Running Head: Superresolution of cortical microtubule dynamics

Jozef Šamaj: Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Cell Biology, Faculty of Science, Palacký University Olomouc, Šlechtitelů 11, 78371 Olomouc, Czech Republic

Tel.: +420 585 634 978

Email: jozef.samaj@upol.cz

Research Area: Cell Biology

Secondary Research Area: Breakthrough Technologies
Dynamics and organization of cortical microtubules as revealed by superresolution structured illumination microscopy

George Komis\textsuperscript{a}, Martin Mistrik\textsuperscript{b}, Olga Šamajová\textsuperscript{a}, Anna Doskočilová\textsuperscript{a}, Miroslav Ovečka\textsuperscript{a}, Peter Illés\textsuperscript{a}, Jiří Bártěk\textsuperscript{b,c}, and Jozef Šamaj\textsuperscript{a,1}

\textsuperscript{a}Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Cell Biology, Faculty of Science, Palacký University Olomouc, Šlechtitelů 11, 78371 Olomouc, Czech Republic

\textsuperscript{b}Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry Palacký University Olomouc, Hněvotínská 5, 779 00 Olomouc, Czech Republic

\textsuperscript{c}Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

Summary: This is the first quantitative study on dynamic organization of plant cell focused on cortical microtubules by using structured illumination superresolution microscopy.

This work was supported by grant P501/11/1764 from the Czech Science Foundation GAČR, by grant LO1204 NPU I Sustainable development of research in the Centre of the Region Haná for Biotechnological and Agricultural Research and by European Commission (project BioMedReg).

Corresponding author:

Jozef Šamaj

Email: jozef.samaj@upol.cz
Abstract

Plants employ acentrosomal mechanisms to organize cortical microtubule arrays essential for cell growth and differentiation. Using structured illumination microscopy (SIM) adopted for optimal documentation of Arabidopsis hypocotyl epidermal cells, dynamic cortical microtubules labeled with GFP-MBD and GFP-TUA6 markers were comparatively recorded in wild type Arabidopsis plants and in mitogen activated protein kinase mutant mpk4 possessing later microtubule marker. The mpk4 mutant exhibits extensive microtubule bundling, due to increased abundance and reduced phosphorylation of microtubule-associated protein MAP65-1, thus providing a very useful genetic tool to record intrabundle microtubule dynamics at subdiffraction level. SIM imaging revealed nano-sized defects in microtubule bundling, spatially resolved microtubule branching and release, and finally allowed quantification of individual microtubules within cortical bundles. Time-lapsed SIM imaging allowed visualizing subdiffraction, short-lived excursions of the microtubule plus end and dynamic instability behavior of both ends during free, intrabundle or microtubule-templated microtubule growth and shrinkage. Finally, short, rigid and non-dynamic microtubule bundles in the mpk4 mutant were observed to glide along the parent microtubule in a tip-wise manner. Conclusively, this study demonstrates the potential of SIM for superresolution time-lapsed imaging of plant cells showing unprecedented details accompanying microtubule dynamic organization.
Introduction

Plant cell growth and differentiation depend on dynamic cortical microtubule organization mechanisms (Ehrhardt, 2008) including branched microtubule formation and release (Murata et al., 2005; Nakamura et al., 2010; Fishel and Dixit, 2013), microtubule-templated microtubule growth (Chan et al., 2009), angle-of-contact microtubule bundling or catastrophe induction (Dixit & Cyr, 2004; Tulin et al., 2012), severing at microtubule cross-overs (Wightman and Turner, 2007) and unique dynamic behavior between steady-state treadmilling and dynamic instability (Shaw et al., 2003).

Cortical microtubule dynamics have been explored in vivo and in vitro with total internal reflection microscopy (TIRFM; Vizcay-Barrena et al., 2011), variable angle emission microscopy (VAEM; Wan et al., 2011), spinning disc microscopy (SD; Shaw & Lucas, 2011), and confocal laser scanning microscopy (CLSM; Shaw et al., 2003). TIRFM and VAEM provide sufficient resolution and speed but at limited depth of imaging (ca. 200 nm; Martin-Fernandez et al., 2013) and inevitably a very narrow field of view when used for in vivo studies (Mattheyses et al., 2010). Dynamic CLSM imaging suffers from field of view limitations while also introducing phototoxicity to the imaged sample. Furthermore, CLSM will require a speed-to-resolution trade off which will necessitate computational extrapolation to bring resolution at affordable levels (Rosero et al., 2014). Finally, SD can provide sufficient depth and speed but otherwise poor resolution owing to aberrations arising from the sample and the properties of the optics commonly used (Shaw and Ehrhardt, 2013).

Microtubule research evolved concomitantly with optical microscopy and the development of fluorescent proteins markers, allowing the resolution of microtubule dynamics and organization at video rates (Marc et al., 1998; Shaw et al., 2013). However, the bulk of plant cells organized in tissues and the optical properties of cell walls hamper microscopic observations, so that the definition of fine details of microtubule organization still relies on laborious transmission electron microscopy (TEM; Kang, 2010).

Alternatively, in vitro assays using TIRF or Allen’s video enhanced contrast - differential interference contrast microscopy (AVEC-DIC; Allen et al., 1981) with purified components have advanced the understanding of microtubule-MAP interactions while providing mechanistic
insight on the function of MAP65 proteins (Tulin et al., 2012; Portran et al., 2013; Stoppin-Mellet et al., 2013), kinesin motors (Song et al., 1997), katanin-mediated microtubule severing (Stoppin-Mellet et al., 1997) and microtubule dynamics (Moore et al., 1997). However, it is explicitly acknowledged that such *in vitro* assays should be addressed in biologically coherent systems with physiological relevance of microtubule dynamics (e.g., see Zanic et al., 2013 and discussion in Gardner et al., 2013). Thus, an ideal approach would be to address microtubule dynamics in the complex cellular environment at spatiotemporal resolutions achieved by *in vitro* assays.

Subdiffraction optical microscopy techniques allow subcellular observations below Abbe’s resolution threshold (Verdaasdonk et al., 2014), circumventing the need for TEM. Such approaches permit dynamic subcellular tracking of appropriately tagged structures within living cells (Tiwari & Nagai, 2013). Practically two superresolution strategies exist. The first involves patterned light illumination, allowing superresolution acquisitions by two fundamentally different methods, i.e., stimulated emission-depletion (STED; Hell, 2007) and structured illumination microscopy (SIM; Gustaffson, 2000). The second interrogates the precision of fluorophore localization and includes stochastic optical reconstruction microscopy (STORM; Kamiyama & Huang, 2012) and photoactivation localization microscopy (PALM; Sengupta et al., 2012). The above regimes differ in translational and axial resolution and their temporal efficiency depends on the size of the imaged area. SIM is probably the best compromise for superresolution live imaging as it offers reasonable lateral resolution (ca. 100 nm; Gustaffson, 2000) which may be reduced to 50 nm (Rego et al., 2012) and sufficient depth of imaging combined with a reasonable axial resolution (ca. 200 nm). SIM allows dynamic imaging in a broader field of view than STED, at biologically meaningful rates compared to PALM and STORM (Kner et al., 2009) and with deeper imaging capacity compared to other superresolution regimes and to TIRFM/VAEM (Leung & Chou, 2011). Superresolution approaches have received limited attention in plant cell biology field (Fitzgibbon et al., 2010, Kleine-Vehn et al., 2011) and their resolution potential during live imaging was not quantified so far.

Hereby, high numerical aperture (NA) objectives are combined with SIM for acquisition and systematic quantification of subdiffraction details of GFP-MBD (Marc et al., 1998; GFP fused to
the microtubule binding domain of mammalian MAP4) and GFP-TUA6-labeled (Shaw et al., 2003) cortical microtubules. For such studies, wild type plants and a *mitogen activated protein kinase 4 (mpk4)* mutant, exhibiting extensive microtubule bundling due to the overexpression and underphosphorylation of MAP65-1 (Beck et al., 2010) were used.

**Results**

**General remarks on sample preparation**

To optimize SIM imaging of *Arabidopsis thaliana* hypocotyl epidermal cells, seedlings were grown in darkness since etiolation promotes the thinning of the outer epidermal wall and reduces the thickness of the cuticular surface (Kutschera, 2008). Thus hypocotyl surfaces can be wetted well with aqueous mounting media used for *in vivo* imaging (1/2 MS medium was used here), excluding air pockets that may introduce additional refractive index mismatches upon imaging. Meanwhile the contour of the etiolated hypocotyl surface is smoother allowing the wider exposure of hypocotyl epidermal cell surfaces underneath the coverslip, as required for high numerical aperture objective with small working distance used hereby. Refractive index mismatches pertaining to fluctuations in coverslip thickness were alleviated by using high performance and low thickness tolerance coverslips. In addition, objective with high numerical aperture (NA 1.57) was used in combination with immersion oil of high refractive index (1.66) in order to obtain maximal possible resolution during SIM, WF, CLSM, TIRF and SD imaging.

**Microtubule organization**

Since details of microtubule organization have been reported using other microscopies, the first task was to characterize qualitatively and quantitatively the resolution potential of SIM and provide relevant comparisons with WF and CLSM with respect to structural features of microtubular arrays. This was particularly important as relevant literature is largely devoid of quantitative resolution data using the above microscopies.

In this respect SIM revealed loop-like defects in GFP-MBD cortical microtubule bundles (Fig. 1A; top inset and Fig. S1A) which were indiscernible by WF (Fig 1A; middle inset and Fig.
S1B) and CLSM (Fig. 1A; bottom inset and Fig. S1C). Visualization of GFP-TUA6 labeled microtubules is inherently compromised by background possibly owing to free tubulin dimers containing GFP-TUA6. With SIM however such problems were significantly ameliorated and GFP-TUA6 cortical microtubules were visualized with high contrast (Fig. 1B and Fig. S1D). Furthermore, it was shown by SIM that GFP-TUA6 is incorporated to the microtubule lattice in a discontinuous manner (Fig. 1B; top inset; Fig. S1D) which was not apparent in WF (Fig. 1B; bottom inset; Fig. S1E). Such discontinuous incorporation of GFP-TUA6 tagged dimers in the microtubule lattice (for mechanism see Waterman-Storer and Salmon, 1998) was best appreciated with linear profiles drawn along GFP-TUA6 labeled microtubules. These showed alternating, bell-shaped fluorescence intensities of variable amplitude, with clear peak-to-peak separation by SIM (Fig. S2A inset and Fig. S2C) as compared to WF (Fig. S2B inset and Fig. S2C). The read-out of such profiles (Fig. S2C) also showed that maximum fluorescence intensities with SIM are well above background levels when compared to WF, further substantiating the use of SIM in generating highly contrasted acquisitions in samples inherently plagued with high background fluorescence as the GFP-TUA6 expressing hypocotyl cells. The speckled appearance of GFP-TUA6-labeled microtubules probably reflects to the stochastic incorporation of GFP-TUA6-containing dimers diluted in a soluble tubulin pool of unlabeled dimers and it might prove a useful source for fluorescence speckled microscopy (Salmon & Waterman, 2011).

Microtubule nucleation through lateral branching from walls of pre-existing microtubules, was also followed by SIM, as it represents a major mechanism of microtubule nucleation in higher plants (Chan et al., 2009). SIM allowed visualizing the very onset of branch formation and release of nascent microtubules (Fig. 1C; top inset and Fig. S1F) while this was not possible with WF (Fig. 1C; bottom inset and Fig. S1G).

In addition, SIM resolved in good detail the composite nature of cortical microtubule bundles, especially in the mpk4 mutant (Fig. 1D; top inset and Fig. S1H), and outperformed WF (Fig. 1D; middle inset and Fig. S1I) and CLSM (Fig. 1D; bottom inset and Fig. S1J) in this respect. The discrimination of very proximal microtubules was best illustrated in orthogonal projections, which provided a clear view on the resolution potential of SIM (Fig. 1E) as compared to WF.
(Fig. 1F) and CLSM (Fig. 1G) making possible to clearly discriminate such adjacent microtubules as individual fluorescent spots with SIM (Fig. 1E) compared to WF (Fig. 1F) and CLSM (Fig. 1G) which showed only one area of diffuse fluorescence.

The quantitative potential of SIM superresolution capacities was measured with the full width at half maximum (FWHM) of normalized, fluorescence intensity profiles drawn perpendicular to individual microtubules. Averaged FWHM values of GFP-MBD and GFP-TUA6 were then compared to respective values obtained with WF and CLSM. During profiling of individual microtubules, care was taken to use identical and co-aligned profiles, meaning that the maximum intensity of all profiles was located at the same position (see Materials and Methods). Since the software used (see Materials and Methods) gives different values for SIM, WF and CLSM, all intensity profiles were normalized to values between 0-1. From the bell curves obtained in this way, the FWHM corresponds to the width of the curve at a normalized intensity value of 0.5. Taking into account the above, SIM outperformed WF and CLSM resolution. The FWHM for GFP-MBD microtubules was 106±20 nm (mean±SD; n=27; Fig. 1H) while for GFP-TUA6 microtubules it was 118±25 nm (mean±SD; n=42; Fig. 1I). The difference between the mean FWHM for GFP-MBD labeled microtubules and for GFP-TUA6-labeled microtubules is not significant (p=0.141). The respective FWHM value for GFP-MBD microtubules visualized by WF was 231±26 nm (mean±SD; n=27; Fig. 1H) and for CLSM it was 235±20 nm (mean±SD; n=27; Fig. 1J), while TUA6-GFP labeled microtubules were resolved at 223±44 nm (mean±SD; n=42; Fig. 1I) by WF and at 325±42 nm (mean±SD; n=47; Fig. 1K) by CLSM.

For some acquisitions a 63×1.40NA oil immersion objective was used to acquire SIM (Fig. 1C, Fig. S3A, S3B) since similar objectives with similar numerical apertures are routinely used for in vivo time-lapsed imaging of plant cells (e.g., Shaw et al., 2003 and van Damme et al., 2004 using 63×1.20NA objectives; Vos et al., 2004 using 63×1.40NA). Although this objective allowed subdiffraction acquisitions with SIM (with an average FWHM of 146±24 nm (mean±SD; n=24; Fig. S3B, S3C) this value was inferior to the 100×1.57NA oil immersion objective which was used exclusively thereon.

Quantitative analysis of cortical microtubule bundles
The most powerful measure of a microscopy platform to resolve intracellular details is the ability to discriminate between very proximal structures. For this reason, it was attempted to quantify the composition of cortical microtubule bundles, taking advantage of the extensive bundling observed in hypocotyl epidermal cells of the *mpk4* mutant. The cortical microtubule bundle composition was approached in both wild type and *mpk4* hypocotyl epidermal cells labeled with GFP-MBD microtubule marker, by two alternative ways: intensity profiling and determination of the Rayleigh criterion.

First, bundles were quantified by means of additive fluorescence intensity. Ideally, this means that the maximum fluorescence intensity of a given bundle would increase linearly upon the successive addition of microtubules. However, it might be expected that according to the specifications of the microscopy used, fluorescence intensities may become saturated after a certain point making it impossible to further quantify microtubule bundles. Thus the second criterion in this approach of quantitation of microtubule bundles was to determine the maximum number of microtubules per bundle before fluorescence intensity was saturated.

By means of absolute fluorescence intensity, bundles visualized by SIM (Figs. 2A to C; Fig. S4A; Figs. 2J, K), were resolvable with very good linear correlation (Figs. 2B, C; n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively; all \( p \) values comparing absolute fluorescence intensity between 1 and 2, 2 and 3, and 3 and 4 microtubules were <0.001). By contrast the linear correlation between microtubule numbers and bundle fluorescence intensity was inferior with WF (Figs. 2D to F; Fig. S4B; Fig. 2K) unable to discriminate between 3 and 4 microtubules (Figs. 2E, F; n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively; \( p=0.158 \) between 3 and 4 microtubules), and such correlations deteriorated even further with CLSM showing saturation after 2 microtubules (Figs. 2G to I; Fig. S4C; Fig. 2L, n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively; \( p=0.057 \) between 2 and 3 microtubules and \( p=0.051 \) between 3 and 4 microtubules). Given the broad variability and the frequent saturation of fluorescence intensity of microtubule bundles observed by WF and CLSM, profiles of such bundles were generated only in cells that were previously observed with SIM.
In fluorescence microscopy, the application of the Rayleigh criterion deems two proximal structures separable when the minimum distance between them (peak-to-peak separation) allows fluorescence intensity drop of ca. 25% of the maximum intensity of either one, provided that the two maximum intensities are nearly equal. In principle, at fluorescence intensity profiles of SIM showing pronounced peak separation (Fig. 3A, D; Fig. S5A) proximal microtubules were indiscriminable by WF (Figs. 3B, E; Fig. S5B) and CLSM (Figs. 3C, F; Fig. S5C). The application of the Rayleigh criterion proved to be particularly cumbersome, especially in the case of WF and CLSM as it was difficult to locate proximal microtubules with nearly equal maximum fluorescence intensities. By applying the Rayleigh criterion comparatively, SIM showed the best discrimination capacity being able to deem adjacent microtubules as separate ones at 131±8 nm (mean±SD; n=32; Figs. 3G, J, M; Fig. S5D), while this distance was 236±17 nm for WF (mean±SD; n=32; Figs. 3H, K, M; Fig. S5E) and 243±16 nm for CLSM (mean±SD; n=32; Figs. 3I, L, M; Fig. S5F).

**Individual microtubule dynamics**

Although SIM provides greatly improved resolution with respect to commonly used descriptive microscopies such as CLSM and WF there are other superresolution approaches including PALM/STORM and STED which can apparently resolve subcellular structures at nearly molecular level. However, PALM/STORM reconstructs superresolution images from raw acquisitions in a time scale of minutes (Cox and Jones, 2013). STED, although much faster, has limitations to time-lapsed imaging of very small fields of view (Westphal et al., 2008), while additionally being phototoxic. Thus the main challenge for SIM would be to evaluate its potential for time-lapsed imaging. It is noteworthy that only few studies of this kind (Kner et al., 2009, Shao et al., 2011, Fiolka et al., 2012) have been published during the 14 year period from first introduction of SIM (Gustaffsson, 2000). Although SIM has been previously demonstrated in plants (e.g., Fitzgibbon et al., 2010, 2013; Linnik et al., 2013; Liesche et al., 2013) its capacity for time-lapsed recordings necessary for quantitative dynamic plant cell studies was never shown before.

To test the efficiency of SIM for time-lapsed imaging, acquisition settings were adjusted to achieve the best possible compromise between spatial and temporal resolution for SIM resulting
in an effective time interval of 2.6 sec (detailed description on SIM adjustments for time lapsed imaging are in Materials and Methods section). This interval is well within accepted published range required to resolve microtubule dynamics (e.g., Shaw et al., 2003; Buschmann et al., 2010) and it was uniformly used as a standard time interval to acquire time lapsed series of cortical microtubule dynamics in all other microscopies used hereby (WF, CLSM, TIRF and SD) as specified in Materials and Methods section. In all cases of imaging the acquisition settings were adjusted towards optimal lateral resolution for the given time frame.

Again the spatial resolution was quantitatively defined by recording the FWHM of a number of averaged, co-aligned and finally normalized transverse intensity profiles centered on individual microtubules. For GFP-MBD labeled microtubules the recorded resolution of SIM was 135±11 nm (mean±SD; n=41; Fig. 4A), while for WF was 226±8 nm (mean±SD; n=41; Fig. 4C), for CLSM was 238±11 nm (mean±SD; n=41; Fig. 4E), for TIRF was 274±14 nm (mean±SD; n=41; Fig. 4G) and for SD it was 323±21 nm (mean±SD; n=54; Fig. 4I). With the SIM module GFP-TUA6 labeled microtubules were resolved at 133±8 nm (mean±SD; n=71; Fig. 4B) while the respective resolution for WF was 225±16 nm (mean±SD; n=71; Fig. 4D), for CLSM was 305±19 nm (mean±SD; n=43; Fig. 4F), for TIRF was 283±19 nm (mean±SD; n=47; Fig. 4H) and for SD was 309±21 nm (mean±SD; n=83; Fig. 4J). The above values correspond to previously published information on the resolution in above mentioned microscopic techniques (e.g., Zucker et al., 1999; Wang et al., 2005). From these data it was concluded that the resolution of SIM, even after the compromises for time-lapsed imaging, significantly exceeded that of WF, CLSM, TIRF and SD. Since SIM clearly outperformed all other techniques, next sections are focused on time-lapsed SIM for quantitative dynamic imaging of cortical microtubules.

In quantitative terms, cortical microtubules of hypocotyl epidermal cells stably transformed with GFP-MBD or GFP-TUA6, exhibited alternating periods of growth succeeded by fast shrinkage (e.g., Figs. 5A, B, E, F; Figs. S6A to S6D and Videos S1 and S2). This behavior of cortical microtubules was best illustrated by respective kymographs (Figs. 5C, D, G, H). Moreover, kymographs of GFP-TUA6 labeled microtubules exhibiting dynamics at both ends, revealed the appearance of successive bright and dark stripes which remained vertical throughout the entire observation time (Figs. 5G, H). Such stripes correspond to the discontinuous incorporation of
GFP-TUA6 in the microtubule lattice as previously described (Figs. 1B, S1D, S2A and S2C; see also Kner et al., 2009). The rates of growth and shrinkage were extrapolated from kymographic analyses of the highly dynamic plus end and the less dynamic minus end. The respective growth and shrinkage rate of the plus end of individual GFP-MBD-labeled microtubules were 6.15±3.06 μm/min (mean±SD; n=65; Fig. 5O; Tables S1 and S2) and 17.65±7.39 μm/min (mean±SD; n=65; Fig. 5O; Tables S1 and S2), while at the minus end GFP-MBD-labeled microtubules were growing and shrinking at 1±0.98 μm/min (mean±SD; n=41; Fig. 5O; Tables S1 and S2) and 0.97±0.95 μm/min (mean±SD; n=33; Fig. 5O; Tables S1 and S2). In the case of GFP-TUA6-labeled microtubules, the plus end was growing and shrinking at 7.84±3.59 μm/min (mean±SD; n=63; Fig. 5O; Tables S1 and S2) and 18.22±5.74 μm/min (mean±SD; n=41; Fig. 5O; Tables S1 and S2) respectively. At the minus end the respective rates were 1.7±1.74 μm/min (mean±SD; n=23; Fig. 5O; Tables S1 and S2) and 1.19±1.12 μm/min (mean±SD; n=26; Fig. 5O; Tables S1 and S2). As illustrated (Fig. 5O) and summarized in Tables S1 and S2, the above measurements are within previously published rates of microtubule growth and shrinkage for both constructs (e.g., Dhonukshe and Gadella, 2003, Shaw et al., 2003, van Damme et al., 2004, Vos et al., 2004) suggesting that SIM can provide a new tool for time lapsed imaging of cortical microtubule dynamics offering significantly higher resolution than commonly used techniques.

Frequently in kymographs from SIM recordings, short length growth and shrinkage events were observed. Such length changes were ca. 200 nm (e.g., Fig. 5C) and they were smaller than the resolution limits reported for time-lapsed WF, CLSM, TIRF and SD. Such events were also taken together for calculating catastrophe and rescue frequencies of individual microtubules according to published procedures (Dhonukshe and Gadella, 2003). Moreover, catastrophe and rescue frequencies were comparatively measured between SIM and WF because such images were acquired simultaneously.

The overall catastrophe frequency of GFP-MBD-labeled microtubule plus ends was 0.020 events/s and the rescue frequency was 0.022 events/s when measured on WF acquisitions. With SIM the catastrophe and rescue frequencies which were measured for the same microtubules as for WF were 0.031 events/s and 0.033 events/s respectively (n=30 microtubules representing 47 minutes of observation).
Intrabundle microtubule dynamics

Microtubule bundles are not static entities and individual microtubules exhibit vigorous dynamic behavior within the crowded environment of a bundle similarly to individual microtubules (Shaw & Lucas, 2011).

As shown previously, SIM offers superior contrast resolution, allowing the nearly linear titration of bundled microtubule numbers by means of fluorescence intensity in the case of overlying microtubules and by means of the Rayleigh criterion for laterally adjacent microtubules. Thus, it was possible to follow and demonstrate intrabundle microtubule dynamics (Figs. 5A, I to K, L to N) by kymographic analyses (Fig. 5J, M) showing that endwise growth and shrinkage rates are comparable to those observed in free microtubules (Figs. 5P; Shaw & Lucas, 2011; Tables S3 and S4). Briefly, plus ends of intrabundle microtubules were growing and shrinking at $7.37 \pm 3.27 \mu m/min$ (mean $\pm$ SD; n=31; Fig. 5P; Tables S3 and S4) and $19.28 \pm 4.86 \mu m/min$ (mean $\pm$ SD; n=33; Fig. 5P; Tables S3 and S4) respectively. Accordingly the respective growth and shrinkage rates of minus ends were $0.89 \pm 0.83 \mu m/min$ (mean $\pm$ SD; n=37; Fig. 5P; Tables S3 and S4) and $1.05 \pm 1.10 \mu m/min$ (mean $\pm$ SD; n=33; Fig. 5P; Tables S3 and S4). Similarly plus end growth and shrinkage rates for GFP-TUA6-labeled intrabundle microtubules were $8.08 \pm 3.98$ (mean $\pm$ SD; n=31; Fig. 5P; Tables S3 and S4) and $21.41 \pm 7.95$ (mean $\pm$ SD; n=35; Fig. 5P; Tables S3 and S4). For the minus ends that were observed the respective values were $0.83 \pm 1.53$ (mean $\pm$ SD; n=31; Fig. 5P; Tables S3 and S4) and $0.89 \pm 1.05$ (mean $\pm$ SD; n=31; Fig. 5P; Tables S3 and S4).

From the kymographs of intrabundle microtubule dynamics it appeared that at the temporal range of observations (which was between 3 and 5 min for all the time-lapsed acquisitions) there were more catastrophe/rescue transitions in intrabundle microtubules than those observed in individual microtubules. In this case small length excursions, resulting from successive rescue/catastrophe transitions, of ca. 200 nm visible by SIM (e.g., Figs. 5J, M, arrowheads) were also considered and hence the overall catastrophe frequency of plus ends of intrabundle microtubules was 0.032 events/s while the rescue frequency was 0.037 events/s (n=112 microtubules representing 321 minutes of observation).
When visible, in most cases minus ends exhibited very slow dynamics and few transitions (Fig. 5J). Exceptionally, very few short microtubules exhibited vigorous minus end dynamics with alternating short length growth and shrinkage resembling dynamic instability (Fig. 5M).

**Microtubule dynamics of the *mpk4* mutant**

The bulk of cortical microtubules of hypocotyl epidermal cells of the *mpk4* mutant reside within bundles therefore it was of interest to provide a preliminary characterization of extra- and intrabundle microtubule dynamics for this mutant.

Thus the dynamic behavior of free (Figs. 6A to E) and bundled microtubules (Figs. 7A to L) was characterized by SIM. Quantitative analysis based on kymographs (Figs. 6C, D) showed that free microtubule ends of GFP-MBD-labeled cortical microtubules grow and shrink at variable rates, while frequently the same microtubule was showing different growth and shrinkage rates during successive events of rescue and catastrophe (Fig. 6D). This resulted in marginal lower rates of growth compared to wild type at 4.14±2.36 μm/min (mean±SD; n=29; Fig. 6E; Tables S1 and S2, by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p=0.0012) but more pronounced decline in shrinkage rates which were measured at 11.68±5.63 μm/min (mean±SD; n=34; Fig. 6E; Tables S1 and S2; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p=0.00056). By contrast, the minus end growth and shrinkage rates were not significantly affected with elongation rated at 0.75±0.75 μm/min (mean±SD; n=36; Fig. 6E; Tables S1 and S2; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p=0.22) and shrinkage at 0.81±0.83 μm/min (mean±SD; n=32; Fig. 6E; Tables S1 and S2; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p=0.50).

Notably, the small length excursions which were reported for free ends of GFP-MBD-labeled cortical microtubules from wild type cells as well as the overall growth/shrinkage transitions appeared to be less frequent in the *mpk4* mutant. Therefore overall catastrophe and rescue frequencies were quantified and their respective values were 0.016 events/s (catastrophe frequency; n=30 microtubules representing 82 minutes of observation) and 0.016 events/s (rescue frequency; n=30 microtubules representing 82 minutes of observation).
Intrabundle dynamics were also addressed in the *mpk4* mutant (Figs. 7A to L; Video S3) and in this case growth and shrinkage rates as well as catastrophe and rescue frequencies were found similarly reduced. Thus, based on kymographs (Figs. 7E to G) intrabundle microtubule plus ends were growing at 4.45±3.27 μm/min (mean±SD; n=22; Fig. 7L; Tables S3 and S4; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p=0.0053) and shrinking at 5.81±5.20 μm/min (mean±SD; n=32; Fig. 7L; Tables S3 and S4; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p<0.0001). The respective rates for discernible minus ends were 0.47±0.63 μm/min (mean±SD; n=37; Fig. 7L; Tables S3 and S4; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p<0.023) and 0.69±0.88 μm/min (mean±SD; n=32; Fig. 7L; Tables S3 and S4; by comparison to plus end dynamics of GFP-MBD-labeled microtubules from wild type cells, p=0.11). The catastrophe and rescue frequencies were 0.015 events/s and 0.020 events/s (n=89 representing 285 minutes of observation).

Side observations on microtubule organization in the *mpk4* mutant showed the formation of short, rigid and non-growing microtubule bundles (e.g., Fig. 8A, B, E) consisting of 3-4 microtubules as judged by their maximum fluorescence intensity (Fig. 8F). These were often positioned free in the cytoplasm but frequently formed tip-wise attachments with the walls of microtubules or microtubule bundles (Fig. 8E). When attached these bundles were either gliding over short distances (e.g., Fig. 8B to D) or swinging around the attachment point (Fig. 8B).
Discussion

General remarks

The temporal resolution of intracellular dynamics always progressed together with advances in microscopy (e.g., Waterman-Storer, 1998). In this respect superresolution techniques that were developed and vigorously upgraded during the past two decades (Hensel et al., 2013) are slowly implemented to biological studies of dynamic subcellular events (e.g., Rego et al., 2012). To date platforms of patterned illumination (e.g., STED and SIM; Hell, 2007, Allen et al., 2014) or precision of localization (e.g., PALM and STORM; Small and Parthasarathy, 2014) superresolution microscopies are commercially available, making superresolution imaging widely accessible.

Knowledge on microtubule dynamics and organization, and their regulation by various MAPs, was largely advanced by *in vitro* imaging of purified components (e.g., Dogterom and Surrey, 2013). In such assays fluorescently labelled or unlabeled microtubules grow in observation chambers, nearly attached to the coverslip and hence they can be very clearly recorded at video rates. However, the *in vitro* acquisition of microtubule dynamics is very frequently conflicting with more physiological *in vivo* studies (Li et al., 2012; Zanic et al., 2013).

In plants a fine example of such discrepancy can be found in the elucidation of the dynamics of *in vitro* assembled and MAP-free carrot tubulin by AVEC-DIC (Moore et al., 1997). Thereby a stunning shortening velocity of 195 μm/min was reported being nearly 10 times higher than the respective rates recorded *in vivo* on microtubules labeled with various markers such as microinjected fluorophore-conjugated brain tubulin, GFP-MBD, GFP-TUA6, GFP-MAP65-1 or GFP-EB1a (e.g., Zhang et al., 1990; Dhonukshe and Gadella, 2003; Shaw et al., 2003; van Damme et al., 2004; Vos et al., 2004).

**Subdiffraction microtubule organization**

Considering the above, SIM was applied to study the organization of cortical microtubule arrays in hypocotyl epidermal cells of *Arabidopsis thaliana* stably transformed with GFP-MBD and GFP-TUA6 markers. Due to the propensity of plant cortical microtubule arrays to form extensive bundles (e.g., Wasteneys and Ambrose, 2009), SIM observations were extended to hypocotyl
epidermal cells of the mpk4 mutant where microtubule bundle formation is more pronounced (Beck et al., 2010).

The point spread function of individual microtubules was determined experimentally for both GFP-MBD and GFP-TUA6 labeled microtubules by means of normalized fluorescence intensity profiling and determination of the FWHM for transverse profiles of such microtubules. As expected from the specifications of the SIM platform used (see Materials and Methods) and the theory behind superresolution imaging with SIM (Gustaffsson, 2000), resolution after high performance imaging (see Materials and Methods for details) was nearly twice as good as the ideal diffraction-limited resolution which cannot exceed 200 nm (Abbe’s limit; e.g., Verdaasdonk et al., 2014), and it reached 106 nm for GFP-MBD labeled microtubules and 117 nm for GFP-TUA6 labeled microtubules. These values are very close to the theoretical 100 nm resolution predicted for linear SIM (Gustaffsson, 2000, Verdaasdonk et al., 2014). In the same manner, high quality acquisitions with WF and CLSM were considerably above Abbe’s limit (roughly between 225 and 325 nm) but nevertheless within the limits reported for such microscopies (e.g., Zucker and Price, 1999; Salmon and Waterman, 2011).

SIM imaging revealed discontinuous incorporation of GFP-TUA6 fusion proteins to the microtubule lattice, leading to the speckled appearance of such labeled microtubules. As previously reported GFP-TUA6 is indistinguishably assembly-competent with unlabeled tubulin dimers incorporating the native TUA6 (Shaw et al., 2003) or other α-tubulin isoforms expressed in hypocotyl cells. Based on the normal phenotypes of GFP-TUA6 transformed seedlings and the similar growth/shrinkage rates of GFP-TUA6-labeled microtubules or microtubules labeled by microinjection of Tradescantia stamen hair cells with fluorophore-conjugated brain tubulin (Zhang et al., 1990), it was concluded that the GFP-TUA6 marker is not affecting microtubule dynamics and function (see Shaw et al., 2003).

The speckled decoration of intracellular filamentous polymeric structures such as actin filaments or microtubules was first reported when such structures were allowed to assemble in the presence of very low levels (e.g. 0.01%-0.25% of labeled molecules with respect to the total pool) of fluorescently labeled monomers diluted in a pool of unmodified monomers (e.g. Salmon and Waterman, 2011). The stochastic incorporation of either labeled or unlabeled tubulin dimers in
microtubule tips led to the development of fluorescence speckled microscopy with significant implications for the in vivo elucidation of microtubule dynamics (e.g., Vallotton et al., 2003). Among others, it provided a tool to discriminate treadmilling-based and motor-driven microtubule translocation (Waterman-Storer and Salmon, 1997; Salmon and Waterman, 2011).

In Arabidopsis thaliana hypocotyl cells labeled with GFP-TUA6 microtubule marker, cortical microtubules were discontinuously labeled leading to their speckled appearance. Visual inspection as well as quantitative fluorescence intensity profiling showed the alternation of bright fluorescent islands and dark areas, separated at variable distances. Since such labeled microtubules exhibited well resolved organization patterns and end-wise dynamics, it can be assumed that the discontinuous microtubule labeling by GFP-TUA6 is owing to the stochastic incorporation of GFP-TUA6-containing tubulin dimers and non-labeled dimers at the microtubule ends.

**Structure of cortical microtubule bundles and other subdiffraction details of the cortical microtubule array**

Microtubule bundle formation in interphase cortical arrays is mediated redundantly by members of the MAP65 family including MAP65-1, MAP65-2 (e.g., Lucas and Shaw, 2012) and possibly MAP65-5 (van Damme et al., 2004). Interestingly, MAP65-1 and MAP65-2 (along with the cytokinesis specific MAP65-3) were previously identified as targets for MAPK phosphorylation (Sasabe et al., 2011) which serves as a negative regulator of MAP65 affinity for the microtubule surface (Smertenko et al., 2006). Importantly, MAP65-1 levels are significantly upregulated in the mpk4 mutant while the levels of phosphorylated MAP65-1 are considerably lower compared to the wild type (Beck et al., 2010). Theoretically, the above observations may explain the mechanism behind extensive cortical microtubule bundling in the mpk4 mutant.

The arrangement of individual microtubules within microtubule bundles was visually and quantitatively very clearly discriminated, suggesting that high performance SIM may be used for mapping the associations of MAPs with microtubules and perhaps resolve intricate microtubular super-structures such as the preprophase microtubule band.
Moreover, in comparison to WF and CLSM, SIM provided subdiffraction details of cortical microtubule organization not resolvable with the other techniques. With high performance SIM it was possible to capture the onset of branched microtubule formation and release. The γ-tubulin-mediated branch initiation (Chan et al., 2009; Nakamura and Hashimoto, 2009; Nakamura et al., 2010) and the katanin-mediated branch release (Nakamura et al., 2010) are key mechanisms supplementing the cortical array with nascent microtubules, thus SIM may provide a subdiffraction tool that will allow the spatiotemporal resolution of this successive nucleation and severing mechanism.

Subdiffraction microtubule dynamics

Time-lapsed imaging by SIM was traded for speed, thus the resolution of individual microtubules was slightly inferior (135 and 133 nm for GFP-MBD and GFP-TUA6, respectively) but still significantly outperformed all other imaging approaches (WF, CLSM, TIRF and SD). Unfortunately, literature on dynamic plant cell studies is largely devoid of resolution data. Thus there is no comparison measure other than the data included hereby. However, the FWHM values for WF, CLSM, TIRF and SD are comparable to the resolutions reported elsewhere (e.g., Zucker et al., 1999; Wang et al., 2005; Salmon and Waterman, 2011), which optimally range within 250-270 nm. If this is also true for studies published previously in plants then the difference between SIM and any other microscopic techniques is ca. 120 nm, corresponding to roughly 195 tubulin subunits ([120 nm × 13 protofilaments]/8 nm tubulin dimer size) in terms of microtubule length.

Microtubule dynamics at the faster plus end or the slower minus end evolve with the linear addition or removal of tubulin dimers (Desai and Mitchison, 1997). Since the addition of tubulin dimers during growth or the removal during shrinkage are roughly linear function of time the respective rates are not expected to be influenced by the resolution of the microscopy platform used. Indeed this was the case of SIM time-lapsed recordings of microtubule growth and shrinkage rates, which were within previously published ranges for both molecular markers (GFP-MBD; e.g. van Damme et al., 2004; Vos et al., 2004; and GFP-TUA6; e.g. Dhonukshe and Gadella 2003; Shaw et al., 2003). Similarly the elongation and shrinkage rates measured for...
intrabundle microtubules did not differ significantly from those previously published (Shaw and Lucas, 2011).

However, SIM imaging revealed that at the standard time interval used hereby (2.6 s), there were short length transitions resulting in microtubule length changes of ca. 200 nm which were smaller than the resolutions of WF, CLSM, TIRF and SD. Such small length transitions were more frequently observed in intrabundle than in independent microtubules and their occurrence contributed to the higher catastrophe and rescue frequencies which were calculated for intrabundle microtubules.

In line with speckled appearance of GFP-TUA6 labeled microtubules in SIM, the kymographic analyses of such labeled microtubules showed alternating bright and dark vertical stripes which remained throughout the entire course of observations. If minor or major translocations of the entire microtubule as frequently observed in plants (this study; e.g., Shaw et al., 2003) were owing to motor-driven gliding then the stripes appearing in kymographs would appear inclined since the stably incorporated GFP-TUA6 speckles would translocate together with the entire microtubule. Since the stripes in SIM kymographs remain vertical (i.e., the GFP-TUA6 speckles remain immotile through time), it can be deduced that such translocations owe to the hybrid treadmilling mechanism described before (Shaw et al., 2003). This mechanism was proven by generating a marker on the wall of growing GFP-TUA6-labeled microtubules by means of photobleaching creating a dark spot, which was translated as a dark stripe on the respective kymograph (Shaw et al., 2003). The photobleaching approach can be applied to a single or few microtubules at a time, thus it is time-demanding to generate the amount of data necessary for quantitative evaluation. On the contrary, application of fluorescence speckle microscopy by means of SIM allows the simultaneous recordings of large populations of microtubules at once because it is a widefield method (Gustaffsson, 2000).

**Microtubule dynamics in the mpk4 mutant**

In the mpk4 mutant, it was found that growth and shrinkage rates were reduced for both extra- and intrabundle microtubule dynamics. Since these rate reductions occurred in both microtubule classes, it is likely that overabundant but underphosphorylated MAP65-1 in the mpk4 mutant (Beck et al., 2010) is not related to end-wise microtubule dynamics. This is somewhat expected
since at least MAP65-1 does not bind to soluble tubulin dimers (Smertenko et al., 2004) while using *in vitro* assays, the addition of MAP65-1 is not affecting growth and shrinkage rates (Stoppin-Mellet et al., 2013). Quite contradictory results were obtained from *in vivo* microtubule dynamic measurements in tobacco BY-2 suspension culture cells stably transformed with *proCaMV35S::GFP:AtMAP65-1* construct showing marginal increase of growth rates compared to cells transformed with *proCaMV35S::GFP-MBD* (probably insignificant; see Table I from van Damme et al., 2004), but rather steep reduction of depolymerization rates (nearly 50%; see Table I from van Damme et al., 2004).

Considering: (a) the inability of MAP65-1 to bind to tubulin dimers and affect their incorporation to microtubule ends (Smertenko et al., 2004, 2008), (b) the similar polymerization/depolymerization rates measured for extra- and intrabundle microtubules (this study; see also Shaw and Lucas, 2011) and (c) the similar reduction of intra- and extrabundle microtubule dynamic rates in the *mpk4* mutant, it is probable that the overall downregulation of growth and shrinkage rates observed in the *mpk4* mutant is owing to a MAP65-1-independent mechanism.

In the *mpk4* mutant, the transition frequencies were also decreased leading to smaller number of both catastrophes and rescues. However, catastrophe and rescue frequencies were similarly reduced for both free and intrabundle microtubules. *In vitro* experiments showed that MAP65-1 caused decreased catastrophe frequency and amplitude and increased rescue frequency and amplitude (Stoppin-Mellet et al., 2013). These *in vitro* frequencies are again contradictory to *in vivo* observations (van Damme et al., 2004) showing that in tobacco BY-2 cells heterologously overexpressing AtMAP65-1-GFP fusion protein, catastrophe frequency is comparable to that of GFP-MBD labeled microtubules but rescue frequency is nearly half of the respective frequency of the GFP-MBD-labeled microtubules.

In this context the downregulation of both catastrophe and rescue frequencies in the *mpk4* mutant in combination with the downregulation of polymerization/depolymerization rates, indicate more complex mechanisms of microtubule regulation in the *mpk4* mutant as the simple straightforward involvement of MAP65-1. Elucidation of this mechanism is out of the scope of the present work and deserves further attention. Nevertheless, it was already predicted (Šamajova et al., 2013) that
other cytoskeletal proteins involved in various aspects of microtubule organization and dynamics
may be targeted and regulated by MAPK-dependent phosphorylation.

Concluding remarks and perspectives

Successful applications in live cell biology studies lag behind current advancements of
superresolution microscopy, owing to physical burdens imposed by the size and contour of plant
cells as well as by optical properties of cell walls. Herein one promising superresolution
technique, SIM, is quantitatively assessed and shows the potential to follow spatio-temporal
evolutions of cortical microtubules.

Present findings indicate the potential of SIM to unravel subdiffraction details of plant cortical
microtubule organization. Using SIM with high resolution optics and imaging setup, individual
microtubules were resolved well below Abbe’s limit. It was possible to describe the complexity
of microtubule bundle structure and characterize extra- and intrabundle microtubule dynamics
with length fluctuations below the limits of other techniques such as WF, CLSM, TIRF and SD.
Live SIM imaging will allow to shed new light on interactions of microtubules with MAPs that
induce bundling and bias the parallel arrangement of cortical arrays (Tulin et al., 2012, Stoppin-
Mellet et al., 2013), but also to track microtubule nucleation processes in plant acentrosomal
cells (Binarová et al., 2006, Nakamura et al., 2010, Kirik et al., 2012, Stoppin-Mellet et al.,
2013). Finally, the resolution of dynamic features of microtubule structures such as the
preprophase microtubule band (Müller et al., 2009), the mitotic spindle (Zhang &Dawe, 2011),
and the phragmoplast (Smertenko et al., 2011) could be the challenging task for future live SIM
imaging in the plant field.
Materials and Methods

Plant material and sample preparation for microscopy

Seedlings of *Arabidopsis thaliana* ecotype Columbia (Col-0) carrying either GFP-MBD or GFP-TUA6 constructs as well as *mpk4* mutant (in Col-0 background) carrying GFP-MBD construct were used. Plants were stably transformed by the floral dipping method (Clough & Bent, 1998) with either a cauliflower mosaic virus 35S promoter driven GFP fusion of the microtubule binding domain of the mammalian microtubule associated protein MAP4 (*proCaMV35S::GFP-MBD*) (Marc et al., 1998), or with a similarly expressed fusion of GFP with the TUA6 alpha-tubulin isoform (*proCaMV35S::GFP-TUA6*) provided by Dr. Sidney Shaw. Seeds were surface sterilized and placed on half-strength MS culture medium (pH 5.7) without vitamins containing 1% (w/v) sucrose and 0.4% (w/v) phytagel. The plates were stored at 4°C for 48 h to break dormancy, and then kept vertically under 22°C in darkness for 5 days. Seedlings expressing fusion constructs visualizing microtubules were transferred to microscopic slides that had been modified into thin chambers using high precision and low thickness tolerance Nexterion round cover slips (SCHOTT CR, a.s. Zašovská 850, 75701 Valašské Meziříčí, Czech Republic, HI, D=0.17 mm ± 0.003 mm, diameter=25mm for objective "Plan-Apochromat" 100x/NA1.57 Oil, DIC Corr, 000000-1787-996, very dense flint N-SSK2, refractive index n = 1.62229, Abbe numbers Vd = 53.27, Ve = 52.99, reflectance at 0.5876 µm R = 0.05632). The chambers were filled with the liquid half-strength MS medium either using one layer of parafilm as spacer or without this spacer. Microchambers with seedlings were sealed with silicone paste to prevent evaporation of liquid medium and stabilize samples during microscopy examinations.

Microscopy setup, optics and image acquisition

For WF, SIM, CLSM and TIRF all samples were examined in a Zeiss Axioimager Z.1 platform equipped with the Elyra PS.1 super-resolution system for SR SIM and the LSM780 module for CLSM using Zeiss objectives Alpha Plan Apochromat 63x/NA1.40 oil objective (tot. mag. 1008x) and Alpha Plan Apochromat 100x/NA1.57 oil objective (tot. mag. 1600x) with appropriate oils (Immersol 518F with refractive index of 1.518 for 63x/NA1.40 objective, and Immersol HI with refractive index of 1.66 for 100x/NA1.57 objective). In few cases longer imaging was done on a Zeiss LSM710 platform with a 63x/NA1.40 objective. Light source for
SR SIM and WF included diode laser 488-100 (488nm). Images were captured with an EM-CCD camera (Andor iXON EM+; 1004x1002 px, cooled at -64°C, 16-bit) at typical exposure times varying between 80-200 ms and with gain values between 20-25. High performance SR-SIM setup included 5 rotations and 5 phases of the grated pattern for each image layer. Gratings for patterned illumination were spaced by 42 µm for the 100x/NA1.57 oil immersion objective. Up to 7 (usually 3) Z-stacks were acquired per image with a slice thickness of 110nm for the 100x/NA1.57 objectives. Light source for CLSM included Argon-Neon Laser (458, 488 and 514 nm). Images were captured using GaAsP spectral detector, scanning speed 4, line averaging 4 and pinhole: Airy unit. CLSM was setup to meet the SR-SIM magnification (1008x and 1600x depending on the objective) in the optimum pixel resolution (according to Nyquist sampling criteria).

For time lapsed imaging with SIM the Alpha Plan Apochromat 100x/NA1.57 oil objective was exclusively used and images were acquired from a single optical section. Grid rotations were reduced from 5 to 3 and the exposure time of the EM-CCD was reduced to minimum. In this way, time interval was 2.6 seconds and this value was used as a standard for WF (acquired simultaneously with SIM), CLSM, SD and TIRF in order to ensure similar temporal resolution employing the same objective.

SIM, WF, CLSM and TIRF platforms are integrated in the Zeiss Elyra PS.1 system used hereby, therefore it was possible to acquire sequential time-lapsed series exactly from the same cell with the aforementioned microscopies. Spinning disc microscopy was conducted using an independent microscopy platform equipped with the Zeiss Axio Observer inverted microscope combined with a Yokogawa CSU-X1 scanning head. The same Alpha Plan Apochromat 100x/NA1.57 oil objective, cover slips and immersion oil (Nexterion high precision coverslips and high refractive index Immersol HI as used for acquisition of SIM images) were used for bioimaging with this system. Images were acquired by high resolution camera Evolve 512 black-thinned EM-CCD (Photometrics).

**Image processing and quantitative analysis**

Raw CLSM, WF and SR SIM images were acquired with Zeiss Zen 11 software (Zen Blue version, Carl Zeiss Microscopy GmbH, Jena). Measurements involving intensity profiles of
individual microtubules or microtubule bundles were directly conducted in Zen 11. For multiple measurements, the original line selected for intensity profiling was cloned with the appropriate Zen 11 tool to different positions of the same or other individual or bundled microtubules in the image. To alleviate for differences in absolute intensity values between WF, SIM and CLSM images, raw values were exported to MS Excel, normalized to a range between 0 and 1 and plotted against distance. Scatter plots of normalized intensity values vs distance were used to measure FWHM with Image J.

To quantitatively address bundle structure by means of maximum absolute fluorescence intensity, line profiles were drawn perpendicular to cortical microtubule bundles acquired by SIM (as depicted in Figs. 2A, D, G), based on visual inspection of the actual individual microtubules converging to such bundles, to avoid user bias on placing profiles based on intensity values. The respective profiles were then exactly copied to the corresponding WF image and the sequentially acquired CLSM image (appx. 2 min after the simultaneous acquisition of the SIM/WF image). This was done because absolute fluorescence intensities of individual microtubules visualized by WF and CLSM showed broad variability, thus absolute fluorescence intensity could not serve as a predictor to decide the placement of the profile as to correspond the microtubule number independently for WF and CLSM.

Kymographs of dynamic microtubules were generated by three alternative ways: either by using the time profile tool of the Zen 2011 blue version, or in Image J, on line selections of time stacks subsequently resliced, or by using the MultipleKymograph plugin for Image J (http://www.embl.de/eamnet/html/body_kymograph.html) developed by J. Rietdorf and A. Seitz (European Molecular Biology Laboratory, Heidelberg, Germany). Quantitative analysis of kymographs was done again in ImageJ using the Kymoquant plugin (http://cmci.embl.de/downloads/kymoquant) written by Koto Miura (European Molecular Biology Laboratory, Heidelberg, Germany).

Catastrophe and rescue frequencies were calculated according to previously published work (Dhonukshe and Gadella, 2003). Briefly, total number of events (summed from as many individual microtubules as reported in text) was divided by total amount of time required for
such events. In this was cumulative $f_{\text{cat}}$ and $f_{\text{res}}$ are calculated and they are devoid of standard deviation.

For resolution studies via fluorescence intensity profiles and microtubule dynamic parameters measurements were carried out on the designated microtubule numbers (reported in both main text and figure legends) from 64 hypocotyl epidermal cells from 26 different etiolated seedlings (for SIM/WF/CLSM/TIRF/SD microscopic acquisition of GFP-MBD labeled microtubules); from 50 hypocotyl epidermal cells from 26 etiolated seedlings (for SIM/WF/CLSM/TIRF/SD microscopic acquisition of GFP-TUA6 labeled microtubules); and from 21 hypocotyl epidermal cells from 12 etiolated seedlings (for SIM/WF/CLSM/TIRF/SD microscopic acquisition of GFP-MBD labeled microtubules).

**Statistical analysis**

Unpaired, two-tailed Student’s t-test assuming unequal variance was applied to deem statistical significance particularly between different dynamic values of microtubules from the different experimental conditions described. Data (mean values, standard deviations, sample number and summary of p-values from pairwise t-tests are shown and explained in Supplementary Tables 1, 2 for individual, unbundled microtubules and in Supplementary Tables 3,4 for intrabundle microtubules). All descriptive data in main text and graphs are mentioned and depicted as mean±standard deviation.

**Supplemental Material**

**Table S1:** Summary of extrabundle microtubule dynamics in wild type expressing GFP-MBD or GFP-TUA6 markers and *mpk4* mutant expressing GFP-MBD marker.

**Table S2:** Pairwise Student’s t-test comparing growth and shrinkage rates from Table S1.

**Table S3:** Summary of intrabundle microtubule dynamics in wild type expressing GFP-MBD or GFP-TUA6 markers and *mpk4* mutant expressing GFP-MBD marker.

**Table S4:** Pairwise Student’s t-test comparing growth and shrinkage rates from Table S3.

**Figure S1:** Full view of unprocessed images used in Figure 1.
Figure S2: The discontinuous incorporation of GFP-TUA6 marker in the microtubule lattice of hypocotyl epidermal cells of Arabidopsis thaliana transformed with the CaMVpro35S::GFP-TUA6 construct.

Figure S3: Full view of hypocotyl epidermal cell expressing GFP-MBD marker, showing SIM performance using 63×/NA1.40 oil immersion objective.

Figure S4: Full view of the image used in Fig. 2.

Figure S5: Full view of the images used in Fig. 3.

Figure S6: Full view of the images used for kymographs of Figs. 5D, H.

Figure S7: Longitudinal line profiles of absolute fluorescence intensity along bundle shown in Fig. 7B corresponding to selected time frames from a hypocotyl epidermal cell of mpk4 mutant expressing GFP-MBD microtubule marker.

Video S1: Video of epidermal hypocotyl cell transformed with GFP-MBD, corresponding to Fig. 5D and Figs. S2A, S2B.

Video S2: Video of epidermal hypocotyl cell expressing GFP-TUA6 marker, corresponding to Figs. 5E to G.

Video S3: Video of epidermal hypocotyl cell of mpk4 mutant expressing GFP-MBD marker corresponding to Figs. 7A to G and 8A to E.

Acknowledgements

We thank Dr. Tomáš Takáč for useful suggestions, Dr. Klaus Weisshart (Carl Zeiss Microscopy) for validating our raw data, and Dr. Sidney Shaw for seeds with GFP-TUA6 construct.
References


Gustafsson MG (2000) Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J Microsc 198: 82-87


Waterman-Storer CM (1998) Microtubules and microscopes: how the development of light microscopic imaging technologies has contributed to discoveries about microtubule dynamics in living cells. Mol Biol Cell 9: 3263-3271


**Figure legends**

**Figure 1:** Comparison of SIM, WF and CLSM in resolving fine details of cortical microtubule organization in hypocotyl epidermal cells of *Arabidopsis thaliana* expressing either GFP-MBD or GFP-TUA6 microtubule markers. (A) Overview of bundled GFP-MBD-labeled microtubules in a hypocotyl epidermal cell of wild type Arabidopsis thaliana (see also Fig. S1A). The boxed area includes a loop within a bundle which is readily visible by SIM (top inset, arrow), but not by WF (middle inset, arrow; Fig. S1B) or CLSM (bottom inset, arrow; Fig. S1C). (B) Overview and detail of a cortical microtubule bundle in an epidermal cell expressing TUA6-GFP (Fig. S1D). Top inset shows the discontinuous incorporation of the tagged tubulin revealed by SIM which is however not discernible by WF (bottom inset; see also Fig. S2A to S2C). (C) Overview and details of microtubule branch formation and release in a hypocotyl epidermal cell expressing GFP-MBD microtubule marker (Fig. S1F) acquired with a 63×1.40NA oil immersion objective. A nascent branch (top inset, arrow) and a newly released branch (top inset, arrowhead) are clearly visible by SIM, but not by WF (bottom inset, respective arrow and arrowhead). (D) Microtubule bundle complexity in a hypocotyl epidermal cell of the mpk4 mutant as visualized by SIM (Fig. S1J). Such bundles (white boxed area in D) can be resolved in detail by SIM (top inset) but not by WF (middle inset) or CLSM (bottom inset). (E to G) Details (left column) and orthogonal projection (right column) of three closely proximal microtubules (lines in E to G) from the dotted boxed area in (D). By SIM (E; Fig. S1J) the three microtubules are clearly separated as three fluorescent spots, while in the respective WF (F; Fig. S1K) and CLSM (G; Fig. S1L) images the three microtubules appear as a fuzzy fluorescent area. (H to K) Quantitative analysis of the resolution of individual GFP-MBD (H, J) or GFP-TUA6 (I, K) labeled microtubules by means of SIM and WF (H, I) or CLSM (J, K). The graphs represent averaged, co-aligned and normalized intensity profiles (as described in Materials and Methods section) of individual cortical microtubules (n= 27 and 42 for SIM (GFP-MBD and GFP-TUA6 respectively), 27 and 42 for WF (GFP-MBD and GFP-TUA6 respectively) and 27 and 47 for CLSM (GFP-MBD and GFP-TUA6 respectively)). Scale bars: 5 μm (A to G), 2 μm (insets of A to C).
Figure 2: Quantitative analysis of GFP-MBD-labeled cortical microtubule bundles by means of absolute fluorescence intensity comparing the resolution capacity of SIM, WF and CLSM. (A to C) SIM overview (A; Fig. S4A) of an area with microtubule bundles with different microtubule numbers (1, blue line; 2, green line; 3, red line and 4, purple line) quantified by means of fluorescence intensity profiling (B) corresponding to the colored lines in (A) and quantitative evaluation of microtubule number per bundle (C; mean±SD; R², linear correlation coefficient; *, p<0.001; n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively). (D to F) The same area shown in (A) after WF acquisition (D; Fig. S4B) with the representative (E) and the averaged maximum fluorescence intensities (F; mean±SD; R², linear correlation coefficient; *, p<0.001 comparing 1 with 2 and 2 with 3 microtubules; N.S. non-significant difference (p=0.158 between 3 and 4 microtubules); n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively). (G to I) CLSM imaging (G; Fig. S4C) of the same area depicted in (A) showing the representative (H) and the averaged (I; mean±SD; R², linear correlation coefficient; *, p<0.001 comparing 1 with 2 microtubules; N.S. non-significant difference (p=0.057 between 2 and 3 microtubules and 0.051 between 3 and 4 microtubules); n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively) fluorescence intensities corresponding to increasing microtubule numbers per bundle. (J to L) Detailed view (J) of the boxed area in (A) and the fluctuation of absolute fluorescence intensities along the longitudinal profile (represented by red, tan and orange brackets in J; white arrow points to fluorescence intensity depression between red and tan brackets) by SIM, WF (K) and CLSM (L). Brackets in (K, L) correspond to the fluorescence of the respective brackets in (J). Note that the intensity along the red and the tan bracket is well discriminated in SIM, declining incrementally (K), less discriminated in WF declining nearly linearly (K) and not discriminated at all in CLSM (L). Black arrows in (K, L) correspond to the intensity drop marked with white arrow in (J). Scale bars: 5 μm (A, D, G) and 2.5 μm (J). For clarity the colored line bars in A, D, G, corresponding to the fluorescence intensity profiles plotted in B, E, H are twice as long as the actual profile length.
Figure 3: Resolution of proximal cortical microtubules labeled with GFP-MBD microtubule marker, comparing SIM, WF and CLSM. (A to C) The same cortical area of a hypocotyl epidermal cell expressing GFP-MBD fusion protein showing two adjacent microtubules by SIM (A; full view in Fig. S5A), WF (B; full view in Fig. S5B) and CLSM (C; full view in Fig. S5C). (D to F) Normalized fluorescence intensity profiles corresponding to white lines in (A to C), showing clear discrimination of the two adjacent microtubules as two peaks in SIM (D) in contrast to the fluorescence intensity profiles obtained from the respective WF (E) and CLSM (F) acquisitions showing only one peak. (G to I) Different areas from the same cell visualized with SIM (G; full view in Fig. S5D), WF (H; full view in Fig. S5E) and CLSM (I; full view in Fig. S5F). White lines across adjacent microtubules represent the position from which fluorescence intensity profiles were acquired and correspond to the Rayleigh resolution limit (i.e., peak-to-peak discrimination at ca. 25% depression of maximum fluorescence intensity). (J to L) Quantitation of the corresponding intensity profiles shown in (G to I). Brackets denote the peak-to-peak separation at the Rayleigh limit. (M) Graph depiction of the averaged Rayleigh limits measured for SIM, WF and CLSM (mean±SD, n=32 measurements in all cases; *, p<0.001 comparing Rayleigh limits from SIM with WF and CLSM). Scale bars for all Figures: 5 μm.

Figure 4: Resolution of individual cortical microtubules of Arabidopsis thaliana hypocotyl epidermal cells, labeled with GFP-MBD (A, C, E, G, I) or GFP-TUA6 (B, D, F, H, J) during time-lapsed imaging with SIM (A, B), WF (C, D), CLSM (E, F), TIRF (G, H) and SD (I, J) microscopies. Scatter plots represent averaged weighted profiles of many individual microtubules. (A, B) Resolution of individual cortical microtubules labeled with GFP-MBD (A; n=40) and GFP-TUA6 (B; n=71) after SIM imaging. (C, D) Resolution of individual cortical microtubules labeled with GFP-MBD (C; n=40) and GFP-TUA6 (D; n=71) after WF imaging. (E, F) Resolution of individual cortical microtubules labeled with GFP-MBD (E; n=41) and GFP-TUA6 (F; n=43) after CLSM imaging. (G, H) Resolution of individual cortical microtubules labeled with GFP-MBD (G; n=41) and GFP-TUA6 (H; n=47) after TIRF imaging. (I, J) Resolution of individual cortical microtubules labeled with GFP-MBD (I; n=54) and GFP-TUA6 (J; n=83) after SD imaging. Black lines are positioned to a normalized fluorescence
intensity (0.5) corresponding to the FWHM of each respective curve. Scale bars for all Figures: 5 μm.

**Figure 5:** Independent and intrabundle dynamics of GFP-MBD and GFP-TUA6-labeled cortical microtubules visualized by SIM. (A to C) Overview (A) of a hypocotyl epidermal cell expressing GFP-MBD microtubule marker during time-lapsed imaging with SIM, selected stills of a free microtubule visible at both ends (right boxed area in A; B) and respective kymograph of the same microtubule exhibiting short length transitions of growth and shrinkage (full arrowheads; C). (D) Kymograph representing dynamics of free cortical microtubule (corresponds to Figs. S6A, B; Video S1) visible at one end showing smooth, uninterrupted growth (solid lines) and shrinkage (catastrophe; dotted line). (E to G) Overview (E) of a hypocotyl epidermal cell expressing GFP-TUA6 microtubule marker, selected stills of a fully visible independent microtubule (boxed area in E; F; arrowheads point to plus end; Video S2) and respective kymograph showing dark and bright striations owing to the inhomogeneous incorporation of GFP-TUA6 in the microtubule lattice (G). (H) Another kymograph of a GFP-TUA6-labeled microtubule where minus end behavior can be followed (corresponds to Figs. S6C, S6D). (I) Selected stills of a microtubule growing within a bundle from the top right boxed area in (A). (J) The respective kymograph showing vigorous plus end dynamics and short length excursions in the plus end (arrowheads) and minimal dynamics at the minus end. (K) Longitudinal fluorescence intensity profile along the bundle encompassing the microtubule tracked in (I). (L) selected stills of a short intrabundle microtubule (arrowheads) selected from the left boxed area of (A). (M) The corresponding kymograph of the microtubule in (L) showing frequent and short length transitions in the plus end (full arrowheads) and dynamic instability at the minus end (open arrowheads). (N) The longitudinal fluorescence intensity profile harboring the short intrabundle microtubule depicted in (L). (O, P) Graphs depicting average plus and minus end growth and shrinkage rates reported for GFP-MBD and GFP-TUA6 labeled extrabundle (O) and intrabundle (P) microtubules from values summarized in Tables S1 and S3 for free and intrabundle microtubules respectively. All full arrowheads point to plus ends and all open arrowheads point to minus ends. Scale bars: 5 μm (A, E), 2 μm (B, F, G, L), 1 μm (C, D, I, J, M) and 0.5 μm (H). Time bars in kymographs: 127.85s (C), 80s (M), 60s (J) 34.5s (D), 30s (G) and 10s (H). (+) indicate microtubule plus ends and (-) indicate microtubule minus ends. Numbers in
(B, F, I, L) are in min:sec.msec. In all kymographs (C, D, G, H, J, M) time evolution is from top to bottom.

**Figure 6:** Overview of independent cortical microtubule dynamics of hypocotyl epidermal cells of the *mpk4* mutant expressing GFP-MBD fusion protein, as visualized by SIM and quantified accordingly. (A to C) Widefield SIM view (A), selected stills (B) from the boxed area in (A) and respective kymograph showing a shrinkage (C, dotted line) and a successive growth event (C, full line). (D) Another kymograph of a free growing microtubule showing two growth phases with different rate (full lines) interrupted by one catastrophe (dotted line). Arrowheads denote minor such transitions. (E) Graphic illustration of plus and minus end dynamics of free microtubules observed by SIM in hypocotyl epidermal cells of GFP-MBD-transformed *mpk4* mutants as summarized in Table S1. Scale bars: 5 μm (A), 2 μm (B), and 1 μm (C, D). Time bars in (C, D) 60 s. (+) indicate microtubule plus ends. Numbers in (B) are in min:sec.msec. In all kymographs (C, D) time evolution is from top to bottom.

**Figure 7:** Intrabundle microtubule dynamics of the GFP-MBD expressing *mpk4* mutant as acquired with SIM. (A) Overview (A; see also Video S3) of an *mpk4* hypocotyl epidermal cell with GFP-MBD-labeled microtubules, exhibiting extensive cortical microtubule bundling (boxed areas). (B) Selected stills of a short microtubule (from left bottom boxed area in A; open arrowheads) growing on the tip of a cortical microtubule bundle. From the same bundle a free microtubule extends away by its plus end (red arrowhead). Temporal changes of the longitudinal profile illustrated in H, can be seen in Fig. S (C) Selected stills from the right boxed area in (A) of a microtubule bundle showing two discrete short microtubules (denoted by orange and white full arrowheads respectively). Red full arrowhead shows depolymerizing microtubule in the same bundle and full and open blue arrowheads show a short gap visible as a steep fluorescence intensity depression in (I). (D) Selected stills of a short microtubule growing (full arrowheads) upon a bundle from the top left boxed area in (A). (E to G) Kymographs corresponding to B (E), C (F) and D (G) respectively. Arrowheads in (E to G) are tracking the respective positions shown in (B to D). (H to J) Longitudinal fluorescence intensity profiles drawn along the microtubule bundles depicted in the last frames of B (H), C (I) and D (J). (K) Vertical fluorescence intensity profiles corresponding to the colored lines in the last frame of (B; blue line, 1 microtubule; green
line, 2 microtubules; red line, three microtubules) demonstrating the complexity of the respective bundle. (L) Graph depiction of plus and minus end intrabundle microtubule dynamics in the mpk4 mutant as summarized in Tables S3 and S4. Scale bars: 5 μm (A), 2 μm (B to D), 1 μm (F) and 0.5 μm (E, G). Time bars in kymographs: 35 s (E to G). Numbers in B to D min:sec.msec. In all kymographs (E to G) time evolution is from top to bottom.

**Figure 8:** Lateral-tip interaction between aberrant, short and rigid microtubule bundles with the walls of individual microtubules labeled with GFP-MBD marker in mpk4 mutant as visualized by SIM. (A to D) Overview of hypocotyl epidermal cell of mpk4 mutant expressing GFP-MBD fusion protein as in Fig. 7A (A; see also Video S3), and time series of two short and rigid microtubule bundles (from boxed area in A; (B) First bundle (full arrowhead) shows short range rapid gliding along the mother microtubule. Second bundle (open arrowhead) swings freely and randomly around its stable anchorage on the site of the mother microtubule. (C, D) Respective kymographs of the short, rigid bundles shown with full and open arrowheads in (B) to demonstrate their rapid, random motility while maintaining stable attachments (C, arrowhead shows the tip of the rigid bundle pointed by open arrowhead in B as it approaches an opposing microtubule (arrow in B, asterisk in C); arrowhead shows to back and forward gliding transitions). (E, F) Absolute fluorescence intensity profiling (dark grey in E and F) of the microtubule bundle (open arrowhead in B) referenced against single microtubule (light grey in E and F). Fluorescence intensity of bundle is about 3-fold increased compared to that of the individual microtubule suggesting it comprises of three microtubules. Scale bars: 5 μm (A), 1 μm (B, E), 0.5 μm (C, D). Numbers on time frames time in min:sec.msec. Time bars in kymographs (35.16s). In all kymographs (C, D) time evolution is from top to bottom.
Figure 1: Comparison of SIM, WF and CLSM in resolving fine details of cortical microtubule organization in hypocotyl epidermal cells of *Arabidopsis thaliana* expressing either GFP-MBD or GFP-TUA6 microtubule markers. (A) Overview of bundled GFP-MBD-labeled microtubules in a hypocotyl epidermal cell of wild type *Arabidopsis thaliana* (see also Fig. S1A). The boxed area includes a loop within a bundle which is readily visible by SIM (top inset, arrow), but not by WF (middle inset, arrow; Fig. S1B) or CLSM (bottom inset, arrow; Fig. S1C). (B) Overview and detail of a cortical microtubule bundle in an epidermal cell expressing TUA6-GFP (Fig. S1D). Top inset shows the discontinuous incorporation of the tagged tubulin revealed by SIM which is however not discernible by WF (bottom inset, see also Fig. S2A to S2C). (C) Overview and details of microtubule branch formation and release in a hypocotyl epidermal cell expressing GFP-MBD microtubule marker (Fig. S1F) acquired with a 63×1.40NA oil immersion objective. A nascent branch (top inset, arrow) and a newly released branch (top inset, arrowhead) are clearly visible by SIM, but not by WF (bottom inset, respective arrow and arrowhead). For example of time-lapped imaging of branched microtubule nucleation see Fig. S1F, S1G depicting a branching event from cell shown in Fig. 6A. (D) Microtubule bundle complexity in a hypocotyl epidermal cell of the *mpk4* mutant as visualized by SIM (Fig. S1J). Such bundles (white boxed area in D) can be resolved in detail by SIM (top inset) but not by WF (middle inset) or CLSM (bottom inset). (E to G) Details (left column) and orthogonal projection (right column) of three closely proximal microtubules (lines in E to G) from the dotted boxed area in (D). By SIM (E, Fig. S1J) the three microtubules are clearly separated as three fluorescent spots, while in the respective WF (F, Fig. S1K) and CLSM (G, Fig. S1L) images the three microtubules appear as a fuzzy fluorescent area. (H to K) Quantitative analysis of the resolution of individual GFP-MBD (H, J) or GFP-TUA6 (I, K) labeled microtubules by means of SIM and WF (H, I) or CLSM (J, K). The graphs represent averaged, co-aligned and normalized intensity profiles (as described in Materials and Methods section) of individual cortical microtubules (n= 27 and 42 for SIM (GFP-MBD and GFP-TUA6 respectively), 27 and 42 for WF (GFP-MBD and GFP-TUA6 respectively) and 27 and 47 for CLSM (GFP-MBD and GFP-TUA6 respectively)). Scale bars: 5 μm (A to G), 2 μm (insets of A to C).
Figure 2: Quantitative analysis of GFP-MBD-labeled cortical microtubule bundles by means of absolute fluorescence intensity comparing the resolution capacity of SIM, WF and CLSM. (A to C) SIM overview (A; Fig. S4A) of an area with microtubule bundles with different microtubule numbers (1, blue line; 2, green line; 3, red line and 4, purple line) quantified by means of fluorescence intensity profiling (B) corresponding to the colored lines in (A) and quantitative evaluation of microtubule number per bundle (C; mean±SD; R², linear correlation coefficient; * p<0.001; n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively). (D to F) The same area shown in (A) after WF acquisition (D; Fig. S4B) with the representative (E) and the averaged maximum fluorescence intensities intensities (F; mean±SD; R², linear correlation coefficient; * p<0.001 comparing 1 with 2 and 2 with 3 microtubules; N.S. non significant difference (p=0.158 between 1 and 4 microtubules); n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively). (G to I) CLSM imaging (G; Fig. S4C) of the same area depicted in (A) showing the representative (H) and the averaged (I; mean±SD; R², linear correlation coefficient; * p<0.001 comparing 1 with 2 microtubules; N.S. non significant difference (p=0.657 between 2 and 3 microtubules and 0.051 between 3 and 4 microtubules); n=119, 99, 33, and 26 measurements for one, two, three, and four microtubules, respectively) fluorescence intensities corresponding to increasing microtubule numbers per bundle. (J to L) Detailed view (J) of the boxed area in (A) and the fluctuation of absolute fluorescence intensities along the longitudinal profile (represented by red, tan and orange brackets in J; white arrow points to fluorescence intensity depression between red and tan brackets) by SIM, WF (K) and CLSM (L). Brackets in (K, L) correspond to the fluorescence of the respective brackets in (J). Note that the intensity along the red and the tan bracket is well discriminated in SIM, declining incrementally (K), less discriminated in WF declining nearly linearly (K) and not discriminated at all in CLSM (L). Black arrows in (K, L) correspond to the intensity drop marked with white arrow in (J). Scale bars: 5 μm (A, D, G) and 2.5 μm (J). For clarity the colored line bars in A, D, G, corresponding to the fluorescence intensity profiles plotted in B, E, H are twice as long as the actual profile length.
Figure 3: Resolution of proximal cortical microtubules labeled with GFP-MBD microtubule marker, comparing SIM, WF and CLSM. (A to C) The same cortical area of a hypocotyl epidermal cell expressing GFP-MBD fusion protein showing two adjacent microtubules by SIM (A; full view in Fig. S5A), WF (B; full view in Fig. S5B) and CLSM (C; full view in Fig. S5C). (D to F) Normalized fluorescence intensity profiles corresponding to white lines in (A to C), showing clear discrimination of the two adjacent microtubules as two peaks in SIM (D) in contrast to the fluorescence intensity profiles obtained from the respective WF (E) and CLSM (F) acquisitions showing only one peak. (G to I) Different areas from the same cell visualized with SIM (G; full view in Fig. S5D), WF (H; full view in Fig. S5E) and CLSM (I; full view in Fig. S5F). White lines across adjacent microtubules represent the position from which fluorescence intensity profiles were acquired and corresponds to the Rayleigh resolution limit (i.e., peak-to-peak discrimination at ca. 25% depression of maximum fluorescence intensity). (J to L) Quantitation of the corresponding intensity profiles shown in (G to I). Brackets denote the peak-to-peak separation at the Rayleigh limit. (M) Graph depiction of the averaged Rayleigh limits measured for SIM, WF and CLSM (mean±SD, n=32 measurements in all cases; * p<0.001 comparing Rayleigh limits from SIM with WF and CLSM). Scale bars for all Figures: 5 μm.
Figure 4: Resolution of individual cortical microtubules of *Arabidopsis thaliana* hypocotyl epidermal cells, labeled with GFP-MBD (A, C, E, G, I) or GFP-TUA6 (B, D, F, H, J) during time-lapsed imaging with SIM (A, B), WF (C, D), CLSM (E, F), TIRF (G, H) and SD (I, J) microscopies. Scatter plots represent averaged weighted profiles of many individual microtubules. (A, B) Resolution of individual cortical microtubules labeled with GFP-MBD (A; n=40) and GFP-TUA6 (B; n=71) after SIM imaging. (C, D) Resolution of individual cortical microtubules labeled with GFP-MBD (C; n=40) and GFP-TUA6 (D; n=71) after WF imaging. (E, F) Resolution of individual cortical microtubules labeled with GFP-MBD (E; n=41) and GFP-TUA6 (F; n=43) after CLSM imaging. (G, H) Resolution of individual cortical microtubules labeled with GFP-MBD (G; n=41) and GFP-TUA6 (H; n=47) after TIRF imaging. (I, J) Resolution of individual cortical microtubules labeled with GFP-MBD (I; n=54) and GFP-TUA6 (J; n=83) after SD imaging. Black lines are positioned to a normalized fluorescence intensity (0.5) corresponding to the FWHM of each respective curve. Scale bars for all Figures: 5 µm.
Figure 5: Independent and intrabundle dynamics of GFP-MBD and GFP-TUA6-labeled cortical microtubules visualized by SIM. (A to C) Overview (A) of a hypocotyl epidermal cell expressing GFP-MBD microtubule marker during time-lapse imaging with SIM, selected stills of a free microtubule visible at both ends (right boxed area in A, B) and respective kymograph of the same microtubule exhibiting short length transitions of growth and shrinkage (full arrowheads; C). (D) Kymograph representing dynamics of free cortical microtubule (corresponds to Figs. S6A, B, Video S1) visible at one end showing smooth, uninterrupted growth (solid lines) and shrinkage (catastrophe; dotted line). (E to G) Overview (E) of a hypocotyl epidermal cell expressing GFP-TUA6 microtubule marker, selected stills of a fully visible independent microtubule (boxed area in E; F, arrowheads point to plus end; Video S2) and respective kymograph showing dark and bright striations owing to the inhomogeneous incorporation of GFP-TUA6 in the microtubule lattice (G). (H) Another kymograph of a GFP-TUA6-labeled microtubule where minus end behavior can be followed (corresponds to Figs. S6C, S6D). (I) Selected stills of a microtubule growing within a bundle from the top right boxed area in (A). (J) The respective kymograph showing vigorous plus end dynamics and short length excursions in the plus end (arrowheads) and minimal dynamics at the minus end. (K) Longitudinal fluorescence intensity profile along the bundle encompassing the microtubule tracked in (I). (L) Selected stills of a short intrabundle microtubule (arrowheads) selected from the left boxed area of (A). (M) The corresponding kymograph of the microtubule in (L) showing frequent and short length transitions in the plus end (full arrowheads) and dynamic instability at the minus end (open arrowheads). (N) The longitudinal fluorescence intensity profile harboring the short intrabundle microtubule depicted in (L). (O, P) Graphs depicting average plus and minus end growth and shrinkage rates for GFP-MBD and GFP-TUA6 labeled extrabundle (O) and intrabundle (P) microtubules from values summarized in Tables S1 and S3 for free and intrabundle microtubules respectively. All full arrowheads point to plus ends and all open arrowheads point to minus ends. Scale bars: 5 μm (A, E), 2 μm (B, C, D, F, G, H, I, J, L) and 10 μm (A, B, C, D, F, G, H, I, J, L, N). (+) indicate microtubule plus ends and (-) indicate microtubule minus ends. Numbers in (B, F, I, L) are in min/sec/msec. In all kymographs (C, D, G, H, J, M) time evolution is from top to bottom.
**Figure 6**: Overview of independent cortical microtubule dynamics of hypocotyl epidermal cells of the *mpk4* mutant expressing GFP-MBD fusion protein, as visualized by SIM and quantified accordingly. (A to C) Widefield SIM view (A), selected stills (B) from the boxed area in (A) and respective kymograph showing a shrinkage (C, dotted line) and a successive growth event (C, full line). (D) Another kymograph of a free growing microtubule showing two growth phases with different rate (full line) interrupted by one catastrophe (dotted line). Arrowheads denote minor such transitions. (E) Graphic illustration of plus and minus end dynamics of free microtubules observed by SIM in hypocotyl epidermal cells of GFP-MBD-transformed *mpk4* mutants as summarized in Table S1. Scale bars: 5 μm (A), 2 μm (B), and 1 μm (C, D). Time bars in (C, D) 60 s. (+) indicate microtubule plus ends. Numbers in (B) are in min:sec:msec. In all kymographs (C, D) time evolution is from top to bottom.
**Figure 7**: Intrabundle microtubule dynamics of the GFP-MBD expressing *mpk4* mutant as acquired with SIM. (A) Overview (A; see also Video S3) of an *mpk4* hypocotyl epidermal cell with GFP-MBD-labeled microtubules, exhibiting extensive cortical microtubule bundling (boxed areas). (B) Selected stills of a short microtubule (from left bottom boxed area in A; open arrowheads) growing on the tip of a cortical microtubule bundle. From the same bundle a free microtubule extends away by its plus end (red arrowhead). Temporal changes of the longitudinal profile illustrated in H, can be seen in Fig. S (C) Selected stills from the right boxed area in (A) of a microtubule bundle showing two discrete short microtubules (denoted by orange and white full arrowheads respectively). Red full arrowhead shows depolymerizing microtubule in the same bundle and full and open blue arrowheads show a short gap visible as a steep fluorescence intensity depression in (I). (D) Selected stills of a short microtubule growing (full arrowheads) upon a bundle from the top left boxed area in (A). (E to G) Kymographs corresponding to B (E), C (F) and D (G) respectively. Arrowheads in (E to G) are tracking the respective positions shown in (B to D). (H to J) Longitudinal fluorescence intensity profiles drawn along the microtubule bundles depicted in the last frames of B (H), C (I) and D (J). (K) Vertical fluorescence intensity profiles corresponding to the colored lines in the last frame of B; blue line, 1 microtubule; green line, 2 microtubules; red line, three microtubules) demonstrating the complexity of the respective bundle. (L) Graph depiction of plus and minus end intrabundle microtubule dynamics in the *mpk4* mutant as summarized in Tables S3 and S4. Scale bars: 5 μm (A), 2 μm (B to D), 1 μm (F) and 0.5 μm (E, G). Time bars in kymographs: 35 s (E,F,G). Numbers in B to D min:sec.msec. In all kymographs (E to G) time evolution is from top to bottom.
**Figure 8:** Lateral-tip interaction between aberrant, short and rigid microtubule bundles with the walls of individual microtubules labeled with GFP-MBD marker in *mpkd* mutant as visualized by SIM.  

(A to D) Overview of hypocotyl epidermal cell of *mpkd* mutant expressing GFP-MBD fusion protein as in Fig. 7A (A; see also Video S3), and time series of two short and rigid microtubule bundles (from boxed area in A); (B) First bundle (full arrowhead) shows short range rapid gliding along the mother microtubule. Second bundle (open arrowhead) swings freely and randomly around its stable anchorage on the site of the mother microtubule. (C, D) Respective kymographs of the short, rigid bundles shown with full and open arrowheads in (B) to demonstrate their rapid, random motility while maintaining stable attachments (C; arrowhead shows the tip of the rigid bundle pointed by open arrowhead in B as it approaches an opposing microtubule; arrow in B, asterisk in C; arrowhead shows back and forward gliding transitions). (E, F) Absolute fluorescence intensity profiling (dark grey in E and F) of the microtubule bundle (open arrowhead in B) referenced against single microtubule (light grey in E and F). Fluorescence intensity of bundle is about 3-fold increased compared to that of the individual microtubule suggesting it comprises of three microtubules. Scale bars: 5 μm (A), 1 μm (B, E), 0.5 μm (C, D). Numbers on time frames time in min:sec.msec. Time bars in kymographs (35.16s). In all kymographs (C, D) time evolution is from top to bottom.