanical roles of actin structures and, in particular, of microtubules throughout this process remain unclear. Mostly, force generation is ascribed to actin–myosin-based structures such as filopodia bundles and the dynamic polymer gel within the lamellipodium. Our analysis of microtubule buckling and deformation in motile growth cones reveals that extending microtubule filaments contribute significantly to the overall protrusion force. In this study, we establish a relationship of the local variations in stored bending energy and deformation characteristics to growth cone morphology and retrograde actin flow. This implies the relevance of microtubule pushing and deformation for general neurite advancement as well as steering processes.

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1. Introduction

At the tip of outgrowing neuronal processes (axons), a highly complex sensory-motile structure termed a growth cone (GC) develops to perform guided growth of the neurite driven by the dynamic cytoskeleton. The GC converts external signals (e.g. gradients of chemo-attractants/-repellants, electrical and mechanical stimuli) into internal rearrangements of the self-assembling biopolymers of the cytoskeleton. These are mainly actin filaments (F-actin) and tubulin microtubes (microtubules (MTs)). This in turn influences GC morphology and enables directional changes. It is well established that the interplay of actin and MT dynamics in the GC is crucial for the directed outgrowth of neuronal extensions [1–4]. Since actin filaments are rather flexible biopolymers with a persistence length of $l_P \approx 17 \mu m$ [5, 6], only structures built from multiple filaments are mechanically relevant for GC dynamics. These are found in the peripheral domain (P-domain) of the GC where a dense sheet ($\sim 500–600$ nm in height) of dynamically cross-linked actin filaments (often referred to as actin gel) is interspersed with radially oriented bundles of aligned filaments termed filopodia. MTs, in contrast, have a comparatively high rigidity with $l_P > 700 \mu m$ [7] and thus individually influence local GC dynamics. They are usually bundled within the neurite shaft from which a subpopulation extends into the P-domain where they splay apart in all angular directions.

These exploring MTs encounter various obstacles on their polymerization path formed by the aforementioned actin structures. During all phases of GC motility, the actin gel polymerized at the leading edge is transported toward the central domain (C-domain), mainly through myosin motor proteins that attach to F-actin and act as contractile force dipoles [8]. This retrograde flow (RF) opposes the growth of exploring MTs [9, 10] and can lead to their deformation and back-transport within the P-domain [11]. However, while the retrograde movement of the actin network obstructs MT outgrowth, radial filopodia can provide optimal polymerization pathways for these filaments [12]. In regular growth phases, the actin bundles in filopodia...
Figure 1. GC structure. F-actin in the P-domain polymerizes and pushes against the leading edge. At the same time it is moving backward in a RF driven by motor protein activity in the T-zone. In the C-domain, filaments depolymerize and replenish the pool of monomers available for (re-) polymerization. Fluctuations in RF and polymerization lead to a net advancement or retraction of the edge. MTs originate in the axon and push into the GC where their advancement is opposed by the flow of actin material.

are predominantly aligned with the RF. MTs attached to and oriented along those bundles reduce the flow-exposed area to their cross-section and thus minimize the forces opposing their advancement. In a way, dynamic actin structures both hinder and promote the exploration of peripheral regions by MTs. This already indicates a system of highly complex interactions. Not all MTs that are able to reach the P-domain of a GC have to be aligned with filopodia [13], but due to the reduced structural support, free MTs have a higher tendency to buckle under the forces they exert against the flow of actin material. An overview of the complex GC structure is shown in figure 1. For further reading regarding GC structure and function, we recommend the review by Lowery and Vactor [14].
Previous studies have shown that GCs with reduced myosin activity contain a larger number of MTs that reach the P-domain [4]. Axons exposed to actin depolymerizing agents are still able to grow out [15] and even reach higher lengths [16]. These early findings already point out that the actin machinery is not the only motor driving neurite outgrowth. In fact, the competition of two counteracting systems seems necessary for maintaining a regulated balance. Contractile forces of the active actin–myosin network are opposed by a dynein–MT-based counterpart driving extension by pushing the whole machinery from within [10, 17]. The forward forces of polymerizing MTs \textit{in vitro} are in the pN range [18, 19]. In combination with dynein or other MT-based motors, it is more than likely that they also contribute to the overall forces required for GC advancement.

MTs that explore areas outside the C-domain without the mechanical support of filopodia tend to buckle due to the compressive forces originating from counteracting MT and actin dynamics. Forces exceeding the critical buckling limit $F_{\text{crit}}$ result in the transition from a straight to a deformed configuration [20, 21]. After the buckling event, MTs get further deformed and (like springs) are able to store substantial amounts of bending energy. In the situation given, one has to consider that these MTs are mechanically supported by the actin gel they are embedded in. This additional support allows MTs to bear and exert even higher forces without buckling than one would expect for ‘free’ filaments, e.g. in an aqueous solution [22, 23]. The fact that extending MTs are at the threshold between un-deformed and buckled states makes them excellent inherent tools to investigate the forces acting in cellular motility processes.

Our analysis of MT deformation shows that forces generated by MTs invading the GC periphery are in the same order of magnitude as the overall GC protrusion force. Hints at a correlation between MT energy density and GC protrusion as well as RF dynamics support the idea that MT distribution and deformation rate are linked to neurite advancement and turning, which might also have implications for mechanical path-finding.

2. Results

2.1. Microtubule curvature analysis in growth cones

We analyzed the deformed shape of MTs in the GCs of NG-108 15 cells transfected with a MT-binding fluorescent marker using a semi-automated detection algorithm based on that described in [24] (see also section 4). From the fluorescent images, the position of the MT center line was extracted and the local curvature was calculated. The cells were additionally transfected with RFP LifeAct plasmids to visualize actin structures and GC morphology. Figure 2 shows a laser scanning image (a) and an overlay of the actin cytoskeleton channel with the color-coded result of the MT curvature analysis (b) for a section of a GC. The full time lapse series of a large stationary GC can be seen in supplementary movie M1 (online supplementary data available from stacks.iop.org/NJP/15/015007/mmedia). In agreement with previous studies we could observe dynamic instability behavior, the bending and looping of single MTs or bundles as well as numerous cases of MTs aligned with filopodia in the P-domain (see examples in figures 2(c)–(f)). The curvature of MTs we analyzed typically ranged from 0 (straight) to 2 $\mu$m$^{-1}$ (corresponding to a radius of curvature of 0.5 $\mu$m). With this tool at hand we were able to further investigate the deformation of buckled MTs in the P-domain as well as the overall relation between MT curvature and GC dynamics.
Figure 2. Results of MT curvature analysis. The MT channel (green) of a combined laser scanning image (a) is processed to detect MT position and curvature. The corresponding overlay of detected MT curvature and actin signal is shown in (b). (c) Parallel MTs often get deformed in similar ways. (d) The majority of MTs in the P-domain is aligned with filopodia; some grow independently and are able to traverse actin bundles. (e), (f) RF forces act over long distances and buckle less stabilized sections of MTs in the filopodia-free C-domain. All scale bars: 2 μm.
Figure 3. Buckling analysis. After the center line (yellow line in (a)) of an individual MT is extracted from the original scan, the locations of highest curvature are detected (red dots in (a)). Their distance measured along the center line corresponds to half the initial buckling wavelength $\lambda_b$. The histogram of detected $\lambda_b$ (b) features two distinct peaks corresponding to different buckling conditions.

For the calculation of the critical force $F_{\text{crit}}$ required to initiate buckling, individual MTs in the P-domain of GCs were selected and separately analyzed. Rods embedded in an elastic medium tend to buckle in higher modes of deformation with smaller amplitudes reducing the energy dissipated in medium deformation. Hence, instead of the total length of the rod (as is the case for classical Euler buckling) the wavelength of buckling $\lambda_b$ is the characteristic length scale to determine the critical force [22]. This wavelength was measured by detecting the positions on the filament with the highest curvature (red circles in figure 3(a)). Assuming a sine wave as the underlying function for buckling deformation, the distance along the contour between these points corresponds to half the buckling wavelength. Note that the distance along the filament contour corresponds to the axial distance between these points at the beginning of buckling. This is important since we may analyze MTs at different post-buckling states and need to approximate the initial buckling wavelength. Applying this method we found an average buckling wavelength of $\lambda_b = 4.02 \pm 1.48 \mu m$ ($n = 51$; all the values presented are means±standard deviation unless otherwise stated). The buckling event can be described within a constrained buckling theory to compare our result with theoretical considerations. This delivers the following dependence between $\lambda_b$, the flexural rigidity $\kappa = EI$ of the rod and the elastic modulus $G$ of the surrounding medium [23]:

$$\lambda_b = 2\pi \left( \frac{\kappa}{\alpha} \right)^{1/4} \approx \frac{4\pi G}{\ln(100)}$$

Using an elastic modulus of $G \approx 27 \text{ Pa}$ [25] and a flexural rigidity $\kappa$ between 0.4 and $2.15 \times 10^{-23} \text{ N m}^2$ for MTs [5, 7], the theoretical wavelength would be between 3.03 and $4.62 \mu m$. Having confirmed that the wavelengths observed are very well in the expected range, the corresponding forces were computed using $F_{\text{crit}} = \frac{8\pi^2 \kappa}{\lambda_b} [21, 23]$. This results in an average critical buckling force of $147.34 \pm 89.82 \text{ pN per MT}$. However, $F_{\text{crit}}$ is the force necessary to buckle a previously straight MT, which might not always be given in the dynamic environment.
of a motile GC. Thus, to avoid over-estimation of the forces, we additionally calculated the (smaller) restoring force $F_{\text{res}}$ that is exerted by an already buckled rod against further axial compression. $F_{\text{res}}$ depends on the contour length $L$ of the buckled rod (in our case $L \approx \lambda_b$) and the distance $d$ between its endpoints in the deformed state [26]:

$$F_{\text{res}} = \frac{1}{2} F_{\text{crit}} \left(\frac{d}{L}\right)^{1/2}.$$

This yields $F_{\text{res}} = 69.49 \pm 43.97 \text{pN}$ for a single MT.

Interestingly, the measured values of $\lambda_b$ (and thus the calculated values for $F_{\text{crit}}$ and $F_{\text{res}}$) are not normally distributed but produce two distinct peaks (figure 3(b)). These are located at $2.81 \pm 0.64$ and $4.39 \pm 0.39 \mu\text{m}$ (combined normal fits) indicating two populations of MTs buckled under different conditions. The corresponding force peaks are located at 76.77 and 208.90 pN for $F_{\text{crit}}$ and at 35.25 and 101.40 pN for $F_{\text{res}}$.

2.2. Bending energy stored in deformed microtubules

As a measure for MT deformation in different areas of GC, the bending energy per unit length $dU/ds$ was calculated in terms of the elastic beam bending theory [20, 21]. The energy stored in the infinitesimal element $ds$ of a deformed elastic filament with a circular cross-section can then be approximated by

$$dU = \frac{\kappa}{2} \left(\frac{d\Theta}{ds}\right)^2 ds,$$

with $d\Theta/ds$ being the local curvature which can be derived from our image analysis.

In total we analyzed 13 image series of active GCs with recording times between 100 and 600 s resulting in curvature map series as shown in supplementary time lapse movie M1 (online supplementary data available from stacks.iop.org/NJP/15/015007/mmedia). The mean values for $d\Theta/ds$ in each frame of each series were calculated and then averaged over time. The overall mean for the bending energy per length stored in the GCs’ MTs is $(1.00 \pm 0.54) \times 10^{-19} \text{J}\mu\text{m}^{-1}$ ($n = 13$) corresponding to $(23.35 \pm 12.61)k_B T$ per $\mu\text{m}$ or $(0.0142 \pm 0.0077)k_B T$ per tubulin dimer in the tube wall lattice (based on approximately $1640 \text{dimers}\mu\text{m}^{-1}$ [27]).

The total amount of bending energy stored in all detected MTs of a GC ranges from 607 to 9409 $k_B T$ with a mean value of 2568 $k_B T$. The scatter in these total energy values partially results from large variations in total MT mass. The P-domain of large GCs can hold tens of MTs while smaller versions only contain a few single filaments. Moreover, not all MTs present in the GC are detectable with our algorithm. Hence, these numbers only constitute a lower boundary for MT bending energies in GCs.

Having in mind the large forces we could ascribe to single deformed MTs, one would expect a correlation between the protrusion of certain areas of the cone and the local deformation of MTs in that area. This was investigated by dividing GCs into different regions of interest (ROIs) and comparing the average bending energy per length to the overall lamellipodium area change in the corresponding ROI. An example of ROI selection can be seen in figure 4(a).

Typically, the GC was divided into two hemispheres and the actin-covered area within these ROIs was evaluated. For a better comparison between GCs of different sizes, all values of one sequence were normalized to the initial area in the first frame recorded. For the GC in figure 4(a)
Figure 4. MT bending energy and GC protrusion. Different areas (ROIs) of the GC show different motility behavior. White lines in (a) mark lamellipodium edges at the beginning of recording ($t = 0$). The actin signal and MT curvature data (color coded) underneath represent the last observation frame ($t = 187$ s). (b) Shows relative area changes during recording. While ROI2 exhibits area loss (edge retraction), ROI1 is in a stationary/protruding state. (c) The bending energy per unit length $dU/ds$ in ROI1 increases over time while in the stationary region after an initial drop it remains constant at a lower level. Solid lines in (c) represent the data smoothed with a fivefold moving average. (d) Plotting the average ROI area increase/decrease (relative area) over the relative bending energy per $\mu m$ (normalized to the overall average in this particular GC) in the respective ROI indicates a relation between higher MT curvature and GC area changes. The red dashed line in (d) corresponds to a linear regression illustrating the trend in the data. Error bars in (d) represent the standard deviation, thus larger bars correspond to stronger fluctuations during the observation period.

The respective relative area versus time plot can be found in figure 4(b). MT curvature was again measured in terms of bending energy per $\mu m$ (figure 4(c)). Subsequently, the time-averaged $dU/ds$ in each ROI was normalized to the overall mean found in the GC under investigation. Thus, relative energy values $>1$ ($<1$) correspond to the above-average (below-average) MT curvature. As shown in figure 4(d), a clear trend evolves connecting lower MT bending energy with higher area losses (lamellipodium retraction) while regions with higher MT energy remain stationary or gain area (advancement).
2.3. Relating retrograde actin flow to microtubule deformation

In all states of GC motility (advancing, stationary, retracting), retrograde actin flow is directed against MT extension. Thus, it is likely that the deformation of MTs is directly related to the local speed of retrograde actin movement. A comparison of subsequent frames of a time series using feature-tracking and cross-correlation algorithms allows one to determine RF velocity within the P-domain of a GC. In three of the GCs under investigation, we were able to detect RF velocities ranging from 1 to 5 \( \mu \text{m min}^{-1} \), which is in good agreement with values previously measured in GCs of the same cell type [28, 29]. We found that RF varies temporally and spatially and often increases for tens of seconds in confined areas. We evaluated the MT behavior in GC sections where such bursts of RF activity occurred and observed an increase in MT deformation as a response to RF velocity increases. Figures 5(a) and (b) display a pair of MTs exposed to RF. While one filament is stabilized by a filopodium, the other gets deformed after a temporal increase in RF velocity. This deformation process is generally observed after RF bursts. In total, we were able to identify seven similar events, all relating higher MT deformation to RF bursts. An example can be seen in figure 5(c) where three bursts are followed by delayed deformation peaks. The relaxation of deformed MTs is accompanied by phases of decreased RF. The time delay between the bursts that mostly occur in the distal part of the P-domain and the corresponding peak in MT deformation ranges between 10 and 25 s and can be ascribed to transport phenomena transmitting peripheral actin flow activity to more centrally located MTs.

3. Discussion

Important functions in neurite outgrowth have often been ascribed to the distribution of rigid MTs in the rather soft periphery of neuronal GCs [1, 30]. In this section, we address the question of what contribution MTs can make to the overall forces generated by an advancing GC.

3.1. Microtubule buckling

Comparing the lower estimate values \( F_{\text{res}} \approx 30 \text{ pN} \) of our study with the \( \approx 100 \text{ pN} \) that a GC can exert against an AFM cantilever (effective cross section: \( 1.15 \mu \text{m}^2 \)), Fuhs et al [25] clearly highlight the relevance of single MT pushing forces for the advancement of the whole GC. Unexpectedly, the distribution of measured buckling wavelengths features two distinct peaks corresponding to two different forces responsible for the buckling events. If we assume constant MT stiffness, the two different buckling conditions must originate from variations in the surrounding actin gel. The lamellipodium is generally not homogeneous since two different states (on/off) of actin polymerization regulate edge dynamics [28, 31] and result in different types of networks. These differ strongly in actin network structure and density [32] which very likely leads to two typical values for network elasticity. Calculating the respective actin network shear moduli with a constant MT bending rigidity of \( 2.15 \times 10^{-23} \text{ N m}^2 \) [5] yields \( G_1 \approx 33 \text{ Pa} \) for the first peak and \( G_2 \approx 200 \text{ Pa} \) for the second peak. The latter, higher value, however, has to our knowledge never been reported in studies investigating GC elasticity [25, 33]. Treating the lamellipodium as an active medium appears to be a more promising approach when aiming to explain the bimodal distribution. In their theoretical work on MTs in active actin gels, Kikuchi et al [34] show that a contractile environment can either weaken or strengthen an embedded stiff filament. The effect depends on the orientation of actin fibers and contractile elements relative to
Figure 5. Correlation between retrograde actin flow and MT deformation. (a) Actin and MT deformation overlay. A MT that is supported by filopodial actin bundles (filled arrowhead) withstands RF while the free filament (empty arrowhead) gets deformed. Scale bar: 2 µm (b) RF and MT deformation overlay. The deformation of the free filament is caused by a temporal increase (burst) of RF velocity. The color bar to the right encodes both the MT deformation in (a) (left scale) and the RF velocity in (b) (right scale). For better visualization different colors for MT deformation were chosen in (b). White arrows in (b) indicate the direction of RF. (c) Example for RF-deformation correlations. The background color represents the average RF velocity in the evaluated area. After RF bursts (pink) MT deformation and thus locally stored bending energy (dashed line) increases. The time delay between burst and MT deformation peaks is about 20 s.
the MT (anchoring). While perpendicular anchoring leads to an effective weakening of the MT, predominantly parallel alignment creates additional mechanical support and impedes buckling. In relation to our findings, this implies that MTs buckled at longer wavelengths (smaller forces) are embedded in actin networks with fibers mainly oriented perpendicular to the MT and those buckled at small wavelengths (higher forces) are effectively stiffened by parallel aligned fibers in their environment. Such populations of aligned and more randomly oriented actin filaments were observed earlier in electron microscopy studies of GCs [35]. Unfortunately, the orientation of actin filaments within the lamellipodium is not accessible with the applied techniques and requires further investigation.

3.2. Microtubule pushing as a unique motility mechanism

In comparison with other motile cell types the protrusion forces of GCs are rather low. Two studies employing identical AFM setups report \( \approx 450 \text{ Pa} \) forward pressure for epidermal fish keratocytes\(^4\) [36] and \( \approx 90 \text{ Pa} \) for NG108-15 GCs [25]. In the context of our results, this means that already three MTs per \( \mu \text{m}^2 \) at the leading edge of a GC are sufficient to generate the aforementioned pressure. In addition, the lamellipodium of GCs is particularly soft [33]. Considering this, the contribution of pushing MTs appears even more significant for GC motility. In the dynamic lamellipodium of keratocytes, MTs are completely absent and these fast and persistently moving cells rely exclusively on the actin–myosin machinery to generate forces [37] which indicate that MTs are not essential for large forward forces but rather help to increase the system’s versatility. GCs apparently have modified motility mechanisms that incorporate pushing MTs as important contributors to directed force generation. This in turn can be related to the fundamentally different biological functions of GCs and keratocytes. While the latter perform stable directed motility with a little reorientation and are optimized for high protrusion velocities, the former undergo frequent morphological changes related to sensitive path-finding, branching and pausing processes. The strong influence of MT dynamics on GC motility has direct implications for mechano-sensitive growth mechanisms as they have been described for various cell types [38–40]. An additional pushing component essentially alters the initial conditions for any force-dependent guidance mechanism. This may also be relevant for the repeatedly observed but still controversially discussed ‘inverse’ durotaxis of neurons. This phenomenon describes the tendency of neuronal processes to advance faster and branch more frequently on softer substrates [41, 42], whereas other cell types (e.g. fibroblasts) preferentially migrate toward stiffer regions [38]. In GCs the traction generated by substrate coupled retrograde actin flow is complemented by anterograde MT pushing. This generally facilitates the forward motion of neuronal extensions in the soft environment of brain and nerve tissue and could be a possible explanation for their unique preference for highly compliant substrates \textit{in vitro}.

3.3. Relating microtubule deformation to growth cone morphology and advancement

We also found indications of a correlation between local MT deformation rates (measured as stored bending energy per MT length \( dU/ds \)) and morphological changes of the GC. Our observations indicate a correlation between higher MT deformation and GC advancement that raises some questions. As mentioned above, previous studies report that straight MTs aligned

\(^4\) Keratocytes are specialized cells that (upon activation) migrate toward lesions and perform wound healing.
with filopodia support advancement and, in the case of non-uniform distribution, turning of GCs [4, 12, 43, 44]. Our data suggest that the occurrence of aligned and straight MTs in the P-domain is not the only indicator of directional changes. Apparently, a higher rate of bending can also be related to an increase in protrusion of certain areas of the GC. It is known that local forces can be transmitted over tens of micrometers via MT deformation [45]. Insight into whether actin dynamics are influenced by highly curved MTs or the increase in bending energy is a result of an otherwise triggered boost in RF or MT–actin cross-links (e.g. proteins of the spectraplakin family [46]) can be gained from the time correlations shown in figure 5(c). Temporal increases in actin flow velocity (‘bursts’) can be related to time-delayed increases in MT deformation followed by a drop in RF velocity. It is reasonable to assume that strongly deformed MTs which expose larger cross-sectional areas to RF and require increasing forces for further deformation hinder actin back-transport, which would explain the transient nature of bursts. RF is simply slowed down by highly deformed MT filaments. This in turn would result in accelerated edge protrusion, which is regulated by the balance between actin polymerization (forward) and RF. Eventually, this means that both filopodia-aligned MTs that directly push and a population of strongly deformed MTs that slow down actin back-transport support edge protrusion, and contribute to directed GC motility. The suggested interplay of actin and MT dynamics is schematically represented in figure 6.

3.4. Concluding remarks

Over the last decades, neuronal research has become a fast progressing interdisciplinary field and many details of GC motility and the underlying molecular processes have been revealed. Nevertheless, many interesting aspects remain to be studied including the influence of microtubule deformation on mechano-sensitive regulation processes and the details of actin-MT cross-linking throughout GC protrusion. The data presented in our study add a new point of view to the understanding of MTs as mechanical components in neuronal GCs and, in particular, their role in force generation and distribution. Adaptations of existing concepts will be required to incorporate the often neglected MT deformation energies and pushing forces resulting in a more complete picture of GC mechanics.

4. Materials and methods

4.1. Cell culture and image acquisition

The NG108-15 hybrid cell line exhibits certain characteristic features of nerve cells, such as differentiation and the spurting of neurite-like processes, which are known to form synapses that are functional on the presynaptic side [47]. We chose this cell line since these cells readily respond to transfection treatments and their well-pronounced GCs constitute ideal model systems for investigations of the underlying cytoskeleton. NG108-15 neuroblastoma cells were purchased from ATCC (Manassas, VA, USA) and cultured in standard growth medium composed of Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (all purchased from PAA, Pasching, Austria). For image acquisition, cells were seeded on custom-made glass bottom Petri dishes or μ-slide 18-wells (IBIDI, Martinsried, Germany) and supplied with phenol red-free Leibovitz’s L-15 medium with 2% B-27® supplement (both from Invitrogen, Darmstadt, Germany). Cells were
Figure 6. Actin–MT interactions. MTs push from within the C-domain and fulfill two functions in the periphery: direct pushing (when aligned) and obstructing RF (when deformed). In area 1 MTs exert large pushing forces $\vec{F}_{MT}$, get deformed and store bending energy (springs). This slows down RF and a larger portion of actin polymerization forces at the edge is converted into forward motion. This area thus is less likely to retract resulting in net edge advancement. In region 2 with short, un-deformed MTs that do not reach the periphery, $F_{MT}$ is small, RF persists and the edge more likely retracts.

transiently co-transfected with mCherry-LifeAct plasmids (IBIDI, Martinsried, Germany) for F-actin visualization and pCS2+/EMTB–3XGFP plasmids (kindly provided by the group of Ewa Paluch, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) for MT visualization. Transfections were performed 24–72 h prior to image acquisition with liposome-based Metafectene®Easy (Biontex, Martinsried, Germany) according to their standard protocol. All images were captured on a Leica TCS SP5 confocal laser scanning microscope equipped with an HCX PL APO CS 63.0 × 1.40 oil immersion objective (Leica Microsystems, Wetzlar, Germany).

4.2. Microtubule curvature analysis

MT deformation analysis was performed using custom written Matlab (MathWorks, Natick, MA, USA) software. The script is based on suggestions by Brangwynne et al [24] for a semi-automated MT tracking algorithm. Briefly, the user manually marks the approximate MT contour which is then used as a basis for refined position determination. The algorithm evaluates multiple image intensity line profiles perpendicular to the estimated MT contour and searches
for Gaussian intensity peaks which are interpreted as filament centers. The set of refined center locations is subsequently fitted by either a modified spline model (complex MT contours in the GC) or by sine functions (buckling analysis). From these fits, the local curvature at each point of the filament can be derived for further analysis.

4.3. Retrograde actin flow analysis

RF detection was performed using custom-written Matlab (MathWorks, Natick, MA, USA) algorithms. Briefly, the image is rasterized and each image tile is compared with a somewhat larger area in the subsequent frame of the series through cross-correlation methods. Thus, prominent actin features can be traced over multiple frames and their velocity is the base for later interpolation steps that deliver the RF maps depicted in figure 5(b). The algorithm was initially developed by Timo Betz and is described in greater detail in [48].

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