

1 **Increased microtubule lattice spacing correlates with selective binding** 2 **of kinesin-1 in cells**

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9

10 **Abstract**

11 Within the cell cargo is transported via motor proteins walking along microtubules. The affinity of
12 motor proteins for microtubules is controlled by various layers of regulation like tubulin isoforms, post-
13 translational modifications and microtubule associated proteins. Recently, the conformation of the
14 microtubule lattice has also emerged as a potential regulatory factor, but to what extent it acts as an
15 additional layer of regulation has remained unclear. In this study, we used cryo-correlative light and
16 electron microscopy to study microtubule lattices inside cells. We find that, while most microtubules
17 have a compacted lattice (~41 Å), a significant proportion of the microtubule cores have expanded
18 lattice spacings and that these lattice spacings could be modulated by the microtubule stabilizing drug
19 Taxol. Furthermore, kinesin-1 predominantly binds microtubules with a more expanded lattice spacing
20 (~41.6 Å). The different lattice spacings present in the cell can thus act as an additional factor that
21 modulates the binding of motor proteins to specific microtubule subsets.

22

23 **Introduction**

24 Microtubules are highly dynamic, polarized cytoskeletal structures along which motor proteins move
25 to transport cargos throughout the cell. To achieve efficient cargo transport, the binding of motor
26 proteins to specific subsets of microtubules is highly regulated. The multiple layers of regulation and
27 modifications that mark the different populations of microtubules and influence the downstream
28 behaviour of motor proteins have together been termed the tubulin code (Verhey and Gaertig, 2007).
29 Many of the components of this code, like tubulin isoforms, post-translational modifications (PTMs)
30 and microtubule associated proteins (MAPs), have been identified (Gadadhar et al., 2017; Janke and
31 Magiera, 2020; Roll-Mecak, 2020). However, the tubulin code is potentially still incomplete as it does
32 not explain all motor protein behaviour observed *in vivo*.

33 Specifically, the motor protein kinesin-1 is known to be regulated by various components of
34 the tubulin code (Janke and Magiera, 2020). For example, the binding properties of kinesin-1 are
35 modulated by MAPs. Binding of MAP7 leads to activation of kinesin-1, namely by increasing its run

36 length, while tau has the opposite effect (Ferro et al., 2022; Hooikaas et al., 2019; Monroy et al., 2018).
37 Furthermore, kinesin-1 binds with greater affinity to a subset of stable microtubules (typically enriched
38 in the PTMs acetylation and detyrosination) than to microtubules without these modifications (Cai et
39 al., 2009; Dunn et al., 2008; Guardia et al., 2016; Konishi and Setou, 2009; Liao and Gundersen, 1998;
40 Tas et al., 2017). However, *in vitro* experiments to date, where different levels of these PTMs were
41 investigated, have not reproduced the behaviour of kinesin-1 that is observed *in vivo* (Kaul et al., 2014;
42 Sirajuddin et al., 2014; Walter et al., 2012). In addition, upon treatments that lead to acetylation or
43 detyrosination of most cellular microtubules, kinesin-1 still binds to a subset of stable microtubules
44 (Jansen et al., 2021). This indicates that the tubulin code for kinesin-1 is not completely understood.

45 An additional layer of regulation might come from the structure of the microtubule itself, as
46 several studies have shown that proteins are sensitive to its lattice spacing and/or nucleotide state
47 (Manka and Moores, 2018a; Zhang et al., 2018). Upon polymerization of free $\alpha\beta$ -tubulin dimers into
48 microtubules, GTP bound to β -tubulin is hydrolysed to GDP and the structural conformation of the $\alpha\beta$ -
49 tubulin dimer changes (Alushin et al., 2014). This GDP-bound state, which makes up the bulk of the
50 microtubule core, generally has a compacted average monomer spacing of ~ 41 Å that is distinct from
51 that of the GTP-bound state at the tip (GTP-cap), which has an expanded lattice spacing of ~ 42 Å as
52 determined for hydrolysis deficient mutants and the slowly-hydrolysable GTP analogue guanylyl-(α,β -
53 methylene-diphosphonate (GMPCPP) (Hyman et al., 1995; LaFrance et al., 2021). Besides the
54 nucleotide state, drugs, like the anti-cancer drug Taxol, can alter the lattice spacing of the microtubule
55 (Kellogg et al., 2017). Some MAPs can differentiate between these different lattice spacings. For
56 example, end binding proteins and doublecortin preferentially bind to the GTP-cap of the microtubule
57 (Bechstedt and Brouhard, 2012; Tirnauer and Bierer, 2000), while Tau (Duan et al., 2017) and MAP2
58 preferentially bind to microtubules in the GDP-bound state (Siahaan et al., 2021). A similar lattice
59 sensitivity might partially explain the observed microtubule-subset specificity of kinesin-1.

60 *In vitro* data show that kinesin-1 binds expanded microtubules with a higher affinity than
61 microtubules in the compacted state and that kinesin-1 binds microtubules assembled with GMPCPP at
62 a higher frequency than GDP-bound microtubules (Shima et al., 2018). Moreover, GDP-microtubules,
63 when heavily decorated with kinesin-1, were reported to be more expanded than undecorated
64 microtubules (Shima et al., 2018) and saturating concentrations of kinesin-1 induced microtubule lattice
65 expansion (Peet et al., 2018). However, these experiments were all performed in reconstituted systems
66 using brain tubulin that contains a diverse mixture of PTMs and tubulin isoforms (Schwarz et al., 1998).
67 Additionally, the observed effects depended on the kinesin-1 concentrations used, but the relationship
68 to *in vivo* concentrations was not established. It is thus still unclear to what extent the microtubule lattice
69 spacing plays a regulatory role within the cell.

70 To investigate if subsets of microtubules with varying lattice states exist within cells, and to
71 determine if kinesin-1 preferentially binds a particular lattice, we established a cryo-correlative light
72 and electron microscopy (cryo-CLEM) workflow. We show here that microtubules can have diverse
73 lattice spacings in cells and that Taxol treatment greatly expands the lattice. Furthermore, we find that
74 kinesin-1-decorated microtubules are predominately expanded in cells.

75

76 **Results and discussion**

77 **Microtubule lattice spacings within cells are diverse but mainly compacted**

78 To examine the lattice spacing of microtubules in cells, we cultured U2OS cells on EM grids, vitrified
79 them, and prepared ~150 nm thick lamellae using cryo-focused ion beam (FIB) milling for subsequent
80 cryo-electron tomography imaging (Rigort et al., 2012). To measure the lattice spacing of cellular
81 microtubules, we used a layer line approach based on Fourier analysis of microtubule segments (Figure
82 1A). Building from typical 2D analysis of *in vitro* microtubules (Mandelkow et al., 1977), the protocol
83 masks out microtubule density from surrounding cellular material and aligns the segments in 3D (Figure
84 1B), prior to projection and computation of the 2D Fourier transforms (Figure 1C and Supplemental
85 Figure 1). Summed power spectra of the aligned microtubule segments were then used to measure the
86 lattice periodicity based on maxima in a line profile plot (Figure 1G).

87 We first assessed the lattice spacing of microtubules from untreated U2OS cells using this *in*
88 *situ* layer line analysis. In line with *in vitro* results of GDP-bound microtubules, 74% (23 out of 31) had
89 a compacted average monomer spacing of 40.8-41.1 Å (Figure 1H). The remaining microtubules had
90 an expanded spacing of 41.5-42.7 Å. These distinct lattice spacings could even be detected within the
91 same tomogram (Supplemental Figure 2). Our results indicate that microtubule lattice spacings are
92 diverse even far away from microtubule tips, where an altered lattice spacing is expected (Hyman et al.,
93 1995; LaFrance et al., 2021). Within the field of view of our data, which is approximately 0.8 µm, we
94 did not observe any microtubule ends. In contrast to the changes in lattice spacing at microtubule ends,
95 which are important for the regulation of microtubule growth dynamics (Manka and Moores, 2018b),
96 the different lattice spacings that we observe in the core of the microtubule likely play a role in
97 microtubule stability, MAP binding and motor protein kinetics, as has been previously postulated
98 (Cross, 2019).

99 To confirm that our *in situ* layer line analysis is sensitive to changes in lattice spacing, we set
100 out to modulate the microtubule lattice and test whether this change could be detected. Microtubules
101 that are polymerized *in vitro* in the presence of Taxol are structurally altered compared to the GDP-
102 bound, compacted state (Alushin et al., 2014; Kellogg et al., 2017; Rai et al., 2019). We therefore
103 hypothesized that Taxol might expand microtubules inside cells as well. Indeed, in U2OS cells treated
104 with Taxol we observed that most microtubules (92%, 11/12) have an expanded lattice spacing of 42.3-

105 43.2 Å (Figure 1D-H), significantly different from the untreated distribution (p-value = 0.0006).
106 Together, these data show that changes in lattice spacing can be detected with our *in situ* layer line
107 analysis and that the overall distribution of microtubule lattice spacings can be altered by Taxol.

108 Remarkably, the Taxol-induced expansion to 41.2-42.0 Å observed *in vitro* is considerably
109 smaller than the hyper-expansion measured using our *in situ* approach (Alushin et al., 2014; Estevez-
110 Gallego et al., 2020; Kellogg et al., 2017; Rai et al., 2019; Vale et al., 1994). A complex interplay
111 between Taxol, MAPs and PTMs might exist as up or downregulation of MAPs, like Tau, can affect
112 the sensitivity of cells to Taxol (Orr et al., 2003). Additionally, Taxol treatment leads to an increase in
113 PTMs like acetylation (Hammond et al., 2010). These components are usually completely or partially
114 absent from *in vitro* systems, which may explain why this hyper-expansion has thus far remained
115 unreported.

116

117 **Fluorescence microscopy-guided cryo-FIB milling**

118 Having established that different microtubule lattice spacings can be monitored within the cell, we next
119 set out to determine the lattice spacing distribution of the subset of microtubules to which kinesin-1
120 selectively binds (Burute and Kapitein, 2019). To this end we used rigor-2xmNeonGreen, a
121 fluorescently tagged mutant of kinesin-1 that has a very low rate of microtubule unbinding and binds to
122 a subset of stable microtubules (Jansen et al., 2021). We set up a two-step cryo-CLEM workflow,
123 wherein fluorescence microscopy (FM) data of U2OS Flp-In T-Rex cells expressing rigor-
124 2xmNeonGreen were used to target lamella preparation sites in the first correlation step (Figure 2A),
125 and then used to distinguish the kinesin-1 bound microtubules from unbound microtubules in the
126 lamella visible in the transmission electron microscope (TEM) in the second step (Figure 3A). For the
127 first step, the extracellular beads visible in both the FM stack and the SEM image were used to overlay
128 the FM stacks of the rigor-2xmNeonGreen (Figure 2B) and endocytosed fBSA-Au⁵ beads (Figure 2C)
129 (Fermie et al., 2022) with the SEM image (Figure 2D). The resulting overlay (Figure 2E) was used for
130 targeted milling (Figure 2F).

131 The FM-SEM localization accuracy was estimated using a “leave-one-out” metric (see
132 materials and methods) and showed an FM-SEM correlation error with a standard deviation of 121 nm
133 in x and of 116 nm in y (Figure 2G). The slight increase in accuracy that we obtained in comparison to
134 the previous accuracy measurements obtained by Arnold et al. might be due to the increased number of
135 beads we used for calculating the transform (Arnold et al., 2016). This estimated localization error
136 confirmed that the FM-SEM correlation was sufficiently accurate for our CLEM approach.

137

138 **Kinesin-1-bound microtubules inside cells are expanded**

139 Next, the correlated FM data were overlaid with the TEM overview image of the lamella (Figure 3A).
140 To confirm that the FM-TEM correlation was successful, fBSA-Au⁵ beads present in the FM data were

141 localized in the TEM image of the lamella (Figure 3B-E) and fine adjustments based on the higher
142 resolution TEM data were performed. This was critical as the microtubules alone did not allow for
143 assessment or optimization of the correlation (Figure 3F). When the correlation was considered
144 unambiguous and reliable, the rigor-2xmNeonGreen data was used to localize the kinesin-1 bound
145 microtubules within the lamella (Figure 3G-I). Analysis of their lattice spacings revealed a clear shift
146 towards a more expanded lattice distribution, centred around 41.5-41.9 Å (p-value = 0.045), in
147 comparison to the dominant compacted lattice observed for untargeted microtubules (Figure 3J). The
148 observed distribution was also significantly different from the lattice distribution observed for Taxol
149 treated cells (p-value = 0.0107). Our results indicate therefore that kinesin-1 bound microtubules inside
150 cells are predominately more expanded than those not bound by kinesin-1.

151 The observed preference for an expanded lattice might relate to the structure of kinesin-1 or
152 result from cooperative behaviour with other MAPs. The specific length of the neck linker of kinesin-
153 1 results in a notably high processivity compared to other kinesin motors (Shastry and Hancock, 2010;
154 Yildiz et al., 2008). An expanded lattice might better accommodate the step size and increase
155 processivity further. In these experiments the structural changes that occur upon microtubule expansion
156 may result in an increased interaction and binding affinity between the L11- α 4 junction of kinesin-1
157 and the H3 helix of α -tubulin (Morikawa et al., 2015; Shima et al., 2018). Alternatively, additional
158 interactions with MAPs, such as MAP7 that may be sensitive to the lattice spacing, could ultimately be
159 responsible for recruiting kinesin-1 (Ferro et al., 2022; Hooikaas et al., 2019; Monroy et al., 2020).

160 Despite the additional correlation accuracy gained with the intracellular beads, we cannot
161 exclude that a proportion of the kinesin-1 bound microtubules were identified incorrectly due to the
162 limited resolution of our cryo-FM setup. This fractional misassignment might contribute to the large
163 spread observed in lattice spacings for the kinesin-1-bound microtubule-subset. Technical FM
164 improvements such as cryo-MINFLUX (Gwosch et al., 2020), engineered point spread functions (Zhou
165 et al., 2019) or integrated FM-FIB solutions (Bieber et al., 2021) may improve the cryo-FM resolution
166 (lateral and/or axial) and resulting CLEM localization accuracy, as well as the throughput of future
167 cryo-CLEM workflows. Nevertheless, the current workflow, which uses an additional independent
168 marker (the fBSA-Au⁵ beads) for the FM-TEM correlation, can readily be used to answer a broad range
169 of biological questions.

170 Taken together, our approach provides an unbiased way to investigate microtubule lattice
171 spacings inside the cell. We found that most microtubules have a compacted lattice of around 41 Å, in
172 line with previous *in vitro* and *in situ* studies (Figure 4, row I and II). Importantly, we observed that a
173 range of expanded lattices can be found within the cell. Thus far, expanded states have only been
174 reported in *in vitro* studies when microtubules are assembled using the GTP analogue GMPCPP or the
175 drug Taxol (Figure 4, row III and IV). The relevance of different lattice spacings for MAP behaviour
176 has only been investigated for a few examples and expanded lattices have mostly been linked to plus-
177 end regulating proteins (Figure 4, row V). However, our data, together with *in vitro* data, indicate that

178 kinesin-1 preferentially binds to an expanded lattice (Peet et al., 2018; Shima et al., 2018). The
179 microtubule lattice might therefore function as an additional mechanism to guide protein binding
180 specificity, on top of the previously established elements of the tubulin code. Similar analysis on other
181 members of the kinesin family may reveal the extent to which these motors are sensitive to the lattice
182 spacing of microtubules.

183 In this paper we present a two-step cryo-CLEM workflow, which we used to study microtubule
184 lattice spacing within unperturbed cells, upon Taxol treatment, and in correlation with kinesin-1
185 binding. The discovery of diverse microtubule lattice spacings within the cell emphasizes the need to
186 further investigate the relationship between the microtubule lattice and protein binding behaviour.
187 Furthermore, this study shows the potential of our cryo-CLEM workflow to gain new insights into well-
188 studied biological processes from intact cells.

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202 **Competing interest**

203 Nothing declared.

204

205 **Materials and Methods**

206 **List of reagents:**

Reagent or resource	Source	Identifier
Q5 HF DNA polymerase	NEB	Cat# M0491L
Gibson Assembly® Master Mix	NEB	Cat# E2611L
DMEM + GlutaMAX-I	GIBCO	Cat# 61965-026
DPBS	Corning	Cat# 21-031-CV
Pen Strep	GIBCO	Cat# 15140-122
Trypsin-EDTA	Gibco	Cat# 25200-056
Fetal Bovine Serum	Sigma-Aldrich, Inc.	Cat# F7525-500ML
Fibronectin	Sigma-Aldrich, Inc.	Cat# F1141-2MG
Blasticidin	Thermo Fisher Scientific	Cat# R21001
Hygromycin B	Corning	Cat# 30-240CR
Paclitaxel/Taxol	Thermo Fisher Scientific	Cat# P3456
Doxycycline	Sigma-Aldrich, Inc.	Cat# 24390-14-5
fBSA-Au⁵	Cell Microscopy Core, Utrecht UMC	N.A.
Dynabeads™ MyOne™ Carboxylic Acid	Thermo Fisher Scientific	Cat# 65011
Cellview cell culture dish	Greiner bio-one	Cat# 627860
Quantifoil 200 mesh holey carbon R2/2 gold grids	Quantifoil Micro Tools	N.A.
Whatman Filter Paper	Whatman	Cat# 10311610

207

208 **Cell lines and cell culture**

209 U2OS wild type (WT) cells were purchased from ATCC and U2OS Flp-In T-Rex cells were a kind gift
 210 from Prof. Alessandro Sartori (Institute of Molecular Cancer Research, University of Zurich). Cells
 211 were confirmed to be free of mycoplasma. The U2OS Flp-In cell line that upon doxycycline-induction
 212 expresses hKif5b(1-560)G234A-mNeongreen-mNeongreen (hereafter referred to as rigor-
 213 2xmNeongreen) (Jansen et al., 2021) was derived from the U2OS Flp-In cell line by transfection with
 214 the pCDN5/FRT/TO vector (Invitrogen) and pOG44 vector (Invitrogen). The U2OS rigor-
 215 2xmNeongreen cell line was cultured in Dulbecco's Modified Eagle's Medium + GlutaMAX™-1
 216 (DMEM-Glu) supplemented with 10% FBS , 100 U/ml penicillin, 100 µg/ml streptomycin, 15 µg/ml
 217 blasticidin S and 0.25 mg/ml hygromycin B. To induce expression of rigor-2xmNeongreen, doxycycline
 218 (10 ng/mL) was added to the cells 24 hrs before plunging. U2OS WT cells were cultured in DMEM-

219 Glu supplement with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were kept at
220 37°C and 5% CO₂.

221

222 **Plasmids and cloning**

223 To generate a stable, isogenic U2OS Flp-In cell line, rigor-2xmNeongreen was subcloned into
224 pCDNA5/FRT/TO (Invitrogen) via Gibson Assembly using the primer set ‘5-GCTCGGATCCACTAG
225 TCCAGTGTGGTGAATTCTGCAGATGCCACCATGGCGGACCT-3’ and 5’-ACGGGCCCTCT
226 AGACTCGAGCGGCCGCGCCACTGTGCTGGATGCGGCCGCTTACTTGTACAG-3’. The G234A
227 rigor mutation was used as initially described (Rice et al., 1999). mNeongreen (Shaner et al., 2013) was
228 provided by Allele Biotechnology. The FLP recombinase expression vector is encoded in pOG44
229 (Invitrogen).

230

231 **Sample preparation**

232 Quantifoil 200 mesh holey carbon R2/2 gold grids (Quantifoil Micro Tools) were glow discharged
233 (PELCO easiGlow, Ted Pella) and placed on 40 µL fibronectin droplets (50 µg/µL) and incubated at
234 37°C for 2-3 hrs. Subsequently, the grids were washed two times by placing them on droplets of 40 µL
235 PBS and put in 35 mm glass bottom dishes (Greiner Bio-One). 90.000 cells in 2 mL medium were
236 seeded on these grids and were left to settle in the hood for ~20 min. before placing them in the
237 incubator. After 24 hrs, the U2OS rigor-2xmNeongreen cells were treated with doxycycline (10 ng/mL)
238 while WT U2OS cells were treated with Taxol (1 µM), if applicable. Plunging was performed 48 hrs
239 after cell seeding. In preparation for vitrification, the cells were incubated with fBSA-Au⁵ (diluted to
240 OD 5) for 3-4 hrs (Fermie et al., 2022). Just before plunging, the media was exchanged for fresh DMEM.
241 The grids were washed by dipping 2 times in PBS (37°C) before 3 µL of 1 µm Dynabeads (Thermo
242 Fisher Scientific: MyOne with 40% iron oxide, carboxylic acid) diluted 1:20 in PBS, was added to the
243 grids. Finally, the cells were vitrified in liquid ethane after manually blotting for 10s. The grids were
244 clipped into autogrids and kept at liquid nitrogen temperature throughout the subsequent experiments.

245

246 **SEM grid screening**

247 To increase efficiency, although at the cost of ice contamination, grids were screened in the cryo-FIB
248 SEM (AquilosTM, Thermo Fisher Scientific), prior to fluorescent imaging. SEM grid overview images
249 were taken using FEI MAPS 3.8 software. Grids with clearly visible grid holes and an appropriate
250 distribution of cells were used in the next steps of the cryo-CLEM workflow.

251

252 **Cryo-fluorescence microscopy**

253 Cryo-FM data were obtained with the FEI CorrSight™ equipped with a cryo-stage, using the
254 Andromeda spinning-disk confocal microscope module. Grid overviews were collected with a 5x/0.16
255 NA air objective using transmitted light. SEM overview images were aligned to the FM overview
256 images via 3-point correlation in the MAPs v3.8 software (Thermo Fisher Scientific). Based on the
257 SEM and FM overlay, candidate cells were selected. Using the EC “Plan-Neofluar” 40x/0.9 NA air
258 objective, z-stacks ranging from 8-14 μm with 300 nm step size were collected. Z-stacks were recorded
259 to capture 3 different fluorescent probes, namely rigor-2xmNeogreen (488 nm), Dynabeads (488 nm)
260 and fBSA-Au⁵ (561 nm). Images were recorded with FEI MAPS v3.8 software and LA FEI Live
261 Acquisition v2.2.0 (Thermo Fisher Scientific). The images were subjected to a mild deconvolution
262 using Huygens Professional software v 21.04 (Scientific Volume Imaging) with classic maximum
263 likelihood estimation algorithm.

264

265 **Targeted cryo-focussed ion beam milling**

266 Cryo-FIB milling was performed in the cryo-FIB SEM (Aquilos™, Thermo Fisher Scientific). Lamellae
267 were prepared as described previously (Wagner et al., 2020). SEM grid overview images were obtained
268 and overlaid with the cryo-FM overview images using 3-point correlation available in the MAPS v3.8
269 software to guide the localization of candidate lamella sites. The grids were coated with platinum to
270 reduce charging effects. Eucentric heights and minimal stage tilt angles (16-18°, corresponding to 9-
271 11° lamella angle), to ensure access of the ions to the milling sites, were determined. High magnification
272 SEM images (0.135 μm/pixel, 1 μs dwell time, 1,536x1,024 pixels, 2 keV, 13 pA) of each target cell
273 were taken and manually overlaid with the corresponding cryo-FM maximum image projection (MIP)
274 using Dynabeads as fiducials. The location of the milling patterns was based on the correlated cryo-FM
275 data. An FM-FIB correlation was not included in our workflow as this is a time-intensive procedure -
276 and the limited z-resolution of our cryo-FM set up meant this correlation did not add significant
277 information when tested.

278 Next, the grids were subjected to organo-platinum deposition for 10 s via an integrated gas
279 injection system to generate a more even surface and thereby reduce curtaining effects and protect the
280 final lamella. Milling was performed with a stepwise decreasing current of 1 to 0.3 to 0.1 nA, and a
281 shrinking milling pattern. The final polishing step was performed at 30 pA to reach a final lamella
282 thickness of 80-140 nm. High magnification SEM images of the polished lamellae were taken (0.135
283 μm/pixel, 300 ns dwell time, 1,536x1,024 pixels, 2 keV, 13 pA) and the grids were coated with platinum
284 for a second time before unloading.

285 **Targeted cryo-electron tomography**

286 In preparation for FM-guided cryo-electron tomography data collection, SEM images of polished
287 lamellae were aligned to the corresponding cryo-FM z-stacks, guided by high-dose SEM images of the
288 lamella sites. Using the 3D Correlation toolbox (Arnold et al., 2016) 6-9 Dynabeads were localized both
289 in the SEM image and the FM z-stack and a transformation matrix was fitted for the two sets of X,Y,Z
290 coordinates, while aiming for an RMSD smaller than 1 pixel for each bead. Z-stacks were transformed
291 according to this matrix with the 3D rigid body transformation of the Pyto python package (Arnold et
292 al., 2016). MIPs of the transformed z-stack of each channel were overlaid with the SEM image of the
293 polished lamella in FIJI (Schindelin et al., 2012) to generate a correlated FM-SEM overlay.

294 Lamellae were imaged on a 200 kV Talos Arctica transmission electron microscope (TEM)
295 (Thermo Fisher Scientific) with a K2 summit direct electron detector (Gatan) or on a 300 kV FEI Titan
296 Krios TEM (Thermo Fisher Scientific) with a K3 summit direct electron detector (Gatan), both
297 equipped with a post-column energy filter aligned to the zero-loss peak and a 20 keV slit width. Using
298 SerialEM (Mastrorade, 2003), lamellae overview images were taken at 7,300x (Arctica, 18.7 Å/pixel)
299 or 4,800x (Krios, 38.6 Å/pixel).

300 The TEM overview image of the lamella and the corresponding FM-SEM overlay were further
301 manually correlated. This last correlation step was guided by the bimodal intracellular fBSA-Au⁵
302 fiducials. In cases where the rigor-2xmNeongreen FM signal overlapped with a microtubule in the cryo-
303 TEM lamella image, a tilt series was collected. The microtubules in the lamellae of untreated and Taxol
304 treated cells were chosen at random. Tilt series of microtubules were recorded at a pixel size of 2.17
305 Å/px, a dose rate of ~5 (Arctica) or 10-20 (Krios) e⁻/pixel/s and a total dose of 90-100 e⁻/Å². All tilt
306 series were collected using a dose symmetric scheme (Hagen et al., 2017), a tilt increment of 2° or 3°,
307 a defocus target of -2.3 μm, and a tilt range of 69° to -51° or 51° to -69°, depending on the lamella
308 orientation in the microscope.

309

310 **Tomogram reconstruction**

311 The tilt series were aligned and dose-weighted using MotionCor2 (Zheng et al., 2017). The tilt series
312 were generally aligned via patch tracking. If fBSA-Au⁵ beads were present, fiducial tracking was used.
313 Tomogram reconstruction was performed in Etomo, part of the IMOD 4.10.29 package (Kremer et al.,
314 1996). Contrast transfer function estimation and correction was performed in IMOD using the *ctfplotter*
315 and *ctfphaseflip* commands and the tomograms were reconstructed using weighted back-projection and
316 a SIRT-like filter with 3 iterations.

317

318 **Layer line analysis**

319 Reconstructed tomograms were loaded in Dynamo (Castano-Diez et al., 2017). The filament model
320 (crop along axis) was used to pick the microtubule backbone. The backbone coordinates were exported

321 from MATLAB and used to generate a soft mask around the microtubule [in house script, available on
322 GitHub at ldejager/InSitu_LayerLine_Analysis]. Particles with a box size of 190 pixels were extracted
323 from the unbinned, masked tomograms. Microtubule particles were aligned in Dynamo using a
324 reference based on a previously deposited microtubule structure (EMD-10896). After alignment, the
325 particles were inspected using the *dGallery* command. Per microtubule, multiple aligned particles were
326 selected to cover the whole microtubule. These particles were extracted from the unbinned tomograms
327 with a box size of 1,030 pixels and summed along the z-axis [in-house script, available on GitHub at
328 ldejager/InSitu_LayerLine_Analysis]. The power spectrum of each particle was calculated in FIJI. The
329 power spectra of the particles from the same microtubule were summed to increase the signal-to-noise
330 ratio. Layer lines were localized by calculating a line profile plot of the power spectrum. The average
331 lattice spacing of the microtubule was calculated using formula 1.

$$332 \quad Lattice \ spacing = \frac{pixel \ size \left(\frac{A}{pix}\right) \times box \ size}{|Equator \ location \ (pix) - Layer \ line \ location \ (pix)|} \quad (1)$$

333

334 **Statistical analysis**

335 Leave-one-out calculations were performed as described by (Schorb and Briggs, 2014). Briefly, a
336 transformation matrix was calculated with a set of beads. This transform was applied to a bead not
337 included in the initial set. The deviation of the predicted bead position from the true bead position was
338 used as an accuracy measurement. P-values for comparison of the different lattice spacing distributions
339 were calculated with a unpaired two-tailed t-test based permutation test with 10,000 iterations. Lattice
340 spacing density distribution (figure 4) was calculated as a gaussian kernel density estimate. Permutation
341 tests were performed in and graphs (histograms and distribution) were created with Jupyter notebook
342 6.0.3 (Kluyver et al., 2016).

343 References

- 344 Alushin, G. M., Lander, G. C., Kellogg, E. H., Zhang, R., Baker, D. & Nogales, E. (2014) High-resolution
345 microtubule structures reveal the structural transitions in alphabeta-tubulin upon GTP
346 hydrolysis. *Cell*, 157(5), 1117-29. 10.1016/j.cell.2014.03.053.
- 347 Arnold, J., Mahamid, J., Lucic, V., de Marco, A., Fernandez, J. J., Laugks, T., Mayer, T., Hyman, A. A.,
348 Baumeister, W. & Plitzko, J. M. (2016) Site-Specific Cryo-focused Ion Beam Sample
349 Preparation Guided by 3D Correlative Microscopy. *Biophys J*, 110(4), 860-9.
350 10.1016/j.bpj.2015.10.053.
- 351 Bechstedt, S. & Brouhard, Gary J. (2012) Doublecortin Recognizes the 13-Protofilament Microtubule
352 Cooperatively and Tracks Microtubule Ends. *Developmental Cell*, 23(1), 181-192.
353 10.1016/j.devcel.2012.05.006.
- 354 Bieber, A., Capitano, C., Schiøtz, O., Smeets, M., Fenzke, J., Erdmann, P. & Plitzko, J. (2021) Precise
355 3D-correlative FIB-milling of biological samples using METEOR, an integrated cryo-CLEM
356 imaging system. *Microscopy and Microanalysis*, 27(S1), 3230-3232.
357 10.1017/S1431927621011132.
- 358 Burute, M. & Kapitein, L. C. (2019) Cellular Logistics: Unraveling the Interplay Between Microtubule
359 Organization and Intracellular Transport. *Annu Rev Cell Dev Biol*, 35, 29-54.
360 10.1146/annurev-cellbio-100818-125149.
- 361 Cai, D., McEwen, D. P., Martens, J. R., Meyhofer, E. & Verhey, K. J. (2009) Single molecule imaging
362 reveals differences in microtubule track selection between Kinesin motors. *PLoS Biol*, 7(10),
363 e1000216. 10.1371/journal.pbio.1000216.
- 364 Castano-Diez, D., Kudryashev, M. & Stahlberg, H. (2017) Dynamo Catalogue: Geometrical tools and
365 data management for particle picking in subtomogram averaging of cryo-electron
366 tomograms. *J Struct Biol*, 197(2), 135-144. 10.1016/j.jsb.2016.06.005.
- 367 Cross, R. A. (2019) Microtubule lattice plasticity. *Curr Opin Cell Biol*, 56, 88-93.
368 10.1016/j.ceb.2018.10.004.
- 369 Duan, A. R., Jonasson, E. M., Alberico, E. O., Li, C., Scripture, J. P., Miller, R. A., Alber, M. S. &
370 Goodson, H. V. (2017) Interactions between Tau and Different Conformations of Tubulin:
371 Implications for Tau Function and Mechanism. *J Mol Biol*, 429(9), 1424-1438.
372 10.1016/j.jmb.2017.03.018.
- 373 Dunn, S., Morrison, E. E., Liverpool, T. B., Molina-París, C., Cross, R. A., Alonso, M. C. & Peckham, M.
374 (2008) Differential trafficking of Kif5c on tyrosinated and detyrosinated microtubules in live
375 cells. *J Cell Sci*, 121(Pt 7), 1085-95. 10.1242/jcs.026492.
- 376 Estevez-Gallego, J., Josa-Prado, F., Ku, S., Buey, R. M., Balaguer, F. A., Prota, A. E., Lucena-Agell, D.,
377 Kamma-Lorger, C., Yagi, T., Iwamoto, H., Duchesne, L., Barasoain, I., Steinmetz, M. O.,
378 Chretien, D., Kamimura, S., Diaz, J. F. & Oliva, M. A. (2020) Structural model for differential
379 cap maturation at growing microtubule ends. *Elife*, 9. 10.7554/eLife.50155.
- 380 Fermie, J., de Jager, L., Foster, H. E., Veenendaal, T., de Heus, C., van Dijk, S., ten Brink, C., Oorschot,
381 V., Yang, L., Li, W., Müller, W. H., Howes, S., Carter, A. P., Förster, F., Posthuma, G.,
382 Gerritsen, H. C., Klumperman, J. & Liv, N. (2022) Bimodal endocytic probe for three-
383 dimensional correlative light and electron microscopy. *Cell Reports Methods*, 100220.
384 doi:10.1016/j.crmeth.2022.100220.
- 385 Ferro, L. S., Fang, Q., Eshun-Wilson, L., Fernandes, J., Jack, A., Farrell, D. P., Golcuk, M., Huijben, T.,
386 Costa, K., Gur, M., DiMaio, F., Nogales, E. & Yildiz, A. (2022) Structural and functional insight
387 into regulation of kinesin-1 by microtubule-associated protein MAP7. *Science*, 375(6578),
388 326-331. doi:10.1126/science.abf6154.
- 389 Gadadhar, S., Bodakuntla, S., Natarajan, K. & Janke, C. (2017) The tubulin code at a glance. *J Cell Sci*,
390 130(8), 1347-1353. 10.1242/jcs.199471.

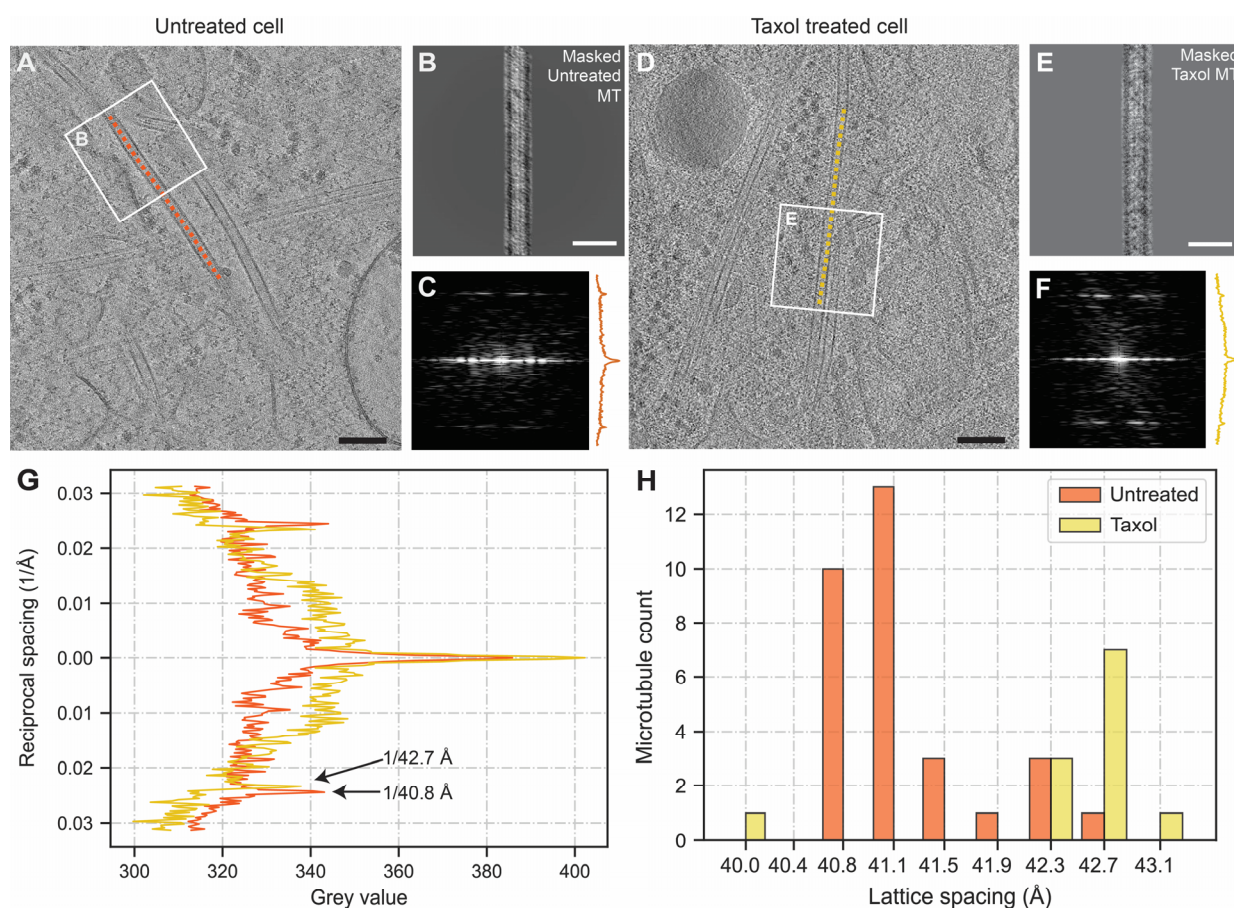
- 391 Guardia, Carlos M., Farías, Ginny G., Jia, R., Pu, J. & Bonifacino, Juan S. (2016) BORC Functions
392 Upstream of Kinesins 1 and 3 to Coordinate Regional Movement of Lysosomes along
393 Different Microtubule Tracks. *Cell Reports*, 17(8), 1950-1961. 10.1016/j.celrep.2016.10.062.
- 394 Gwosch, K. C., Pape, J. K., Balzarotti, F., Hoess, P., Ellenberg, J., Ries, J. & Hell, S. W. (2020) MINFLUX
395 nanoscopy delivers 3D multicolor nanometer resolution in cells. *Nature Methods*, 17(2), 217-
396 224. 10.1038/s41592-019-0688-0.
- 397 Hagen, W. J. H., Wan, W. & Briggs, J. A. G. (2017) Implementation of a cryo-electron tomography tilt-
398 scheme optimized for high resolution subtomogram averaging. *Journal of Structural Biology*,
399 197(2), 191-198. 10.1016/j.jsb.2016.06.007.
- 400 Hammond, J. W., Huang, C.-F., Kaech, S., Jacobson, C., Banker, G. & Verhey, K. J. (2010)
401 Posttranslational Modifications of Tubulin and the Polarized Transport of Kinesin-1 in
402 Neurons. *Molecular Biology of the Cell*, 21(4), 572-583. 10.1091/mbc.e09-01-0044.
- 403 Hooikaas, P. J., Martin, M., Muhlethaler, T., Kuijntjes, G. J., Peeters, C. A. E., Katrukha, E. A., Ferrari,
404 L., Stucchi, R., Verhagen, D. G. F., van Riel, W. E., Grigoriev, I., Altelaar, A. F. M., Hoogenraad,
405 C. C., Rudiger, S. G. D., Steinmetz, M. O., Kapitein, L. C. & Akhmanova, A. (2019) MAP7 family
406 proteins regulate kinesin-1 recruitment and activation. *J Cell Biol*, 218(4), 1298-1318.
407 10.1083/jcb.201808065.
- 408 Hyman, A. A., Chrétien, D., Arnal, I. & Wade, R. H. (1995) Structural changes accompanying GTP
409 hydrolysis in microtubules: information from a slowly hydrolyzable analogue guanylyl-
410 (alpha,beta)-methylene-diphosphonate. *Journal of Cell Biology*, 128(1), 117-125.
411 10.1083/jcb.128.1.117.
- 412 Janke, C. & Magiera, M. M. (2020) The tubulin code and its role in controlling microtubule properties
413 and functions. *Nature Reviews Molecular Cell Biology*, 21(6), 307-326. 10.1038/s41580-020-
414 0214-3.
- 415 Jansen, K. I., Burute, M. & Kapitein, L. C. (2021) A live-cell marker to visualize the dynamics of stable
416 microtubules. *bioRxiv*, 2021.06.23.449589. 10.1101/2021.06.23.449589.
- 417 Kaul, N., Soppina, V. & Verhey, K. J. (2014) Effects of alpha-tubulin K40 acetylation and
418 detyrosination on kinesin-1 motility in a purified system. *Biophys J*, 106(12), 2636-43.
419 10.1016/j.bpj.2014.05.008.
- 420 Kellogg, E. H., Hejab, N. M. A., Howes, S., Northcote, P., Miller, J. H., Diaz, J. F., Downing, K. H. &
421 Nogales, E. (2017) Insights into the Distinct Mechanisms of Action of Taxane and Non-Taxane
422 Microtubule Stabilizers from Cryo-EM Structures. *J Mol Biol*, 429(5), 633-646.
423 10.1016/j.jmb.2017.01.001.
- 424 Kluyver, T., Ragan-Kelley, B., Pérez, F., Granger, B. E., Bussonnier, M., Frederic, J., Kelley, K., Hamrick,
425 J. B., Grout, J. & Corlay, S. (2016) *Jupyter Notebooks—a publishing format for reproducible*
426 *computational workflows*.
- 427 Konishi, Y. & Setou, M. (2009) Tubulin tyrosination navigates the kinesin-1 motor domain to axons.
428 *Nature Neuroscience*, 12(5), 559-567. 10.1038/nn.2314.
- 429 Kremer, J. R., Mastronarde, D. N. & McIntosh, J. R. (1996) Computer visualization of three-
430 dimensional image data using IMOD. *J Struct Biol*, 116(1), 71-6. 10.1006/jsbi.1996.0013.
- 431 LaFrance, B. J., Roostalu, J., Henkin, G., Greber, B. J., Zhang, R., Normanno, D., McCollum, C., Surrey,
432 T. & Nogales, E. (2021) Structural transitions in the GTP cap visualized by cryo-EM of
433 catalytically inactive microtubules. *bioRxiv*. 10.1101/2021.08.13.456308.
- 434 Liao, G. & Gundersen, G. G. (1998) Kinesin is a candidate for cross-bridging microtubules and
435 intermediate filaments. Selective binding of kinesin to detyrosinated tubulin and vimentin. *J*
436 *Biol Chem*, 273(16), 9797-803. 10.1074/jbc.273.16.9797.
- 437 Mandelkow, E., Thomas, J. & Cohen, C. (1977) Microtubule structure at low resolution by x-ray
438 diffraction. *Proc Natl Acad Sci U S A*, 74(8), 3370-4. 10.1073/pnas.74.8.3370.
- 439 Manka, S. W. & Moores, C. A. (2018a) Microtubule structure by cryo-EM: snapshots of dynamic
440 instability. *Essays Biochem*, 62(6), 737-751. 10.1042/EBC20180031.

- 441 Manka, S. W. & Moores, C. A. (2018b) The role of tubulin–tubulin lattice contacts in the mechanism
442 of microtubule dynamic instability. *Nature Structural & Molecular Biology*, 25(7), 607-615.
443 10.1038/s41594-018-0087-8.
- 444 Mastronarde, D. N. (2003) SerialEM: A Program for Automated Tilt Series Acquisition on Tecnai
445 Microscopes Using Prediction of Specimen Position. *Microscopy and Microanalysis*, 9(S02),
446 1182-1183. 10.1017/S1431927603445911.
- 447 Monroy, B. Y., Sawyer, D. L., Ackermann, B. E., Borden, M. M., Tan, T. C. & Ori-McKenney, K. M.
448 (2018) Competition between microtubule-associated proteins directs motor transport.
449 *Nature Communications*, 9(1), 1487. 10.1038/s41467-018-03909-2.
- 450 Monroy, B. Y., Tan, T. C., Oclaman, J. M., Han, J. S., Simó, S., Niwa, S., Nowakowski, D. W., McKenney,
451 R. J. & Ori-McKenney, K. M. (2020) A Combinatorial MAP Code Dictates Polarized
452 Microtubule Transport. *Dev Cell*, 53(1), 60-72.e4. 10.1016/j.devcel.2020.01.029.
- 453 Morikawa, M., Yajima, H., Nitta, R., Inoue, S., Ogura, T., Sato, C. & Hirokawa, N. (2015) X-ray and
454 Cryo-EM structures reveal mutual conformational changes of Kinesin and GTP-state
455 microtubules upon binding. *The EMBO Journal*, 34(9), 1270-1286.
456 10.15252/embj.201490588.
- 457 Orr, G. A., Verdier-Pinard, P., McDaid, H. & Horwitz, S. B. (2003) Mechanisms of Taxol resistance
458 related to microtubules. *Oncogene*, 22(47), 7280-95. 10.1038/sj.onc.1206934.
- 459 Peet, D. R., Burroughs, N. J. & Cross, R. A. (2018) Kinesin expands and stabilizes the GDP-microtubule
460 lattice. *Nat Nanotechnol*, 13(5), 386-391. 10.1038/s41565-018-0084-4.
- 461 Rai, A., Liu, T., Glauser, S., Katrukha, E. A., Estevez-Gallego, J., Rodriguez-Garcia, R., Fang, W. S., Diaz,
462 J. F., Steinmetz, M. O., Altmann, K. H., Kapitein, L. C., Moores, C. A. & Akhmanova, A. (2019)
463 Taxanes convert regions of perturbed microtubule growth into rescue sites. *Nat Mater*,
464 19(3), 355-365. 10.1038/s41563-019-0546-6.
- 465 Rice, S., Lin, A. W., Safer, D., Hart, C. L., Naber, N., Carragher, B. O., Cain, S. M., Pechatnikova, E.,
466 Wilson-Kubalek, E. M., Whittaker, M., Pate, E., Cooke, R., Taylor, E. W., Milligan, R. A. & Vale,
467 R. D. (1999) A structural change in the kinesin motor protein that drives motility. *Nature*,
468 402(6763), 778-84. 10.1038/45483.
- 469 Rigort, A., Bäuerlein, F. J. B., Villa, E., Eibauer, M., Laugks, T., Baumeister, W. & Plitzko, J. M. (2012)
470 Focused ion beam micromachining of eukaryotic cells for cryoelectron tomography.
471 *Proceedings of the National Academy of Sciences*, 109(12), 4449-4454.
472 10.1073/pnas.1201333109.
- 473 Roll-Mecak, A. (2020) The Tubulin Code in Microtubule Dynamics and Information Encoding.
474 *Developmental Cell*, 54(1), 7-20. 10.1016/j.devcel.2020.06.008.
- 475 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S.,
476 Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K.,
477 Tomancak, P. & Cardona, A. (2012) Fiji: an open-source platform for biological-image
478 analysis. *Nat Methods*, 9(7), 676-82. 10.1038/nmeth.2019.
- 479 Schorb, M. & Briggs, J. A. (2014) Correlated cryo-fluorescence and cryo-electron microscopy with
480 high spatial precision and improved sensitivity. *Ultramicroscopy*, 143, 24-32.
481 10.1016/j.ultramic.2013.10.015.
- 482 Schwarz, P. M., Liggins, J. R. & Ludueña, R. F. (1998) β -Tubulin Isotypes Purified from Bovine Brain
483 Have Different Relative Stabilities. *Biochemistry*, 37(13), 4687-4692. 10.1021/bi972763d.
- 484 Shaner, N. C., Lambert, G. G., Chamma, A., Ni, Y., Cranfill, P. J., Baird, M. A., Sell, B. R., Allen, J. R.,
485 Day, R. N., Israelsson, M., Davidson, M. W. & Wang, J. (2013) A bright monomeric green
486 fluorescent protein derived from Branchiostoma lanceolatum. *Nat Methods*, 10(5), 407-9.
487 10.1038/nmeth.2413.
- 488 Shastry, S. & Hancock, W. O. (2010) Neck linker length determines the degree of processivity in
489 kinesin-1 and kinesin-2 motors. *Current biology : CB*, 20(10), 939-943.
490 10.1016/j.cub.2010.03.065.

- 491 Shima, T., Morikawa, M., Kaneshiro, J., Kambara, T., Kamimura, S., Yagi, T., Iwamoto, H., Uemura, S.,
492 Shigematsu, H., Shirouzu, M., Ichimura, T., Watanabe, T. M., Nitta, R., Okada, Y. & Hirokawa,
493 N. (2018) Kinesin-binding-triggered conformation switching of microtubules contributes to
494 polarized transport. *J Cell Biol*, 217(12), 4164-4183. 10.1083/jcb.201711178.
- 495 Siahhaan, V., Tan, R., Humhalova, T., Libusova, L., Lacey, S. E., Tan, T., Dacy, M., Ori-McKenney, K. M.,
496 McKenney, R. J., Braun, M. & Lansky, Z. (2021) Microtubule Lattice Spacing Governs
497 Cohesive Envelope Formation of Tau Family Proteins. *bioRxiv*, 2021.11.08.467404.
498 10.1101/2021.11.08.467404.
- 499 Sirajuddin, M., Rice, L. M. & Vale, R. D. (2014) Regulation of microtubule motors by tubulin isoforms
500 and post-translational modifications. *Nature Cell Biology*, 16(4), 335-344. 10.1038/ncb2920.
- 501 Tas, R. P., Chazeau, A., Cloin, B. M. C., Lambers, M. L. A., Hoogenraad, C. C. & Kapitein, L. C. (2017)
502 Differentiation between Oppositely Oriented Microtubules Controls Polarized Neuronal
503 Transport. *Neuron*, 96(6), 1264-1271.e5. 10.1016/j.neuron.2017.11.018.
- 504 Tirnauer, J. S. & Bierer, B. E. (2000) EB1 proteins regulate microtubule dynamics, cell polarity, and
505 chromosome stability. *The Journal of cell biology*, 149(4), 761-766. 10.1083/jcb.149.4.761.
- 506 Vale, R. D., Coppin, C. M., Malik, F., Kull, F. J. & Milligan, R. A. (1994) Tubulin GTP hydrolysis
507 influences the structure, mechanical properties, and kinesin-driven transport of
508 microtubules. *Journal of Biological Chemistry*, 269(38), 23769-23775. 10.1016/s0021-
509 9258(17)31582-x.
- 510 Verhey, K. J. & Gaertig, J. (2007) The tubulin code. *Cell Cycle*, 6(17), 2152-60. 10.4161/cc.6.17.4633.
- 511 Wagner, F. R., Watanabe, R., Schampers, R., Singh, D., Persoon, H., Schaffer, M., Fruhstorfer, P.,
512 Plitzko, J. & Villa, E. (2020) Preparing samples from whole cells using focused-ion-beam
513 milling for cryo-electron tomography. *Nat Protoc*, 15(6), 2041-2070. 10.1038/s41596-020-
514 0320-x.
- 515 Walter, W. J., Beranek, V., Fischermeier, E. & Diez, S. (2012) Tubulin acetylation alone does not affect
516 kinesin-1 velocity and run length in vitro. *PLoS One*, 7(8), e42218.
517 10.1371/journal.pone.0042218.
- 518 Watanabe, R., Buschauer, R., Bohning, J., Audagnotto, M., Lasker, K., Lu, T. W., Boassa, D., Taylor, S.
519 & Villa, E. (2020) The In Situ Structure of Parkinson's Disease-Linked LRRK2. *Cell*, 182(6),
520 1508-1518 e16. 10.1016/j.cell.2020.08.004.
- 521 Yildiz, A., Tomishige, M., Gennerich, A. & Vale, R. D. (2008) Intramolecular Strain Coordinates Kinesin
522 Stepping Behavior along Microtubules. *Cell*, 134(6), 1030-1041. 10.1016/j.cell.2008.07.018.
- 523 Zhang, R., LaFrance, B. & Nogales, E. (2018) Separating the effects of nucleotide and EB binding on
524 microtubule structure. *Proc Natl Acad Sci U S A*, 115(27), E6191-E6200.
525 10.1073/pnas.1802637115.
- 526 Zheng, S. Q., Palovcak, E., Armache, J. P., Verba, K. A., Cheng, Y. & Agard, D. A. (2017) MotionCor2:
527 anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat*
528 *Methods*, 14(4), 331-332. 10.1038/nmeth.4193.
- 529 Zhou, Y., Handley, M., Carles, G. & Harvey, A. R. (2019) Advances in 3D single particle localization
530 microscopy. *APL Photonics*, 4(6), 060901. 10.1063/1.5093310.

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533

534 **Figure 1. Microtubule lattice spacings within cells are diverse and expand upon Taxol treatment.**

535 (A) Tomogram slice (thickness 10 nm) showing the selected microtubule (MT) backbone of a MT in

536 an untreated U2OS cell (orange). (B) Aligned and masked MT segment from the MT shown in A. (C)

537 Power spectrum of the MT segment and its layer line plot from the MT shown in A. (D-F) Similar MT

538 layer line analysis performed on Taxol-treated U2OS cells (yellow). (G) Layer line plot of the summed

539 power spectra of segments from the same MT in A (orange, untreated) and D (yellow, Taxol), arrows

540 indicate the location of the layer line peak and its related lattice spacing. (H) Histogram showing the

541 untreated (orange, N=31, 12 tomograms) and Taxol-treated (yellow, N=12, 4 tomograms) lattice

542 spacings. Scale bars: 100 nm (A,D), 50 nm (B,E). Untreated distribution is significantly different from

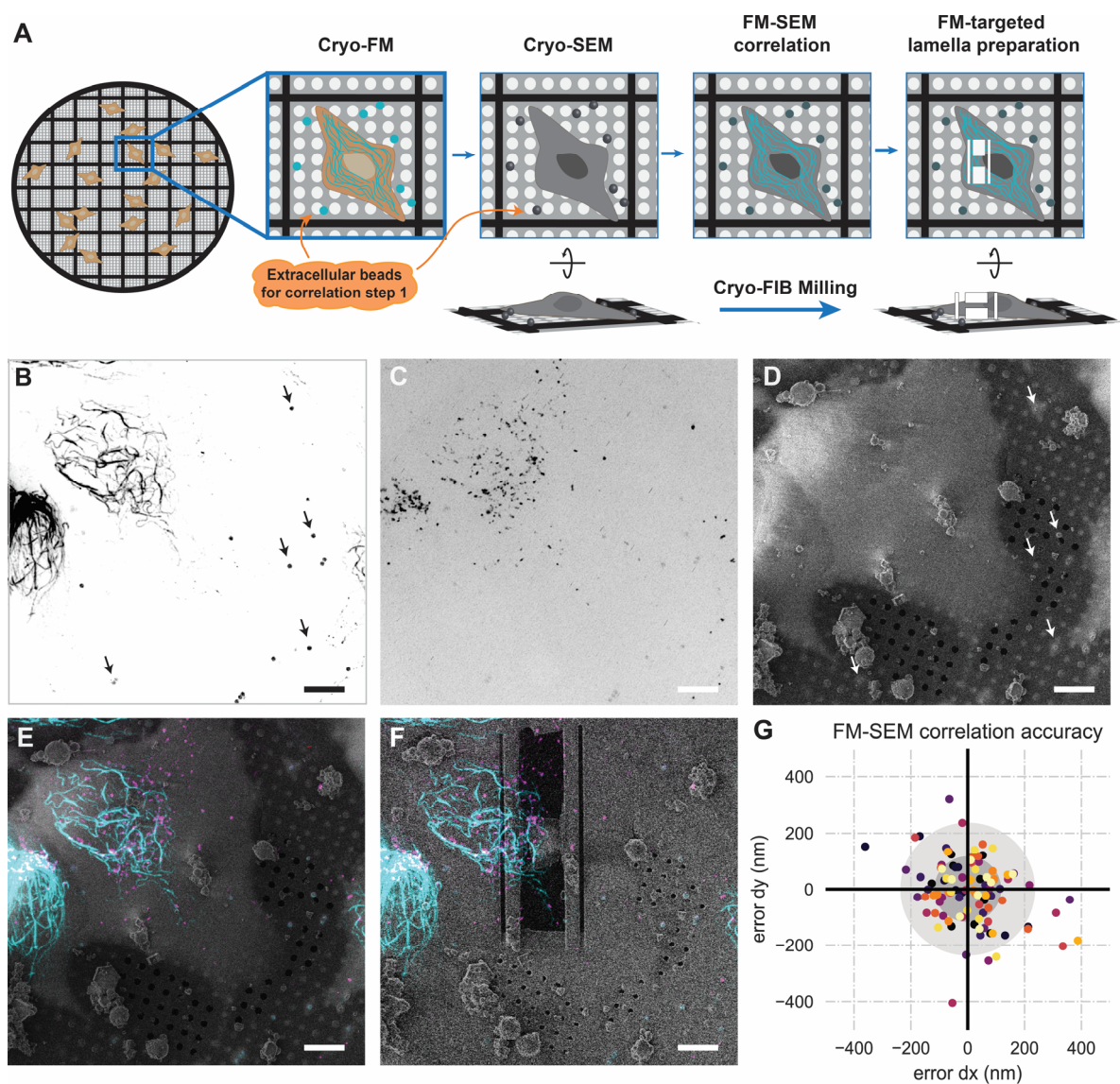
543 Taxol distribution (p-value = 0.0006, unpaired t-test based permutation-test).

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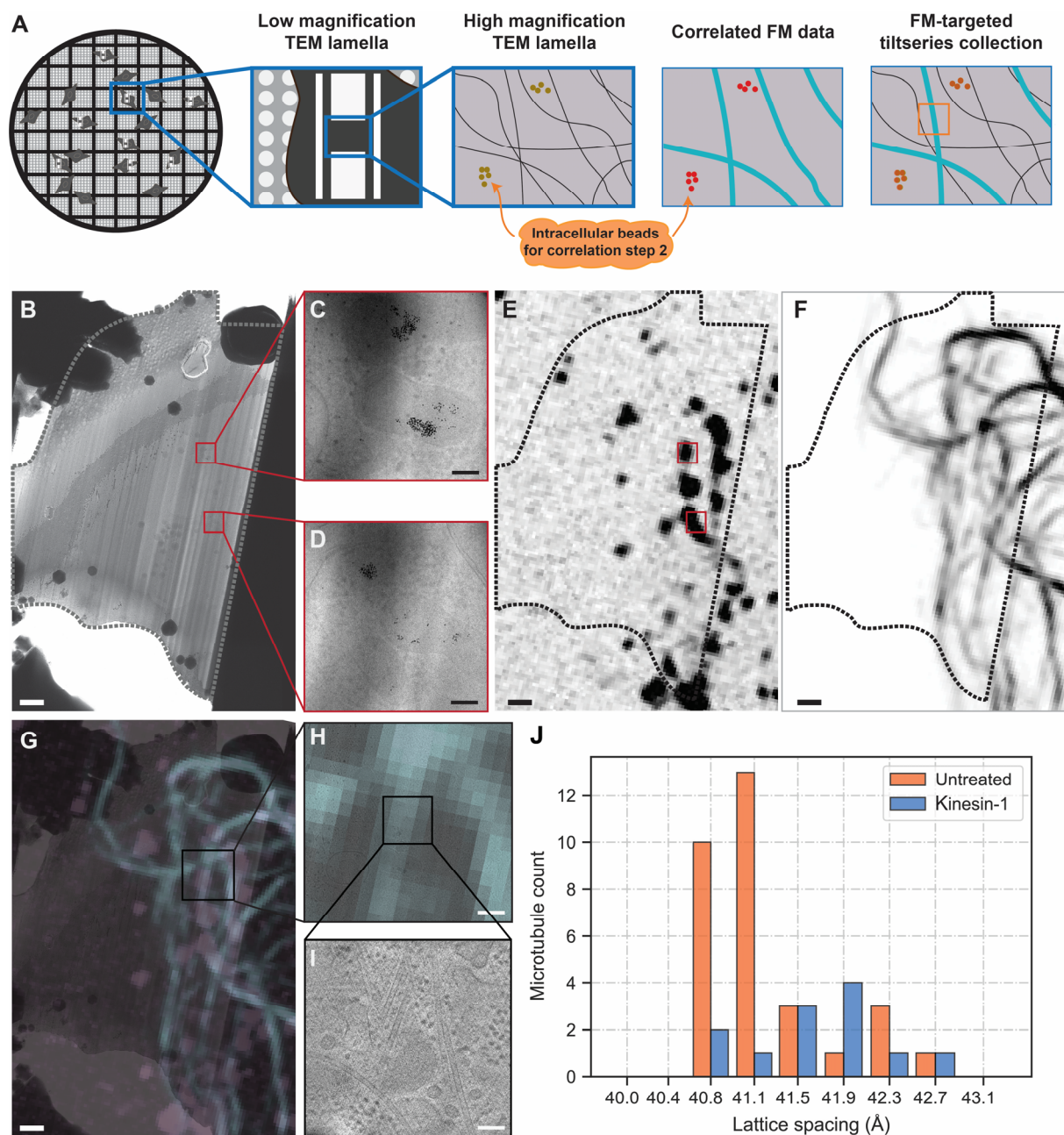


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549 **Figure 2. Correlation of FM to SEM data for targeted cryo-FIB milling.** (A) Cartoon describing the
 550 FM-SEM correlation performed using extracellular beads. (B) MIP of a rigor-2xmNeongreen z-stack,
 551 beads used for 3D correlation are indicated with arrows. (C) MIP of a z-stack with fBSA-Au⁵ beads
 552 used for the subsequent FM-TEM correlation (see Figure 3). (D) Untilted SEM image of the same grid
 553 square as shown in B and C. (E) Correlated rigor-2xmNeongreen and fBSA-Au⁵ overlaid with the
 554 SEM image. (F) Correlated rigor-2xmNeongreen and fBSA-Au⁵ overlaid with the untitled SEM
 555 image of the polished lamella (milled at a 9 degree angle). (G) Scatterplot of correlation errors from
 556 leave-one-out calculations, each dataset has a unique colour, grey circles mark the 1xSD and 2xSD
 557 boundaries (12 datasets, 96 beads). Scale bars: 10 μ m (B-F).

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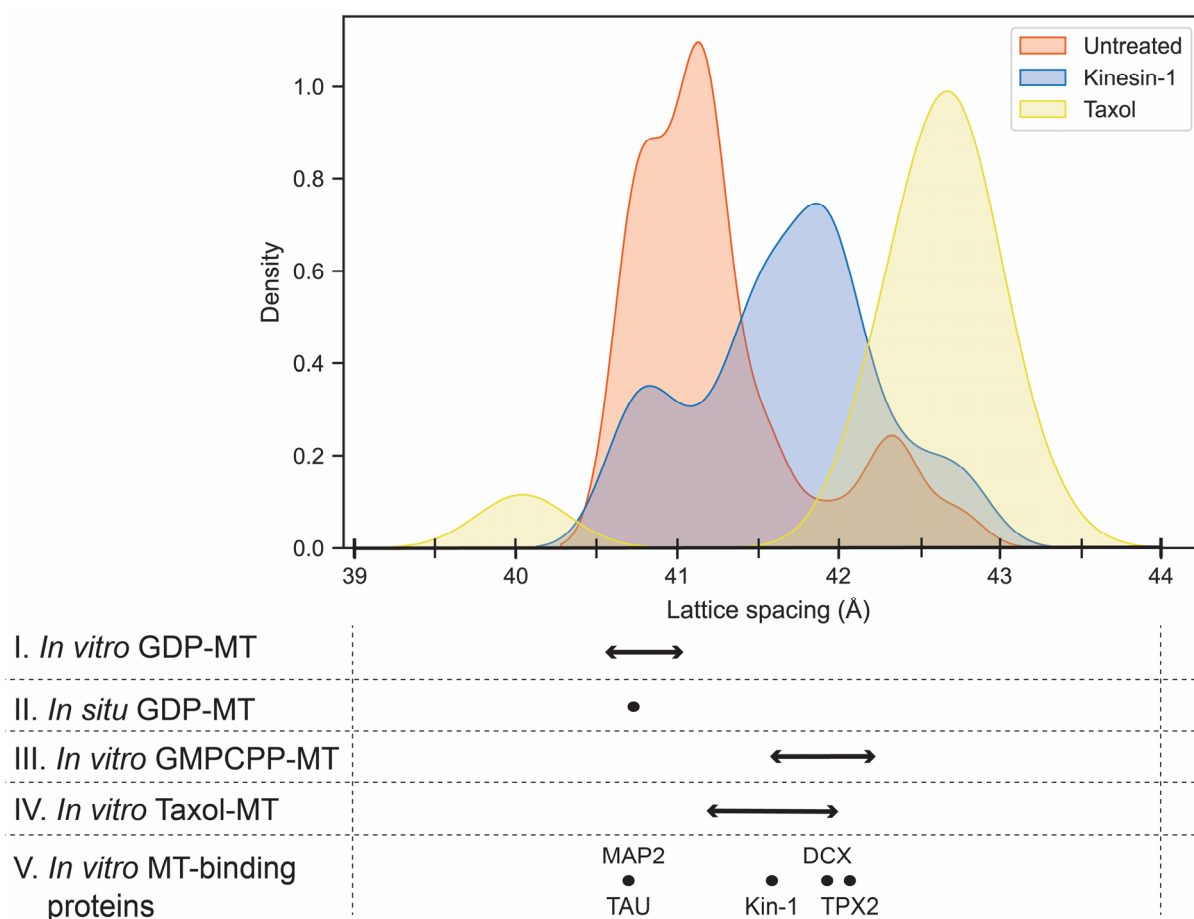


560

561 **Figure 3. Kinesin-1 bound microtubules have a slightly expanded lattice compared to the GDP-**
 562 **compact lattice.** (A) Cartoon describing the FM-TEM correlation performed using intracellular
 563 beads. (B) TEM overview image of a lamella, dotted line indicates outline of the lamella, red squares
 564 the location of the fBSA-Au⁵ beads. (C and D) Zoom in of the fBSA-Au⁵ beads found both in the TEM
 565 lamella in B and in the correlated fBSA-Au⁵ FM data in E. (E) Correlated fBSA-Au⁵ FM data, red
 566 squares indicate fBSA-Au⁵ location. (F) Correlated rigor-bound microtubules. (G) Overlay of TEM
 567 lamella and correlated FM data of both fBSA-Au⁵ (pink) and rigor-bound microtubules (blue). (H and
 568 I) Two step zoom of two microtubules overlapping with the rigor-bound FM data. (J) Histogram
 569 showing the untreated lattice spacings (N=31, 12 tomograms, same data as Fig. 1H, included for
 570 comparison) and the lattice spacings of the kinesin-1 bound subset of microtubules (N=12, 6

571 tomograms). Scale bars: 1 μm (**B, E, F and G**), 100 nm (**C,D and I**), 400 nm (**H**). Untreated distribution
572 is significantly different from kinesin-1 distribution (p-value = 0.045, unpaired t-test based permutation
573 test).

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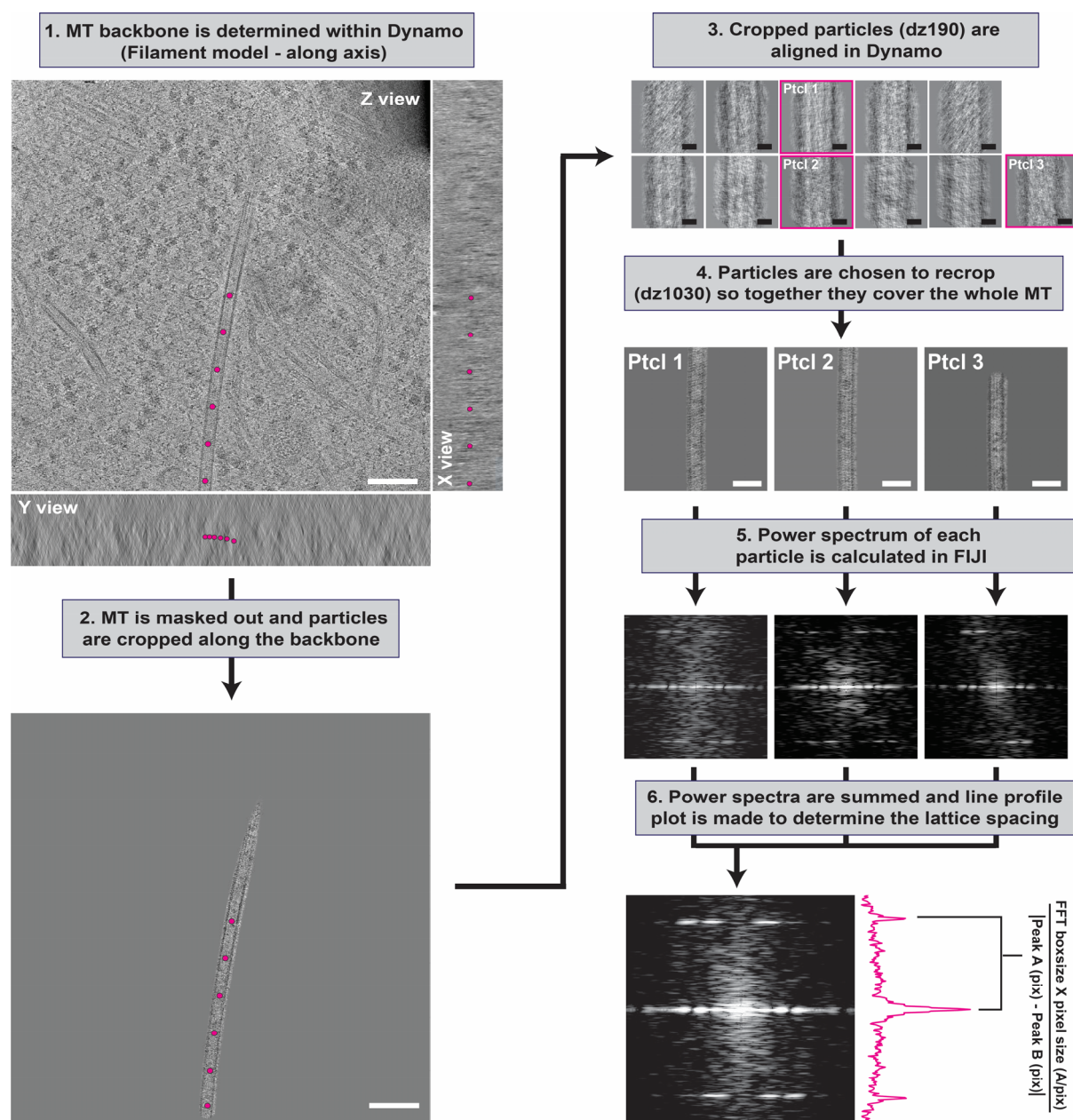
576 Figure 4. ***In situ* microtubule landscape in relation to previously reported lattice spacings.** Upper
 577 panel shows a density distribution calculated from the different lattice spacing datasets measured here.
 578 The table below shows the reported lattice spacings for the GDP lattice both *in vitro* and *in situ* (row I
 579 and II), and for *in vitro* GMPCPP and Taxol-bound lattices (row III and IV). Row V shows MT-binding
 580 proteins for which binding in relationship to the microtubule lattice spacing has been investigated.
 581 References can be found in supplementary table 1.

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584 SUPPLEMENTARY MATERIALS

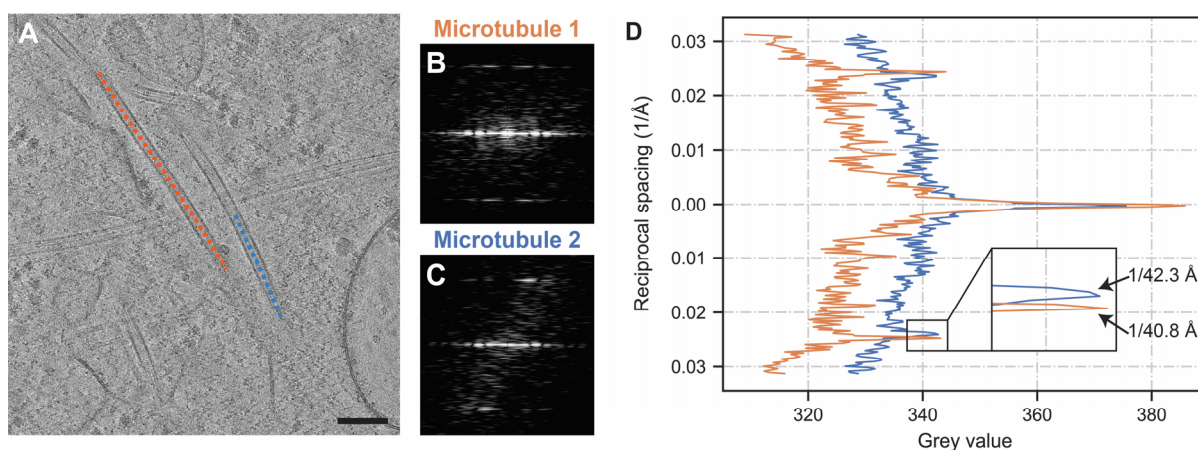
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587 Supplemental Figure 1. *In situ* layer line analysis workflow. Graphical depiction of the steps
 588 performed during the layer line analysis. (Step 1) Microtubule backbone is picked in dynamo. (Step 2)
 589 Using the backbone coordinates, a mask around the microtubule is generated and particles are cropped.
 590 (Step 3) The cropped particles (box size 190) are aligned and picked so that after recropping (step 4)
 591 with a box size of 1030 the whole microtubule is covered. (Step 5) 2D power spectrum of the MIP of
 592 each particle is calculated. (Step 6) power spectra of all particles part of the same microtubule are
 593 summed and the final power spectrum is used to localize the layer line and thereby calculate the lattice
 594 spacing. Scale bars: 100 nm (step 1, 2), 10 nm (step 3), 50 nm (step 4).

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597 Supplemental Figure 2. **Expanded and compacted microtubule lattices within one tomogram.**

598 Tomogram where two MT backbones were analysed in parallel. (A) MT backbones were localized and

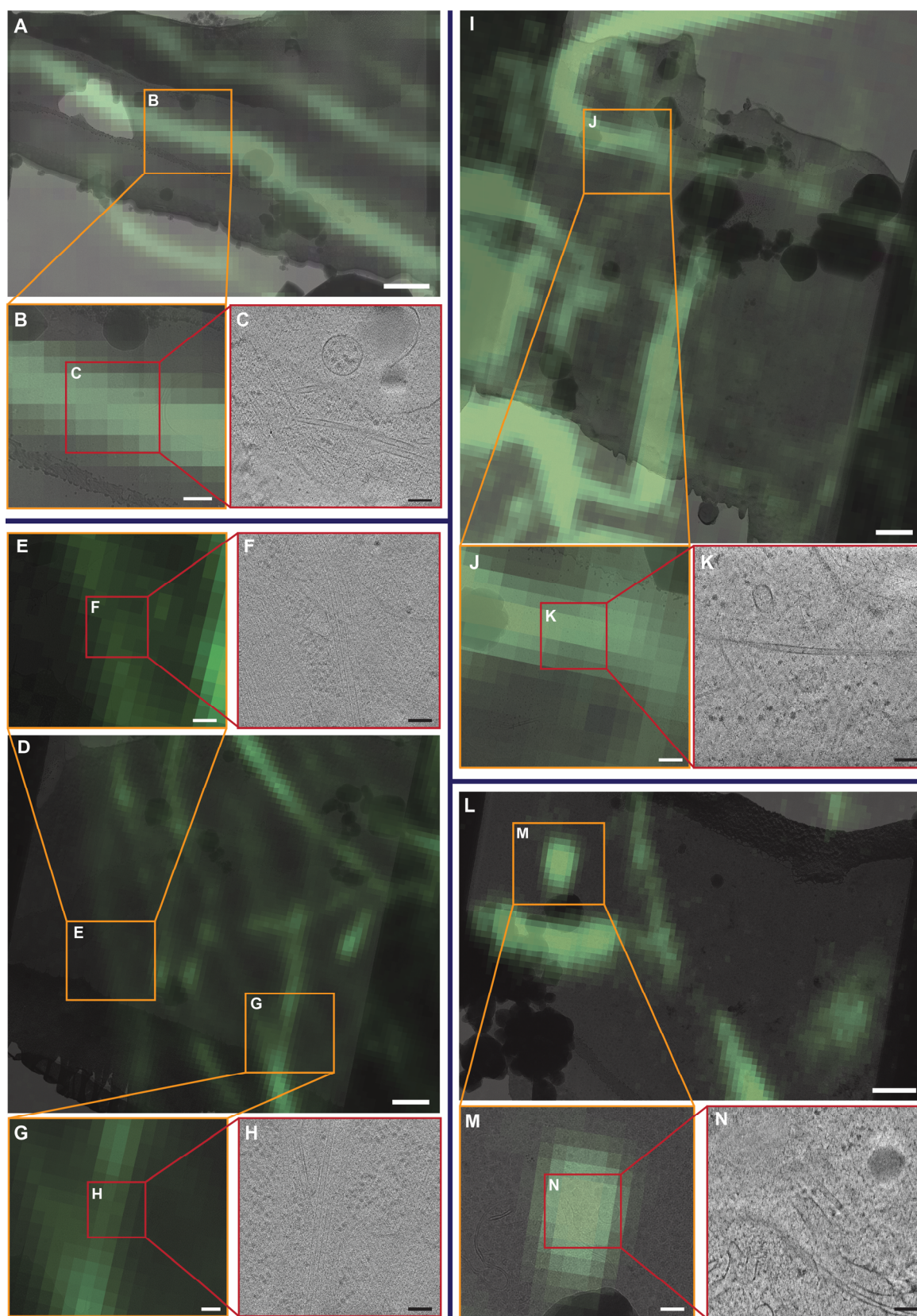
599 processed as explained in supplemental figure 1. (B and C) show the final, summed power spectra of

600 MT1 (orange) and MT2 (blue). (D) Layer line plot of the summed power spectra of MT1 and MT2.

601 Scale bar: 100 nm (A).

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605 Supplemental Figure 3. **Kinesin-1 bound microtubules found via our cryo-CLEM set up.** (A) TEM
606 lamella overlaid with correlated rigorm-2xNeonGreen cryo-FM data. (B and C) One time and two time

607 zoom in of the region of interest with the correlated microtubules. **(D-F, D/G/H, I-K and L-N)** Similar
 608 examples of correlated microtubules. Scale bars: 1 μm (**A, D, I, L**), 300 nm (**B, E, G, J, M**) and 100
 609 nm (**C, F, H, K, N**).

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611 *Supplementary table 1. Literature that investigates the microtubule lattice spacings with respect to*
 612 *nucleotide state, Taxol treatment and MT-binding proteins.*

Microtubule component	References
I. <i>In vitro</i> GDP-MT	(Estevez-Gallego et al., 2020; Hyman et al., 1995; LaFrance et al., 2021; Rai et al., 2019; Vale et al., 1994; Zhang et al., 2018)
II. <i>In situ</i> GDP-MT	(Watanabe et al., 2020)
III. <i>In vitro</i> GMPCPP-MT	(Estevez-Gallego et al., 2020; Hyman et al., 1995; Kellogg et al., 2017; LaFrance et al., 2021; Vale et al., 1994; Zhang et al., 2018)
IV. <i>In vitro</i> Taxol-MT	(Alushin et al., 2014; Estevez-Gallego et al., 2020; Kellogg et al., 2017; Rai et al., 2019; Vale et al., 1994)
V. <i>In vitro</i> MT-binding proteins	(Manka and Moores, 2018b; Peet et al., 2018; Shima et al., 2018; Siahaan et al., 2021; Zhang et al., 2018)

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614 List with abbreviations

Abbreviation	Meaning
CLEM	Correlative light and electron microscopy
GMPCPP	Guanylyl-(α,β)-methylene-diphosphonate
FIB	Focused ion beam
FM	Fluorescence microscopy
MAP	Microtubule associated proteins
MIP	Maximum image projection
MT	Microtubule
PTM	Post-translational modification
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

615 **(Standard abbreviations: GTP, GDP, NA, WT)**

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