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1 **Lysate-based pipeline to characterise microtubule-associated proteins uncovers unique**
2 **microtubule behaviours**

3

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16

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18 microtubule reconstitution, medium-throughput, cell lysate

19

20 **ABSTRACT**

21 The microtubule (MT) cytoskeleton forms complex macromolecular assemblies with a
22 plethora of MT-associated proteins (MAPs) that play fundamental roles in cell architecture,
23 division and motility. Determining how an individual MAP modulates MT behaviour is an
24 important step in understanding the physiological roles of various MT assemblies. To
25 characterise how the ever-increasing number of MAPs control MT properties and functions,
26 we developed an approach allowing for medium-throughput analyses of MAPs in cell-free
27 conditions using lysates of mammalian cells. Our pipeline allows for quantitative as well as
28 ultrastructural analyses of MT-MAP assemblies. Analysing about fifty *bona-fide* and potential
29 mammalian MAPs, we uncovered previously unknown activities that lead to distinct and
30 unique MT behaviours, such as MT coiling or hook formation, or liquid-liquid phase
31 separation along MT lattice that initiates MT branching. We have thus established a powerful
32 tool for a thorough characterisation of a wide range of MAPs and MAP variants, thus opening
33 avenues for the determination of mechanisms underlying their physiological roles and
34 pathological implications.

35

36 MAIN TEXT

37 Introduction

38 The microtubule (MT) cytoskeleton is a key structural and mechanical component of all
39 known eukaryotic cells. MTs assemble into a wide variety of dynamic arrays and structures
40 that are implicated in many different cellular functions, such as cell division, motility, shape
41 and intracellular transport. It has been known for decades that building these meta-structures
42 requires distinct sets of microtubule-associated proteins (MAPs), among which are molecular
43 motors¹⁻³, severing enzymes⁴ and proteins that regulate MT plus or minus ends⁵. However, the
44 term “MAP” is usually reserved to describe non-enzymatic proteins that bind to the MT
45 network, which are also referred to as “structural MAPs”⁶⁻⁸. While the number of known
46 MAPs is steadily increasing⁸ and many novel links to human diseases are being described
47 (Supplementary Table 1 and citations within), most of these proteins remain poorly
48 characterised, thus impeding the understanding of their biological roles.

49 Characterising MAPs is a non-trivial endeavour. Cell-biological approaches, on one hand, are
50 limited by the fact that the overexpression of MAPs often leads to a variety of artefacts, such
51 as excessive MT bundling. In-depth biochemical and biophysical characterisation, on the
52 other hand, classically employs *in vitro* reconstitution assays that require purification of the
53 MAP in a functional form⁹. Despite recent advances in purifying and studying the role of
54 multiple MAPs, such as MAP7, tau, MAP2, DCX, DCLK1 and MAP9¹⁰, or the role of MAP7
55 family in controlling kinesin-1¹¹, such approaches still remain cumbersome and are thus
56 limiting their application to a larger number of MAPs.

57 Here we established a pipeline to observe a wide variety of MAPs in cell lysates without prior
58 protein purification at single-MT resolution, using total internal reflection fluorescence
59 (TIRF) microscopy. The use of cell lysates was inspired by previous work using *Xenopus*
60 *laevis* egg extracts to study complex subcellular structures of the cytoskeleton^{12, 13}, as well as
61 studies employing lysates from yeast¹⁴, or mammalian cells¹⁵⁻²⁰ to study MT motors and their
62 impact on MT dynamics²¹. Out of the 45 proteins we analysed, some are well-known MAPs,
63 while others inferred to be MAPs based on their colocalisation with MTs in cells, or
64 bioinformatic prediction (Supplementary Table 1). As all proteins were invariably soluble in
65 our lysate approach, we discovered a variety of remarkable, so-far unknown activities by
66 which MAPs affect the behaviour of dynamically growing MTs. We further used our
67 approach to characterise these MT phenotypes in a quantitative manner, and demonstrate that
68 MT-MAP assemblies in lysates allow obtaining MT-binding information by cryo-electron

69 microscopy. Finally, we demonstrate how our method can be used to answer long-standing
70 open questions in the field. Based on an early discovery²² we reveal that CLIP170, known as a
71 MT-plus-tip binding protein, forms co-condensates of CLIP170 and tubulin at the MT lattice,
72 which can nucleate new MTs, thereby forming branched MTs. Our lysate-based approach is
73 thus a medium-throughput discovery tool that allows screening for properties and functions of
74 MAPs in MT assembly, and can further be extended towards an in-depth characterisation of
75 MAPs and other complex biological assemblies.

76

77 **Results**

78 A lysate-based pipeline for the characterisation MAPs

79 To enable analyses of any MAP candidate of choice in our pipeline (Fig. 1a), one
80 precondition was to be entirely independent of the availability of pre-existing expression
81 plasmids. Therefore, we based our pipeline on *de-novo* cloning of the open reading frames of
82 proteins of interest using the fast and simple SLIC²³ approach (Step 1, Extended Data Fig. 1).
83 All *bona-fide* and potential MAPs were fused to the fluorescent protein at the carboxy-
84 terminus, except MAPs that had been analysed before, where we followed the tag positioning
85 found in the literature (Supplementary Table 1).

86 The expression of fluorescently-tagged proteins was first tested in U-2 OS cells, allowing to
87 verify the intracellular localisation of the proteins (option to step 2, Fig 1a; Fig. 2a; Extended
88 Data Fig. 2; Supplementary information Fig. 1). For cell-free assays, GFP-tagged MAPs
89 (GFP-MAPs) were expressed in HEK-293 cells (Step 2, Fig 1a), from which soluble cytosolic
90 lysates containing the overexpressed, fluorescently-tagged proteins were generated (Step 3,
91 Fig 1a). Controlling total protein concentrations in all lysates assured similar concentrations
92 of endogenous tubulin in all assays, as tubulin concentrations in cells are strictly regulated in
93 cells²⁴⁻²⁶. Therefore, observed differences in the MT growth behaviour in our assays can be
94 directly attributed to the effect of the GFP-MAPs, which are the sole variable component. Cell
95 lysates were directly introduced into flow chambers where MT polymerisation was recorded
96 by TIRF microscopy (Step 4, Fig 1a). The impact of MAPs on dynamic MTs was observed by
97 recording the GFP-MAPs; MTs and F-actin were visualised by post-staining. Cell lysates
98 were also directly used in ultrastructural studies using cryo-electron microscopy (option to
99 Step 4, Fig 1a).

100 We validated our assay with two well-characterised MT-interacting proteins: the MT-severing
101 enzyme spastin and the end-binding protein 3 (EB3). Purified spastin is known to efficiently
102 sever MTs²⁷. When we incubated taxol-stabilised MTs with lysates of spastin-expressing
103 cells, we observed efficient MT severing (Fig. 1b; Supplementary Video 1a). EB3, which
104 tracks growing MT plus ends²⁸, also did so in lysates (Fig. 1c; Supplementary Video 1b). This
105 demonstrates that our lysate-based approach faithfully reproduces MT-MAP interactions that
106 are known from previous cell-based or *in-vitro* reconstitution assays.

107

108 Systematic characterisation of 45 *bona-fide* or candidate MAPs

109 We next selected a representative panel of 45 *bona-fide*, or candidate MAPs from the
110 literature (Supplementary Table 1). Expression of these 45 proteins in U-2 OS cells (Extended
111 Data Fig. 2; Supplementary information Fig. 1) revealed three principal phenotypes: MAPs
112 that (i) strongly bundle MTs, (ii) decorate MTs and rarely induce visible MT bundles, or (iii)
113 show no MT binding at all (Fig. 2a). We observed some variations within these categories, for
114 instance, among MAPs that bundle MTs some generated thick MT bundles (e.g. Tau, MAP2),
115 while others formed thinner and wavy MT bundles (e.g. GLFND1, MAP7D3). Despite these
116 variations, it was straight-forward to distinguish MT bundling and categorise all MAPs
117 (Fig. 2c; Extended Data Fig. 2; Supplementary information Fig. 1).

118 We next prepared lysates of HEK 293 cells expressing the selected 45 MAPs or MAP
119 candidates and recorded their impact on dynamically growing MTs by TIRF microscopy. We
120 performed at least three independent assays to ensure the reproducibility of our observations.
121 40 out of the 45 tested proteins co-localised with growing MTs in lysates (Fig. 2b, Extended
122 Data Fig. 3-6; Supplementary information Fig. 2; Supplementary Videos 2-13). Most MAPs
123 also induced MT polymerisation independently from the MT seeds (Fig. 2c, grey squares). In
124 some cases, it was difficult to determine whether the newly polymerised MTs were assembled
125 from the seeds (Fig. 2c, grey half-squares). For MAPs with strong nucleation activities, we
126 observed much higher MT densities as compared to control, or lysates expressing MAP
127 candidates that did not bind MTs. This indicates that de-novo MT nucleation and assembly
128 might be a general characteristic of many MAPs than those previously reported^{29, 30}.

129 Labelling of MTs and actin by post-staining revealed that 11 MAPs strongly increased the co-
130 localisation of actin filaments (F-actin) with MTs (Fig. 2c, full red squares), suggesting that
131 they promote alignment of F-actin with MTs. To determine the potential role of such MT-
132 actin interactions in MT behaviour, we prevented actin polymerisation with Latrunculin A³¹,
133 ³². Absence of actin polymerisation in our assays did not impede any MAPs to associate with
134 MTs, nor did it fundamentally change the appearance of the MT networks (Extended Data
135 Fig. 3-6; Supplementary information Fig. 2; Supplementary Videos 2-13). This suggests that
136 the co-alignment of F-actin to MTs by some of the here-tested MAPs has no essential role in
137 the formation of MT arrays by these MAPs.

138 Based on our observations we categorised the 45 candidate MAPs into (i) MAPs that bind
139 MTs, but do not align with F-actin, (ii) MAPs that bind MTs and promote co-alignment of
140 F-actin and MTs, and (iii) proteins that do not bind MTs (Fig. 2b). A combined, schematic
141 representation of MAP behaviour in cells and the lysate-based experiments (Fig. 2c) shows

142 that most MAPs associating with MTs in cells also promote polymerisation and growth of
143 MTs in lysates. This confirms our pipeline as a powerful and versatile tool to analyse the
144 impact of MT-interacting proteins on growing MTs in cell-free conditions.

145

146 The impact of individual MAP-family members on MT behaviour

147 One of the advantages of our pipeline lies in the opportunity to compare side-by-side MAPs
148 that have previously been characterised only in isolated studies. When comparing members of
149 the mammalian EMAP-like protein (EML) family in our assay, we discovered striking
150 differences in their behaviour. While EML1, 3 and 4 associated with growing MTs, no MT
151 polymerisation was observed in the presence of EML2 (Extended Data Fig. 4; Supplementary
152 Video 5), confirming a previous observation that EML2 (also known as EMAP-like protein
153 70) exhibits MT depolymerising activity³³. Among EMLs that bound MTs, EML1 and EML4
154 strongly associated with growing MTs, while EML3 accumulated with a delay at apparently
155 pre-existing MTs (Extended Data Fig. 4; Supplementary Video 5). Finally, EML1 showed a
156 unique feature of accumulation towards growing MT ends. This, however, was visible only at
157 early time points of MT growth but later obscured by uniform accumulation of EML1-GFP
158 all-along MTs (Extended Data Fig. 4a; Supplementary Video 5a). We thus demonstrated that
159 despite their sequence similarity, each of the EML proteins has a distinct type of MT
160 interaction.

161 The MAP7 family comprises four different proteins: MAP7, MAP7D1, MAP7D2 and
162 MAP7D3³⁴⁻³⁶, which were shown to participate to different degrees in linking kinesin to MTs
163 in a thorough *in-vitro* study¹¹. In our lysate approach, MAP7, but none of the other MAP7
164 family members, induced the formation of characteristic aster-like MT arrays (Extended Data
165 Fig. 5; Fig. 3a,b; Supplementary Videos 9,14). This observation is coherent with the function
166 of MAP7 as a mediator of kinesin-MT interactions^{11,37}, and suggests that MAP7 recruits
167 endogenous kinesin motors from the cell lysates, thereby inducing aster formation³⁸. What
168 was striking was not the mere fact that MAP7 induced aster formation, but that none of the
169 other 44 MAPs tested here did so. This unique feature could either reflect the way MAP7
170 binds to MTs in conjunction with kinesin motors, or by recruitment of additional cofactors
171 from the cell lysate that participate in the formation of these unique MT arrays. These
172 findings might therefore provide a framework for deeper mechanistic characterisation of
173 MAP7-induced formation of MT asters.

174

175 Discovery of unique MT behaviours induced by MAPs

176 Some MAPs analysed here showed unique, previously undescribed features with growing
177 MTs in lysates. The centriole, cilia and spindle-associated protein (CSAP), first reported as a
178 MAP specifically colocalising with polyglutamylated MTs on centrioles, mitotic spindles, and
179 cilia³⁹, and later as a recruitment factor of polyglutamylating enzymes to MTs⁴⁰, induced the
180 formation of MT helices in lysates (Fig. 3a,b; Extended Data Fig. 3b; Supplementary
181 Videos 3d,14). To visualise these MT coils, we used the HILO imaging mode, which allows a
182 higher penetration depth than the TIRF mode⁴¹. We measured ~40 CSAP-induced MT coils
183 (Extended Data Fig. 7a) and found a helix width of ~900 nm (Fig. 3c). A similar MT
184 phenotype had so far only been reported for MAP6/STOP⁴².

185 Strikingly, the discovery of previously unknown activities of MAPs in cell lysates was not
186 restricted to scantily studied MAPs such as CSAP. We also discovered a previously
187 undescribed activity for the well-characterised MT-actin crosslinker MACF1⁴³⁻⁴⁵. Because of
188 the large size of MACF1 (~600 kDa), and our focus on MT binders, we used a truncated
189 version of the protein encoding the carboxy-terminal 1023 amino acids (amino acid 4305-end)
190 that include the MT-binding domain⁴⁵, called MACF1_C1023. In lysates, MACF1_C1023-
191 GFP decorates the lattice of growing MTs, which spontaneously formed hooks at their
192 growing ends that prevented further MT elongation (Fig. 3a,b; Extended Data Fig. 3c;
193 Supplementary Videos 7b,14). Measuring >100 individual hooks (Extended Data Fig. 7b)
194 revealed an average diameter of $\sim 1.02 \pm 0.15 \mu\text{m}$ (Fig. 3d). This phenotype appears to be
195 specific to MACF1, as the homologue MACF2 (dystonin) did not form similar structures in
196 our assays (Extended Data Fig. 3d; Supplementary Video 7c).

197 MAP2, a neuronal MAP⁴⁶ expressed either as high-molecular-weight splice isoforms MAP2A
198 and MAP2B (~280 kDa)⁴⁷, or as smaller isoforms MAP2C (~51 kDa) and MAP2D
199 (~54 kDa)^{48,49} strongly promotes MT polymerisation in lysates (Extended Data Fig. 6a,b;
200 Supplementary Videos 7d,8a). Here we observed a previously unknown activity of MAP2C
201 and MAP2D, the induction of faint, hook-like structures that might be transitional
202 deformations of either whole MT lattice, or of a subset of protofilaments. These structures
203 initiate the accumulation of MAP2 into small clusters, or droplet-like assemblies (Fig. 3a,b;
204 Supplementary Video 14). The increase of GFP fluorescence intensity over time showed that
205 these clusters progressively accumulate MAP2. In some cases, MAP2 clusters terminated MT

206 growth (Extended Data Fig. 7c). Our observation that this phenotype was more pronounced
207 for MAP2D as compared to MAP2C (Extended Data Fig. 6a,b; Supplementary Videos 7d,8a)
208 suggests that it directly depends on the interaction strength between MAP2 and MTs, which is
209 stronger for MAP2D due to the presence of an additional MT-binding tandem repeat in this
210 isoform⁴⁸. Strikingly, a similar cluster formation was not observed with the highly similar Tau
211 protein (Extended Data Fig. 6c,d; Supplementary Video 12c,d).

212 Finally, we observed that MTs in lysates were gliding when decorated with DCLK, DCX,
213 MAP2, MAP8, MAP11 and Tau (Supplementary Videos 4,7d,8a,10a,b,12c,d), and to a lesser
214 extent with CKAP2, CKAP2L, EML1 and MAP9 (Supplementary Videos 3b,c,5a,10b). MT
215 gliding activity could occur when a MAP bound to an attached MT captures a motor protein
216 from the cell lysate in a way that activates the motor domain, which in turn would move
217 unattached MTs in the lysate. Uncovering this active motor component could reveal other
218 motor regulatory mechanisms by MAPs, which could be fundamentally different from the
219 way MAP7 is recruiting kinesin¹¹.

220

221 Determining the impact of MAP concentrations in lysates

222 The capacity of MAPs to control MT assembly and dynamics, and their ability to induce the
223 formation of specific MT arrays is concentration-dependent⁵⁰⁻⁵⁴. To determine the impact of
224 MAP concentrations in lysates, we investigated the formation of MT hooks by
225 MACF1_C1023 (Fig. 3a,b; Supplementary Video 14). We estimated the concentration of
226 MACF1_C1023-GFP in the lysate to be 520 nM by immunoblot (Fig. 4a). By performing
227 dilutions with lysate from non-transfected cells, we then prepared lysates in which the
228 concentration of MACF1_C1023-GFP was decreased, while the overall protein concentration
229 of the lysates was maintained at 8 mg/ml. In lysates containing 520 nM MACF1_C1023-GFP,
230 MT growth was rapidly stalled by hook formation, whereas at 260 nM, MTs grew more
231 persistently before forming hooks. At 130 nM, the number of MTs forming hooks was
232 strongly decreased, and no MT hooks at all were formed at 65 nM MACF1_C1023-GFP
233 (Fig. 4b; Supplementary Video 15). The concentration dependency of MACF1-induced MT
234 hook formation suggests that in cells, MT hooks might be induced by local accumulation of
235 MACF1.

236

237 Determining the impact of disease-related mutations of MAPs

238 Whole-genome or exome sequencing of patients with genetic diseases reveals a growing
239 number of MAPs with mutations linked to a spectrum of human disorders (Supplementary
240 Table 1). Being able to rapidly and reliably test the impact of these mutations on the functions
241 of MAPs is essential for the understanding of the molecular mechanisms underlying these
242 diseases. To test whether our lysate-based approach could be used for the functional
243 characterisation of MAP mutations, we analysed an EML1 mutation (EML1^{Thr243Ala}) from
244 patients with subcortical heterotopia, a neurodevelopmental disorder leading to brain
245 malformation^{55, 56}. To directly compare mutant and wild-type proteins, we mixed lysates from
246 cells expressing wild-type EML1-GFP and EML1^{Thr243Ala}-mCherry 1:1 and concomitantly
247 observed the behaviour of both proteins during MT polymerisation (Fig. 5a). While the
248 EML1-GFP strongly decorated the rapidly polymerising MTs, EML1^{Thr243Ala}-mCherry
249 showed no MT decoration (Fig. 5b; Supplementary Video 16a). Experiments with swapped
250 fluorescent tags, i.e. wild-type EML1-mCherry and EML1^{Thr243Ala}-GFP, yielded the same
251 result, thus excluding a potential impact of the fluorescent protein tags (Extended Data Fig. 8;
252 Supplementary Video 16b). This demonstrates that the Thr243Ala mutation of EML1 leads to
253 a strong decrease of its MT-binding capacity, which in turn might affect the overall dynamics
254 and architecture of the MT cytoskeleton in the neurons of patients carrying this mutation^{55, 56}.

255 Apart from a high throughput that allows concomitant screening of a panel of mutations, our
256 lysate-based approach has the capacity to reveal indirect effects of mutations, such as causing
257 the sequestration of a MAP, or a MAP becoming less competitive for binding to a site shared
258 with another MT binder that is likely to be present in the cell lysate. Such indirect effects
259 would not be detected in *in-vitro* binding assays with purified components. This makes our
260 pipeline a promising tool for the medium- to large-scale analyses of disease-related MAP
261 mutations.

262

263 Ultrastructural studies of MAP-MT assemblies in cell lysates

264 One of the greatest challenges to understand MT-MAP interactions at the structural level is
265 that many MAPs are intrinsically unstructured proteins. Recent advances in cryo-electron
266 microscopy have demonstrated that MAPs can adapt defined structures upon their binding to
267 the MT surface, and delivered detailed insight into their mode of binding⁵⁷⁻⁶⁰. Similar to *in-*
268 *vitro* reconstitution experiments, structural studies require purified, functional MAPs, thus
269 encountering the known limitations of protein purification. Having demonstrated that virtually
270 all MAPs characterised here were soluble and functional in cell lysates, we tested whether

271 high-resolution structures of MAPs decorating MTs can be obtained from cell lysates, which
272 had so far only been done for membrane-bound proteins⁶¹.

273 We first adapted our pipeline to cryo-EM requirements. Pre-polymerised GMPCPP-MT seeds
274 were attached to EM grids mounted to the Vitrobot, incubated with cell lysates to allow MT
275 polymerisation, plunge-frozen and imaged (Fig. 6a). Additional tubulin was added to
276 MACF1_C1023-GFP lysates to allow the formation of the MACF1_C1023-induced MT
277 structures within the 2-min time constraint of the freezing approach, as the characteristic MT
278 hooks induced by MACF1_C1023 were mostly observed between 5-10 min by TIRF
279 microscopy (Fig. 3; Supplementary Video 14). Electron micrographs from GMPCPP-MTs
280 assembled from pure tubulin showed an undecorated MT structure; by contrast, MTs
281 assembled in the presence of EML1, EML4 and MACF1_C1023 in lysates showed a regular
282 MT decoration in the micrographs (Fig. 6b). Moreover, while EML1 and EML4 generated
283 straight MTs, MTs formed with MACF1_C1023 showed splayed ends, as well as structures
284 resembling protofilament rings (Fig. 6b). It is possible that the splayed MT ends are
285 precursors for the formation of MT hooks (Fig. 3; Supplementary Video 14).

286 We next performed a helical reconstruction of MTs decorated with full-length EML1, and
287 obtained a high-resolution structure showing densities of EML1 bound along a
288 14-protofilament MT. The MT part of the reconstruction was resolved to 3.7 Å, while the
289 resolution of the electron density corresponding to EML1 was considerably lower. The
290 pattern of decoration resembled a previously determined structure of a 207-amino-acid
291 amino-terminal fragment of EML4 (EML4_N207), obtained with MTs assembled from
292 purified brain tubulin and recombinant EML4_N207⁵⁸ (Fig. 6c; Supplementary Video 17). To
293 directly compare the MT binding modes of both proteins, we fitted EML1 and EML4_N207
294 densities with models for α - and β -tubulin. The overlay images revealed that both proteins
295 bind similarly to the ridges of the protofilaments, where they might interact with flexible
296 carboxy-terminal tubulin tails that were not resolved (Fig. 6d).

297 Our lysate-based approach thus permits the acquisition of electron density maps of MAPs
298 bound to MTs at high resolution, which can be used for reconstruction of high-resolution
299 structures revealing binding modes of MAPs to MTs. The lower resolution of the MAP as
300 compared to the MTs could originate from the higher flexibility of these proteins, in which
301 case the same limitations would be found with purified proteins. Alternatively, lower
302 resolution of a MAP could be caused by sub-stoichiometric binding to the MT lattice. This can
303 be easily overcome with purified components by increasing the MAP concentration, but

304 would require the optimisation of the expression conditions of MAPs in cells to increase their
305 concentrations in the cell lysates. Nevertheless, even low-resolution images of MAPs for
306 which no other structural data are available could provide important information about the
307 mode of binding, and whether competition with other MT interactors could occur.

308

309 Droplet formation by CLIP170 along MTs in lysates

310 Having discovered previously unknown activities for well-characterised MAPs, such as
311 MACF1, MAP2 and MAP7 encouraged us to revisit CLIP170, a protein with extensively
312 characterised MT +TIP-tracking properties⁶²⁻⁶⁵. Intriguingly, however, early observations had
313 shown that CLIP170 can also form patches in cells²², reminiscent of liquid-liquid phase
314 separation, which was confirmed by a recent study⁶⁶.

315 Live-imaging of overexpressed GFP-CLIP170 in U-2 OS cells showed the formation of
316 dynamic GFP patches that emanate from small droplets, which coalesce and deform over
317 time, typical for liquid-like behaviour^{66, 67} (Fig. 7a; Supplementary Video 18a). CLIP170
318 droplets and patches in cells appeared to line MTs and MT bundles (Fig. 7b), suggesting that
319 droplet formation is MT-mediated. We thus examined CLIP170 behaviour in cell lysates,
320 where it indeed formed chains of droplets along MTs. The fluorescence intensity of these
321 droplets (Fig. 7c; Supplementary Video 18b), as well as their diameter (Fig. 7d) increased
322 over time, indicating a progressive accumulation of CLIP170. In some instances, droplets
323 coalesced, which was revealed by an increasing median distance between maximum-
324 fluorescence peaks over time (Fig. 7e).

325 To determine the concentration-dependency of CLIP170-droplet formation, we prepared a
326 series of lysates with decreasing CLIP170, but constant overall protein concentrations.

327 Reduced CLIP170 concentrations led to the formation of smaller droplets (Fig. 7f,g).

328 Intriguingly, not just the size, but also the spacing of the droplets decreased (Fig. 7h;
329 Extended Data Fig. 9), indicating a topological link between these two parameters. We further
330 tested the behaviour of these droplets in the presence of 1,6-hexanediol, known to dissolve
331 protein condensates that are formed by hydrophobic interactions, as it had been shown for
332 condensates of tau on MTs⁶⁸. Our observation that CLIP170 patches were not dissolved by
333 1,6-hexanediol (Extended Data Fig. 10, Supplementary Video 19) indicates that their
334 formation might not result from hydrophobic interactions⁶⁷.

335 Liquid-like droplets of another MT-interacting protein, TPX2, have recently been
336 demonstrated to induce the formation of branched MT networks⁶⁹. To determine whether
337 CLIP170 droplets have a similar activity, we first allowed the formation of MTs decorated
338 with CLIP170 droplets, followed by a second step in which the droplet-decorated MTs were
339 incubated with fresh lysate lacking CLIP170 (Fig. 8a). To visualise MTs in these experiments,
340 lysates were prepared from HeLa Kyoto cells expressing mCherry- α -tubulin⁷⁰. During the
341 initial assembly of CLIP170-decorated MTs, mCherry- α -tubulin accumulated in the CLIP170
342 droplets (Fig. 8b). After lysate exchange, we observed de-novo polymerisation of MTs that
343 almost exclusively originated from CLIP170 droplets (Fig. 8c; Supplementary Video 18c),
344 which was further underpinned by post-staining of MTs (Fig. 8d). This suggests an active role
345 of CLIP170 in the initiation of MT assembly on existing MTs, and thus in the formation of
346 MT branches.

347

348 **Discussion**

349 The MT cytoskeleton is an interaction hub for a multitude of proteins. Understanding how
350 MT-interacting proteins control the properties and functions of MTs is essential for
351 deciphering the working of the MT cytoskeleton. While *in vitro* reconstitution assays of MT
352 arrays from purified components are a gold standard⁹, they critically depend on the successful
353 purification of functional components, which is often a major stumbling stone. As a
354 consequence, only some proteins could so far be thoroughly characterised, which in the light
355 of the constantly growing list of newly discovered MT interactors impedes advances towards
356 a holistic understanding of MT architecture and functions. Moreover, the growing number of
357 links between MAPs and human disorders (Supplementary Table 1)⁸ makes it primordial to
358 establish approaches that allow faster and less constrained characterisation of MAP-MT
359 assemblies in cell-free settings.

360 We demonstrate here that using lysates from mammalian cells overexpressing fluorescently
361 labelled proteins allows to circumvent the most daunting bottleneck of reconstitution assays;
362 the purification of novel proteins. In a set of 45 *bona-fide* or candidate MAPs (Supplementary
363 Table 1), all proteins remained soluble in cell lysates, which allowed a systematic,
364 comparative characterisation of their interactions with individual, dynamically growing MTs
365 (Fig. 2). The fact that all components needed for MT polymerisation, i.e. free tubulin and
366 GTP, come directly from the cell lysates, makes the assay highly reproducible and

367 independent from additional factors, such as the quality of purified tubulin used in classical
368 *in-vitro* reconstitutions. Strikingly, many of the MT-MAP assemblies we observed in assays
369 with cell lysates were not seen on MTs inside cells overexpressing the same MAPs, most
370 likely due to spatial constraints inside the cells, or to the propensity of most MAPs to induce
371 MT bundling. This underpins the unique capacity of the assay to discover previously
372 unknown MAP activities.

373 In contrast to *in vitro* reconstitutions with purified components, lysates cannot be used to
374 determine the minimal set of components needed to build macromolecular assemblies. The
375 presence of cellular proteins in the lysates, however, provides the great opportunity to identify
376 protein complexes interacting with MTs that would never have been discovered by
377 conventional biochemical methods. MTs with associated MAPs and their unknown interactors
378 could be purified and identified by proteomics. To increase the specificity of this approach,
379 proximity-ligation approaches such as bio-ID⁷¹ followed by purification and identification of
380 biotinylated proteins could be employed.

381 A potential limitation of our method is that MAPs concentrations in lysates are limited by
382 efficiency of their expression, which could for instance limit the resolution of MAP in
383 structural studies. For most proteins, this could be overcome by increasing the amount of
384 plasmid transfected into the cells, and/or by increasing expression time. Of the 45 proteins
385 tested here, low expression levels were rather rare, and surprisingly little related to the overall
386 size of the overexpressed proteins. For proteins that are particularly hard to express,
387 additional steps such as changing the position of the fluorescent tag, or optimising the coding
388 sequence to avoid rare codons or the formation of mRNA secondary structures that hinder
389 ribosome progression⁷² could be employed.

390 While determining concentration of MAPs is more difficult in lysates as compared to purified
391 proteins, this does not impede a quantitative evaluation of the concentration-dependency of
392 MAP-induced MT phenotypes, as we demonstrated here for MACF1_C1023 and CLIP170.
393 Whether the concentrations we found to induce specific MT structures correspond to the
394 average intracellular concentration of a particular MAP is not necessarily relevant, given that
395 many MAPs can be locally enriched in cells. MACF1, for instance, might be targeted via its
396 amino-terminal actin-binding domain specifically to actin-rich subcellular compartments such
397 as the leading edge or the neuronal growth cone⁷³, and form MT hooks only at those
398 locations. The MT phenotypes we have described here might thus represent rare, locally
399 restricted MT structures in cells, which have so-far been overlooked. However, they might

400 play important roles in the formation of specific MT meta-structures, and their initial
401 demonstration in lysates becomes an essential prerequisite for the characterisation of their
402 cellular functions.

403 We thus established and validated a reconstitution assay of cytoskeletal assemblies in cell
404 lysates as a fast, straightforward and medium-throughput approach to characterise MAP-MT
405 assemblies at single-MT resolution. Our success in obtaining persistently soluble and
406 functional full-length proteins illustrates the power of this method. By demonstrating the
407 suitability of our assay for reproducible quantitative measurements, ultrastructural analyses,
408 and its capacity to be extended to study cytoskeletal crosstalk and to characterise disease-
409 related mutations, we illustrate how our pipeline can transform research on cytoskeletal
410 mechanisms and their impact on a wide variety of cellular functions and human diseases.

411

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441

442 **AUTHOR CONTRIBUTIONS STATEMENT**

443 JAS established the lysate-based pipeline and performed all experiments related to it. Lysate
444 experiments shown in Extended Data Fig. 10 and Supplementary Video 19 were performed
445 by MG. SB, FM and VH performed molecular cloning and sequence analyses with the help of
446 JAS and MG. SB performed all cell biology experiments. SB and VS performed cell live
447 imaging experiments. MG purified proteins and established quantitative approaches with JAS.
448 MB performed electron microscopy studies and analyses. JAS and LB analysed and
449 quantified imaging data with the help of SB. JAS, SB and CJ analysed data and prepared
450 figures and videos with the help of MB and LB. MS supervised electron microscopy studies
451 and analyses. CJ supervised the study with the help of MMM. JAS, SB, MMM, MS and CJ
452 acquired funding. JAS, SB and CJ wrote the manuscript with the help of MG, MB, MMM and
453 MS.

454

455 **COMPETING INTERESTS STATEMENT**

456 The authors declare no competing interests.

457

458 **FIGURE LEGENDS**

459 **Figure 1: A pipeline to analyse MAP-MT assemblies in cell lysates**

460 **a)** Schematic representation of the pipeline used in this study. In step 1, MAPs of interest are
461 amplified from different cDNA libraries, cloned into expression plasmids, and sequenced. In
462 some cases, different isoforms are identified. In step 2, fluorescently labelled MAPs are
463 expressed in HEK 293 cells for cell lysates, and in U-2 OS cells to determine their
464 intracellular behaviour. In step 3, HEK 293 cells are lysed, and clarified lysates are used in
465 step 4 to perform TIRF assays assembling MTs in the presence of fluorescent MAPs in the
466 lysates. Optionally, GFP-MAP concentrations in lysates can be quantified, and MAP-
467 decorated MTs can be used in cryo-electron microscopy.

468 **b)** Spastin-GFP from cell lysates severs taxol-stabilised MTs. Zoom images highlight the
469 progressive fragmentation of a MT. N = 4 independent TIRF assays conducted using
470 3 independent cell lysate preparations.

471 **c)** EB3-GFP from cell lysates tracks growing MT ends. Zoom images highlight the
472 enrichment of EB3-GFP (white arrow heads) at a growing MT tip. N = 5 independent TIRF
473 assays conducted using 4 independent cell lysate preparations.

474 Numerical data are available in source data.

475

476 **Figure 2: Systematic characterisation of 45 MAP candidates**

477 **a)** GFP-tagged MAPs, or presumed MAPs, expressed in U-2 OS cells show three principal
478 behaviours in cells: strong MT bundling, MT decoration with rare formation of bundles, or no
479 obvious MT binding. Three representative proteins, co-labelled with anti- α -tubulin antibody
480 to visualise MTs, are shown (see Extended Data Fig. 2; Supplementary information Fig. 1 for
481 all 45 proteins analysed). MT bundles are defined as thick fibres that are labelled much
482 stronger with anti- α -tubulin antibody than MTs in control cells. In all observed cases, they
483 co-localise with the overexpressed GFP-MAP. N = 19 cells (Tau-0N-04R), n = 36 cells
484 (MAP9-GFP) and n = 106 cells (MAP11-GFP) from two independent experiments.

485 **b)** GFP-tagged MAPs in lysates observed by TIRF microscopy decorate growing MTs. At the
486 end of the time-lapse imaging (12 min), MTs and actin are visualised with YL1/2 antibody
487 and SiR-actin to determine whether a MAP aligns spontaneously forming actin fibres (F-
488 actin) to the MTs in the lysate. Some presumed MAPs did not decorate MTs in lysates. Three

489 representative examples from 45 proteins are shown (other examples are shown in Extended
490 Data Fig. 3-6; all 45 proteins are shown in the Supplementary information Fig. 2 and
491 Supplementary videos 2-13). N = 14 (MACF1_C1023), n = 4 (DCX) and n = 3 (JPL1)
492 independent TIRF assays from 7, 3 and 2 independent cell lysates.

493 **c)** Summary of the behaviour of 45 proteins in cells (Extended Data Fig. 2; Supplementary
494 information Fig. 1) and in cell lysates (Extended Data Fig. 3-6; Supplementary information
495 Fig. 2; Supplementary Videos 2-13). Grey bars show relative size of proteins, number of
496 amino acids, and the position of the GFP tag (green). Truncated MAPs are indicated.
497 Classification of MAP behaviours uses same colour codes as in (a) and (b). MAPs showing
498 partial, or weak characteristics are visualised by half-squares. MT nucleation was evaluated
499 within the initial 5 min of the experiments. Full squares indicate MAPs leading to massive
500 spontaneous MT nucleation. Half-squares indicate no clearly discernible spontaneous MT
501 nucleation, with most MTs growing from seeds, and only few MTs without visible seed
502 entered the field of observation. Details of the experimental repeats of 45 proteins are
503 provided as source data.

504

505 **Figure 3: Discovery of unique activities of MAPs on growing MTs**

506 **a)** Representative time-lapse images of selected MAPs inducing unique MT behaviour in
507 lysates. MAPs are false-colour-coded (see Supplementary Video 14). Arrowheads indicate
508 growing CSAP-induced MT coils. **b)** Zoom images and schematics showing the typical
509 behaviour induced by the respective MAPs. Tubulin labelling with YL1/2 antibody is shown
510 in white. Growth directions of the MTs are indicated by arrows. All MT phenotypes were
511 observed in at least 3 independent TIRF assays from at least 3 sets of independent lysate
512 preparations. Numerical data for each MT phenotype is available in source data. **c)**
513 Determination of the average width of CSAP-induced MT coils. Quantification of about 40
514 coils (Extended Data Fig. 7a) showed an average width of about 900 nm. Data are presented
515 as scatter plots with mean \pm SD. Quantification was performed from n = 4 independent TIRF
516 assays using 3 independent cell lysate preparations. **d)** Quantification of the diameter of MT
517 hooks induced by MACF1_C1023. Measurements of over 100 MT hooks (Extended Data
518 Fig. 7b) from n = 5 independent TIRF assays using 4 independent cell extracts showed an
519 average hook diameter of about 1 μ m. Data are presented as scatter plots with
520 mean \pm standard deviation (SD). Numerical data for (c) and (d) are available in source data.

521

522 **Figure 4: Determining the impact of MAP-GFP concentrations on MT behaviour in**
523 **lysates**

524 **a)** Determination of the concentration of MACF1_C1023-GFP by quantitative immunoblot.
525 Purified GFP with known concentrations was analysed by immunoblot together with different
526 dilutions of the lysate from HEK 293 cells expressing MACF1_C1023-GFP. The intensities
527 of the bands labelled with anti-GFP antibody was determined, and plotted against the known
528 concentrations of the purified GFP dilution series. The plot shows the linearity of the
529 calibration curve. The three values determined from different dilutions of the
530 MACF1_C1023-GFP lysates were used to determine the concentration of the protein in each
531 sample. The values were then concentration-adjusted and averaged to determine the final
532 concentration of MACF1_C1023-GFP, which was found to be 520 nM in the undiluted lysate.
533 Immunoblotting of MACF1_C1023-GFP quantification was performed once.

534 **b)** The impact of MACF1_C1023-GFP concentration on MT hook formation (red
535 arrowheads) was determined in lysates with four defined concentrations of this MAP. Time
536 lapse images of MACF1_C1023-GFP (GFP shown in inverted grey scale) show that hook
537 formation propensity decreases relative to the number of MTs with decreasing
538 MACF1_C1023-GFP concentrations (see Supplementary Video 15). $N \leq 2$ independent TIRF
539 assays for each concentration from one set of cell lysate having quantified MACF1_C1023-
540 GFP concentration (a).

541 Numerical data and unprocessed gels and immunoblots are available in source data.

542

543 **Figure 5: The impact of a disease-related mutation of EML1 on MT binding**

544 **a)** Schematic representation of the work flow. GFP-MAP encoding plasmids are mutagenized
545 to introduce a disease related mutation, and fluorescent tags are swapped to obtain a GFP and
546 an mCherry version of both, wild-type and mutant MAP. Cell extracts are prepared for each
547 expression construct separately, and cell lysates are mixed before TIRF assays.

548 **b)** Still images from time-lapse (Supplementary Videos 16a) of MTs polymerising in a 1:1
549 mixture of lysates from HEK 293 cells expressing wild-type EML1-GFP and EML1^{Thr243Ala}-
550 mCherry. MT seeds are visualised in the red channel together with EML1^{Thr243Ala}-mCherry.
551 Note that EML1-GFP strongly decorates polymerising MTs, while no MT labelling was

552 detected for EML1^{Thr243Ala}-mCherry. N = 4 independent TIRF assays from 3 sets of
553 independent cell lysate preparation. This result was confirmed with EML1-mCherry and
554 EML1^{Thr243Ala}-GFP (Extended Data Fig. 8; Supplementary Videos 16b).

555

556 **Figure 6: Structural analyses of MAP-MT assemblies in lysates using cryo-electron**
557 **microscopy**

558 **a)** Flow diagram of the pipeline for cryo-electron microscopy (cryo-EM) using MAP-
559 containing lysates. Lysates were initially tested and optimised to show the previously
560 observed MT phenotypes (Extended Data Fig. 3,4) by TIRF microscopy. Lysates were then
561 added onto EM grids with GMPCPP-MT seeds. After 20-120 s incubation, grids were plunge-
562 frozen in liquid ethane, transferred to an electron microscope, and acquired images were
563 processed to obtain 3D reconstructions.

564 **b)** 2D cryo-electron micrographs and false-coloured zoom images of individual MTs in
565 absence of MAPs stabilised with GMPCPP, or in presence of EML1-GFP, EML4-GFP, and
566 MACF1_C1023-GFP. MTs were polymerised from endogenous tubulin in the lysates
567 (additional tubulin was added to MACF1_C1023-GFP lysates). Black arrows show MT-
568 associated densities, blue arrows highlight tubulin ring formations, and crimson arrows show
569 protofilaments about to peel off in the presence of MACF1_C1023-GFP.

570 **c)** Top and lateral views of a symmetrised helical reconstruction (resolution 3.7 Å, low-pass
571 filtered to 15 Å) of 14-protofilament MTs (grey) with extra densities corresponding to EML1-
572 GFP (green). Comparison with a similar helical reconstruction (EMDB 1331; resolution
573 3.6 Å, low-pass filtered to 15 Å) obtained for 13-protofilament MTs (polymerised from brain
574 tubulin) with purified EML4_207 (red)⁵⁸. Note that both MAPs bind along the MT
575 protofilament ridge (see Supplementary Video 17). In total, 1143 micrographs were used for
576 the helical reconstructions of MT decorated with EML1-GFP. Three unprocessed images are
577 shown in Supplementary information Fig. 3.

578 **d)** Densities for a single protofilaments from the symmetrised reconstruction low-pass filtered
579 to 15 Å. The fitted models for α - and β -tubulin (PDB ID 6dpu for EML1-GFP; PDB ID 6i2i
580 for EML4_N207) are shown in light and dark grey as mesh, respectively. The position of
581 associated extra densities corresponding to EML1-GFP (green) and EML4_N207 (red)
582 suggests interactions of the proteins with the flexible carboxy-terminal tails. These tails are
583 not resolved, however their origin at the end of helix 12 is indicated. A superposition of the

584 electron density maps of EML1-GFP and EML4_N207 shows that both MAPs bind at similar
585 positions of the tubulin molecules within the MTs.

586 Numerical data are available in source data.

587

588 **Figure 7: Characterisation of droplet formation by CLIP170**

589 **a)** Time series from a video showing accumulation of overexpressed GFP-CLIP170 in a
590 U-2 OS cell. Cell contours were visualised by bright-field microscopy. Note that initially
591 formed small droplets were later fusing, and GFP patches deform over time (Supplementary
592 Video 18a). N = 24 cells from 2 independent experiments.

593 **b)** U-2 OS cell stained with anti-tubulin antibody show formation of chains of small GFP-
594 CLIP170 droplets along bundled MTs, or bigger patches mostly lined up with MTs. N = 40
595 cells from 4 independent experiments.

596 **c)** Time-lapse of lysates with GFP-CLIP170 (white). GFP-CLIP170 formed regularly spaced
597 droplets along the MTs. Droplet intensity along one MT (orange line) is plotted below (see
598 Supplementary Video 18b). N = 12 independent TIRF assays from 8 independent cell lysate
599 preparations.

600 **d)** Droplet diameters from each time frame shown in (c). Note that the droplet size increases
601 over time. N = 9 - 57 GFP-CLIP170 droplets from each time point from a representative
602 experiment (c) were used. Data are presented as scatter plots with median with 95%
603 confidence interval (CI). Significance was tested using one-way ANOVA followed by
604 Tukey's multiple comparisons test.

605 **e)** Droplet distances were measured as distances between local maxima in intensity profiles
606 (c). Increase of droplet distances over time indicates droplets fusion. N = 17- 48 GFP-
607 CLIP170 droplets from each time point from a representative experiment (c). Data are
608 presented as scatter plots with median with 95% confidence interval (CI). Significance was
609 tested using one-way ANOVA followed by Tukey's multiple comparisons test.

610 **f)** Representative images of MTs assembled in lysates at different GFP-CLIP170 (white)
611 concentrations after 10 min. More images are shown in Extended Data Fig. 9a. N = 3 from 2
612 independent lysate preparations.

613 **g)** GFP-CLIP170 droplet sizes at three different GFP-CLIP170 concentrations in lysates.
614 Droplet size was measured for all droplets indicated in Extended Data Fig. 9a. N = 38 - 57

615 GFP-CLIP170 droplets for each dilution from a representative experiment (f) were used. Data
616 are presented as scatter plots with median with 95% confidence interval (CI). Significance
617 was tested using one-way ANOVA followed by Tukey's multiple comparisons test.

618 **h)** Droplet distance determined as in (e) from all GFP density profiles as indicated in
619 Extended Data Fig. 9b. N = 40 - 103 GFP-CLIP170 droplets from a representative experiment
620 (f). Data are presented as scatter plots with median with 95% confidence interval (CI).
621 Significance was tested using one-way ANOVA followed by Tukey's multiple comparisons
622 test.

623 Numerical data are available in source data.

624

625 **Figure 8: Generation of MTs from CLIP170-tubulin co-condensates**

626 **a)** Schematic representation of the work flow. In step 1, MTs are grown from lysates of HeLa
627 Kyoto cells stably expressing mCherry- α -tubulin, and transfected with GFP-CLIP170. In
628 step 2, after CLIP170 droplet formation, fresh lysate from mCherry- α -tubulin-expressing
629 HeLa Kyoto cells was added and MT polymerisation was observed. MTs were then stained
630 with YL1/2 antibody in step 3.

631 **b)** Images of MTs polymerised in step 1 show that mCherry- α -tubulin accumulates in GFP-
632 CLIP170 droplets. N = 3 independent TIRF assays from 2 independent cell lysate
633 preparations.

634 **c)** Time-lapse images of de-novo MT growth in step 2. Most MTs grow from GFP-CLIP170
635 droplets formed in step 1 (see Supplementary Video 18c). N = 3 independent TIRF assays
636 from 2 independent cell lysate preparations.

637 **d)** At the end of the time-lapse imaging in step 2 (c), MTs were stained with the antibody
638 YL1/2 for better visualisation. Note that MTs have grown almost exclusively from GFP-
639 CLIP170 droplets (merge also shown in Supplementary Video 18d).

640 Numerical data are available in source data.

641

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810

811 **METHODS**

812 **Mouse lines**

813 All the C57BL/6N animals in this study were used in accordance with the recommendations
814 of the European Community (2010/63/UE). Experimental procedures were specifically
815 approved by the ethics committee of the Institut Curie CEEA-IC #118 (authorisation
816 n°04395.03 given by National Authority) in compliance with the international guidelines.

817

818 **Cloning using Sequence and Ligation Independent Cloning (SLIC)**

819 To determine the most complete, annotated open reading frames (ORFs) for proteins of
820 interest, we searched public databases such as NCBI or EMBL-EBI, and compared murine
821 and human ORF variants. The longest identified ORF was chosen for cloning. The amino-
822 and carboxy- terminal boundaries of the MAPs were determined as follows: We first
823 identified the longest annotated open reading frames (ORFs) for the proteins of interest from
824 public databases such as NCBI or EMBL-EBI. As the expression profiles for many MAPs
825 were not well known, we amplified the ORF of interest from a panel of cDNAs from brain,
826 testes and cell lines. Using this approach, we succeeded amplifying most ORFs of interest,
827 and strikingly in some cases our approach allowed us to identify splice isoforms
828 (Supplementary Table 1), as well as previously undescribed ORFs such as for DCX
829 (Supplementary Table 1; DCX_L). Because of the particularly large size of the MT-actin
830 crosslinking factors MACF1 and MACF2 (5328 and 7718 amino acids respectively), we
831 cloned ~1000 amino acids upstream from the carboxy-terminal region, which contain the MT-
832 binding domains⁴³.

833 Primers were designed from the start codon until the stop codon, omitting the stop codon for
834 C-terminal tagging. All PCR primers are listed in Supplementary Table 1. Primers contain
835 standardised homology regions for the insertion into the target vector as shown in Extended
836 Data Fig. 1a. ORFs were PCR-amplified with Q5 High-Fidelity 2× Master Mix (NEB) from
837 cDNA libraries retro-transcribed with First Strand cDNA synthesis kit (Cytiva) from RNA
838 purified from mouse tissue (here we mostly used brain and testis cDNA). As a target vector,
839 we used a pTRIP vector⁷⁴ variant with a CMV-enhanced chicken beta-actin (CAG)
840 promoter⁷⁵ that allows expansion of our pipeline to primary cells, such as neurons. The vector
841 is linearized with NheI for C-terminal tagging, or BsrGI/BamHI for N-terminal tagging
842 (Extended Data Fig. 1a). PCR product and linearized vector were gel-purified with QIAquick

843 gel extraction kit (Qiagen), and fused using the SLIC method²³ (Extended Data Fig. 1b): 2 µl
844 linearized vector was mixed with 7 µl PCR product, 1 µl buffer and 0.3 µl T4 DNA
845 polymerase (NEB) on ice. After 2.5 min incubation at room temperature, it was re-transferred
846 on ice, 100 µl competent E. coli cells were added and incubated for 30 min on ice, heat-
847 shocked for 45 s at 42°C, and re-incubated for 10 min on ice. Bacteria were recovered in LB
848 medium at 37°C, 1 h before spreading onto ampicillin-containing LB-agar. Single colonies
849 were amplified for plasmid DNA purification, and plasmids were digested with restriction
850 enzymes initially used to linearize the plasmid to verify presence of the inserted ORFs.
851 Positive clones were sequenced. In case different sizes of ORFs are detected after cloning, we
852 analysed all variants to identify potential splice isoforms of the protein of interest.

853

854 **Immunostaining and imaging**

855 4×10^4 U-2 OS cells (ATCC HTB-96), seeded on coverslips in 24-well dishes in DMEM
856 complete medium (supplemented with 10% (v/v) FBS, 2 mM L-Glutamine, and $1 \times$ Penicillin-
857 Streptomycin (Pen Strep; Life Technologies)) were transfected with expression vectors
858 (Supplementary Table 1). Cells were fixed 24 or 48 h after transfection with a protocol
859 preserving the cytoskeleton⁷⁶: Cells were incubated 10 min with Hank's Balanced Salt
860 solution (HBSS), 10 min in MT-stabilising buffer (MTSB) containing the crosslinker DSP
861 (dithiobis(succinimidyl propionate); Thermo Fisher Scientific), followed by 4%
862 paraformaldehyde in MTSB (Electron Microscopy Sciences,) for 15 min. Cells were
863 permeabilised with 0.5% Triton X-100 (Sigma-Aldrich) in MTSB for 5 min, followed by
864 blocking in 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.1% Triton
865 X-100 (PBS-T). Primary (anti- α -tubulin antibody 12G10, 1:1,000) and secondary antibodies
866 (goat anti-mouse Alexa Fluor 568, 1:1,000; Thermo Fisher Scientific), both diluted in PBS-T
867 containing 5% BSA, were incubated for 1 h each. Coverslips were mounted using ProLong
868 Gold anti-fade medium (Thermo Fisher Scientific). Images were acquired using a Leica
869 Optigrid microscope equipped with a 63 \times (numerical aperture 1.40) oil immersion objective
870 and the ORCA-Flash4.0 camera (Hamamatsu) set at binning 1 \times 1. The microscope was
871 operated through Leica MM AF imaging software. Images were processed using Fiji⁷⁷ and
872 Adobe Photoshop. Final figures were prepared using Adobe Illustrator.

873

874 **Live-cell imaging**

875 2×10^4 U-2 OS (ATCC HTB-96) cells/well were seeded into 4-well glass-bottomed dishes
876 (Ibidi) and transfected with GFP-CLIP170 (1 μ g of plasmid DNA per well) using jetPEI
877 transfection reagent according to manufacturer's protocol. 8 h after transfection, cells were
878 imaged at Nikon Ti-E spinning disk inverted confocal laser microscope equipped with a 60 \times
879 oil TIRF NA 1.49 objective and an ORCA-Flash 4.0 camera (Hamamatsu) operated through
880 MetaMorph[®] software. Time-lapse images of GFP fluorescence and bright-field images were
881 taken every 5 min for 10 h. The focus plane was maintained by Nikon perfect focus system.
882 During the imaging, the microscope was at 37°C and 5% CO₂. The image stacks were
883 processed with Fiji, and videos were mounted with Adobe After Effects.

884

885 **Preparation of cell lysates**

886 2×10^6 HEK 293 cells (Human embryonic kidney cells; ATCC CRL-1573) were seeded on six
887 22-cm² dishes per plasmid. Cells at ~80% confluency in DMEM complete medium were
888 transfected using jetPEI (5 μ g of DNA and 10 μ l of jetPEI in serum-free DMEM with 2 mM
889 L-Glutamine and 1 \times PenStrep per dish) following the manufacturer's guidelines. After 24 h,
890 GFP expression levels were checked by fluorescence microscopy. At high expression levels
891 (strong GFP fluorescence) cells were harvested, while cells with low-expressing MAPs were
892 harvested after 48 h. The same protocol was followed for generating lysates from HeLa Kyoto
893 cells expressing mCherry- α -tubulin.

894 After media removal, cells were gently washed with 1.5 ml warm PBS per dish, detached with
895 500 μ l of trypsin-EDTA per dish, immediately collected in 2 ml of warm DMEM complete
896 media, and transferred to a falcon tube for centrifugation (453 \times g, 4°C, 10 min; Fig. 1a). the
897 supernatant was removed, and the cell pellet was resuspended in 150-300 μ l cold BRB80
898 buffer (80 mM K-Pipes pH 6.8, 1 mM EGTA and 1 mM MgCl₂) containing 0.05% Triton-
899 X100 and protease inhibitors (10 μ g/ml leupeptin, aprotinin and 4-(2-aminoethyl)-
900 benzenesulfonyl fluoride; Sigma-Aldrich). After transfer to 1.5-ml Beckman ultracentrifuge
901 tubes, cells were lysed by pipetting up down on ice for another 5-8 min and sonicated with
902 three short pulses using the 6.5-mm probe (Branson), setting "Output control" 1, "Duty cycle"
903 10%. Samples were ultra-centrifuged in the Beckman TLA 55 rotor at 33,800 \times g, 4°C for
904 25 min in the Beckman Coulter Optima MAX-XP ultracentrifuge. Supernatants were
905 aliquoted to 5-10 μ l, snap-frozen in liquid nitrogen, and stored at -80°C for up to 1 month. All
906 steps after cell collection are strictly performed on ice.

907

908 **Protein quantification and immunoblot**

909 We controlled overall protein concentrations in lysates to assure consistent tubulin levels in
910 our assays (~3% of the total protein in cultured cells is tubulin²⁶) using Bicinchoninic acid
911 protein assay⁷⁸ (BCA; Thermo Fisher Scientific) following the manufacturer instructions.
912 BCA reagent was freshly prepared for each experiment. A sample-to-reagent ratio of 1:8 was
913 incubated at 37°C for 30 min, and absorbance was measured at 562 nm with TriStar² LB 942
914 Multimode Microplate Reader with MikroWin 2010 software (Berthold). Absorbance values
915 of a BSA standard dilution series were plotted and fitted to a linear equation and obtained the
916 total protein concentration of our samples⁷⁹.

917 The concentration of GFP-MAPs in the lysates was quantified for one specific experiment
918 (Fig. 4) using immunoblot. A dilution series of purified GFP protein of known concentration
919 as standard was run together with a dilution series of lysates expressing MACF1_C1023-GFP
920 (Fig. 4a). The samples were boiled for 5 min with 2× Laemmli sample buffer (4% SDS
921 (VWR), 160 mM Tris-HCl, pH 6.8, 180 mM DTT (Sigma-Aldrich), 20% (w/v) glycerol
922 (VWR) and bromophenol blue), ran on a 10% SDS-PAGE gel, and transferred onto a
923 nitrocellulose membrane with Trans-Blot Turbo system (Bio-Rad) using transfer packs (Bio-
924 Rad). Membranes were blocked for 1 h with 5% non-fat milk in 50 mM Tris-buffered saline
925 (TBS; 50 mM Tris-HCl, pH 7.6, 150 mM NaCl) with 0.1% Tween 20 (Sigma-Aldrich)
926 (TBS-T), followed by 1.5 h incubation in rabbit anti-GFP antibody (1:2,500; Torrey Pines
927 Biolabs TP-401, in 5% non-fat milk in TBS-T). After three washes with TBS-T, the
928 membrane was incubated with secondary HRP-coupled goat anti-rabbit antibody (1:10,000;
929 Thermo Fisher Scientific) for 45 min, followed by three TBS-T washes. The membrane was
930 incubated for 1 min in Clarity Western ECL Substrate (Bio-Rad) and then imaged using the
931 Fusion FX imaging system (Vilber). Values of the known concentration of purified GFP were
932 used to obtain a standard curve⁸⁰, from which concentrations of MACF1_C1023-GFP were
933 deduced (Fig. 4a).

934

935 **Cleaning of glass slides and assembly of flow chambers**

936 TIRF flow chambers were prepared following a modified protocol from reference⁸¹. Glass
937 slides (76×26 mm; Thermo Fisher Scientific) and coverslips (22×22 mm; thickness no. 1.5,
938 VWR 631-0125) were wiped with Kimtech paper, and added one by one into separate clean

939 bottles with 100% acetone, which were sonicated in a water bath (Diagenode Bioruptor) for
940 15 min at room temperature. Acetone was discarded, and slides and coverslips were rinsed
941 with ultra-pure water. The water was exchanged and bottles were sonicated as before, after
942 which the water was exchanged for 100% ethanol, and sonicated as before. Finally, ethanol
943 was exchanged for fresh 100% ethanol, in which the slides and coverslips could be stored for
944 up to one month.

945 TIRF flow chambers were assembled by following protocols from references^{81,82}. Glass slides
946 were removed from ethanol, flamed and placed on a clean surface. Four long strips of double-
947 sided adhesive tape were pasted to obtain three chambers on a single glass slide. The double-
948 sided tape was pressed to the slides with the backside of forceps to fix it firmly, and the
949 protective sheet was peeled off with forceps. A fresh coverslip stored in ethanol was flamed,
950 placed on top of sticky tape strips, and pressed gently with the rear side of the forceps. The
951 typical volume of a single TIRF chamber is around 7-10 μ l. Prepared chambers can be stored
952 at room temperature for a few weeks in a dust-free space.

953

954 **Preparation of GMPCPP seeds**

955 Single-cycled GMPCPP-MT seeds were prepared in 60 μ l of 20 μ M unlabelled pig brain
956 tubulin (purified as in ref.⁸³) and 10-15% rhodamine-labelled brain tubulin, or Fluorescent
957 HiLyte 647 porcine brain tubulin (Cytoskeleton Inc.) in BRB80 buffer. After centrifugation
958 (98,400 \times g, 4°C, 30 min) to remove insoluble components, the supernatant containing soluble
959 tubulin was transferred to a fresh tube, adjusted to 0.5 mM GMPCPP (Jena Biosciences), first
960 incubated on ice for 5 min, followed by 37°C for 1 h. The polymerised GMPCPP MTs were
961 pelleted at 37°C, 20,000 g for 10 min, and subsequently washed with warm BRB80 buffer
962 (20,000 g, 3 min, 37°C). Finally, the GMPCPP-MT pellet was resuspended in warm BRB80
963 buffer (75 μ l) containing 10% (v/v) glycerol, snap-frozen in 2-5 μ l aliquots, and stored at -
964 80°C. For longer storage at -80°C, double-cycled GMPCPP seeds were prepared as described
965 in ref.⁸¹. For this, the GMPCPP-MT pellet obtained after warm centrifugation (20,000 \times g, 3
966 min, 37°C) was resuspended in cold BRB80 buffer, followed by frequent pipetting on ice for
967 20 min to efficiently depolymerise MTs. For the second round of MT polymerisation, tubulin
968 was incubated for 5 min with 0.5 mM of GMPCPP on ice, and then for 30 min at 37°C. The
969 pelleting, washing, resuspension and storage steps are same as for single-cycled MT seeds.

970 For the cryo-electron microscopy, GMPCPP-MT seeds were prepared without labelled
971 tubulin. 50 μ M purified goat brain tubulin (purified as in ref.⁸⁴) in BRB80 buffer was
972 polymerised with 1 mM GMPCPP at 37°C for 2 h, spun on warm 50% BRB80 sucrose
973 cushion in a table-top high-speed centrifuge, and resuspended in two volumes of warm
974 BRB80 buffer.

975

976 **Protein purification**

977 To attach MTs in the imaging chambers, we used a truncated version (the first 555 amino
978 acids) of the mouse kinesin-1 KIF5B carrying the walker mutation Thr92Asn⁸⁵. The protein
979 was expressed in *E. coli* as 6His-mKIF5B_N555^{Thr92Asn86} and was isolated by batch
980 purification as described before⁸⁷. Bacteria were grown at 37°C to an optical density at
981 600 nm (OD⁶⁰⁰) of 0.6-0.8, and protein expression was induced with 0.5 mM IPTG
982 (isopropyl-1-thio- β -D-galactopyranoside) at 20°C for 12 h. Cells were harvested at 4,500 \times g
983 for 15 min, and lysed in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 30 mM imidazole pH 8.0,
984 10% (w/v) glycerol, 0.05% Triton-X100, and 1 \times protease inhibitor cocktail by sonication. The
985 lysate was cleared by centrifugation at 80,000 \times g for 12 min at 4°C, and gently rotated for 2 h
986 (4°C) with chelating sepharose (GE Healthcare) resin functionalised with NiSO₄ (250 μ l resin
987 for 1 l initial *E. coli* culture). The resin was sedimented at 500 \times g for 2 min, and washed 3 \times
988 with 2 ml of wash buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 30 mM imidazole pH 8.0,
989 10% (w/v) glycerol) by gently mixing the resin in the buffer (with a cut pipette tip) followed
990 by a sedimentation step (500 \times g, 2 min). The protein was eluted with 50 mM Tris-HCl pH 8.0,
991 20 mM NaCl, 250 mM imidazole pH 8.0 and 5% (w/v) glycerol.

992 Purification of GFP from HEK 293 cells via Strep-Tag[®] II-Strep-Tactin affinity purification
993 was adapted from a previous protocol¹¹, with minor modifications. Twin-Strep-Tag[®] II (IBA
994 GmbH, Göttingen) was cloned into pTRIP vector as C-terminal fusion to GFP. HEK 293 cells
995 in five 14.5-cm cell culture dishes at ~50% confluency were transfected, and harvested after
996 24 h by subsequent washing with PBS, detachment with 0.05% Trypsin-EDTA and collection
997 in PBS. To remove traces of Trypsin-EDTA, cells were washed one more time with PBS and
998 cold centrifugation. Cells were resuspended in 800 μ l cold lysis buffer (50 mM HEPES
999 pH 7.4, 300 mM NaCl, 1 mM MgCl₂, 0.5% Triton-X-100, 1 mM DTT, 100 mM EGTA, 1 \times
1000 protease inhibitors cocktail) and incubated on ice for 15 min with occasional pipetting to
1001 break cells. The lysate was centrifuged at 18,500 \times g for 15 min at 4°C. The supernatant was

1002 added to 250 μ l pre-equilibrated and blocked Streptactin Sepharose[®] High Performance beads
1003 (Cytiva) and incubated for 45 min at 4°C by slow rotation. The beads were washed 3 \times in
1004 high-salt wash buffer (50 mM HEPES pH 7.4, 300 mM NaCl, 1 mM MgCl₂, 0.5% Triton-
1005 X100, 1 mM DTT), 3 \times in pre-elution-wash buffer (50 mM HEPES pH 7.4, 150 mM NaCl,
1006 1 mM MgCl₂, 0.05% Triton-X100, 1 mM DTT, 1 mM EGTA), and eluted with 100-250 μ l
1007 elution buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.05% Triton-X100,
1008 1 mM DTT, 1 mM EGTA, 2.5 mM d-desthiobiotin; Sigma-Aldrich).

1009 Eluted proteins were aliquoted, snap-frozen in liquid nitrogen and stored at -80°C. The
1010 protein purity was examined via SDS-PAGE and Coomassie Brilliant Blue staining.

1011

1012 **MT reconstitution and TIRF microscopy**

1013 Flow chambers were first blocked for 5 min with 50 μ g/ml of β -Casein (Sigma-Aldrich) in
1014 BRB80, and subsequently incubated with 0.75 μ M of 6His-mKIF5B_N555^{Thr92Asn} for 5 min.
1015 GMPCPP seeds resuspended in warm BRB80 were flushed into the chamber and incubated
1016 for 5 min for immobilisation. Excess unattached seeds were washed out using warm BRB80
1017 buffer, after which the density of the seeds in the chamber was verified under the TIRF
1018 microscope.

1019 A fresh tube of cell lysate stored at -80°C was thawed, adjusted (if required) to 8 μ g/ μ l total
1020 protein concentration in BRB80 buffer, flushed into the prepared imaging chamber and
1021 immediately transferred to a Nikon TiE azimuthal TIRF using an iLas-2 system (Gataca
1022 Systems) on an inverted microscope with a 100 \times TIRF NA 1.49 oil objective equipped with
1023 Nikon perfect focus system. The imaging area was 68.1 \times 68.1 μ m and images were captured
1024 by an Evolve EM-CCD camera using MetaMorph[®] software with a Modular interface for
1025 TIRF. Flow chambers were illuminated at 488 nm (Stradus[®] Vortran, 150 mW) for GFP-
1026 tagged proteins, and at 561 nm (Jive[™] Cobolt, 100 mW) for GMPCPP seeds (rhodamine-
1027 labelled brain tubulin; Cytoskeleton Inc.), or mCherry-tagged proteins for a total duration of
1028 11.612 min with a frame interval of 1.98 s. These settings were strictly followed in all TIRF
1029 microscopy assays shown here.

1030 After completion of time-lapse imaging, MTs and actin were visualised. Chambers were
1031 washed with warm BRB80 buffer, followed by a 5-min incubation with YL1/2 antibody
1032 (1:100 in BRB80). Chambers were washed again, and Alexa 565-labelled anti-rat antibody
1033 (1:100 in BRB80; Thermo Fisher Scientific) was flushed in for 5 min. After another wash,

1034 500 nM SiR-Actin (Spirochrome) was flushed in for 5 min incubation, and subsequently
1035 washed out with BRB80. Still images of GFP-tagged protein (at 488 nm), YL1/2-labelled
1036 MTs (at 561 nm) and the SiR-Actin-stained actin filaments (at 642 nm; StradusTM Vortran,
1037 165 mW) were acquired with the TIRF microscope. All above-described steps were repeated
1038 in the presence of 11 μ M Latrunculin A (Sigma-Aldrich), to prevent actin polymerisation³².
1039 For the experiments with 1,6-Hexanediol (Sigma-Aldrich), GFP-CLIP170 cell lysates were
1040 initially incubated in the microscopy chamber containing GMPCPP MT seeds, and the
1041 formation of droplet-like assemblies was imaged for 3 min at 1 fps. Subsequently, BRB80
1042 with 0% (control), 5% and 10% (w/v) 1,6-Hexanediol was flushed into the chambers, and the
1043 acquisition was continued for another 3 min at 1 fps.

1044

1045 **Image and data analysis for TIRF assays**

1046 TIRF images were processed using Fiji software⁷⁷. Some raw images and videos underwent
1047 shading correction with a homemade macro (<https://doi.org/10.5281/zenodo.5648066>) by
1048 Gaussian smoothing with a standard deviation of 30 sigma to approximate a shading image.
1049 Flat-field corrected images were obtained by subtracting Gaussian-smoothed from raw
1050 images. Videos are 20 \times accelerated for mounting with Adobe After Effects. Still images were
1051 treated and quantitatively analysed with Fiji software, adjusted and mounted with Adobe
1052 Photoshop and Illustrator.

1053 The width of CSAP-induced MT coils (Fig. 3c; Extended Data Fig. 7a) and diameter of
1054 MACF1_C1023-induced MT hooks (Fig. 3d; Extended Data Fig. 7b) were determined by
1055 manually drawing lines over these structures, and extracting their length in Fiji.

1056 CLIP170 droplet diameters was determined by drawing lines across droplets perpendicular to
1057 the MT, along which fluorescence-intensity distribution was measured, and Gaussian fitting
1058 was used to extract full width at half maxima (FWHM; Fig. 7d,g). This was repeated for all
1059 droplets individually. For CLIP170 droplet spacing (Fig. 7e,h), we obtained GFP-CLIP170
1060 intensity profiles from line scans along individual MTs (Fig. 7c; Extended Data Fig. 9).
1061 Droplet spacing was determined by automatically detecting local maxima with the Fiji BAR
1062 plugin (<https://imagej.net/BAR>), with a variable value as minimum peak amplitude that
1063 covers all local maxima. Coordinates of maxima in 1D were transferred to Microsoft Excel
1064 for sorting and calculating distances. All data were finally transferred to GraphPad Prism
1065 software for plotting and statistical analyses.

1066

1067 **Electron microscopy**

1068 Quantifoil gold holey carbon grids R 1.2/1.3 were glow-discharged in a GloQube Glow
1069 Discharge (Quorum Technologies) system for 90 s. Sample application and vitrification were
1070 carried out in a Vitrobot Mark IV (ThermoFisher Scientific) set to 25°C and 100% humidity
1071 using a Whatman No. 1 filter paper for blotting.

1072 Cell lysates with overexpressed MAPs stored at -80°C were thawed and warmed only before
1073 application on the grids. In case of MACF1_C1023-GFP, purified HeLa tubulin⁸⁸ was added
1074 to accelerate MT formation.

1075 1 µl GMPCPP-MT seeds in warm BRB80 was applied to the grid mounted in the Vitrobot.
1076 After 10-20 s for seed adsorption, 3 µl of pre-warmed lysate was applied to the grid, which
1077 were blotted after 20-120 s with blot force zero and blot time 3 s before plunging into liquid
1078 ethane.

1079 Frozen grids were transferred to an Autoloader under liquid nitrogen and mounted into a Titan
1080 Krios (FEI) G3 transmission electron microscope operating at 300 kV and attached to the
1081 Falcon III detector. Data were collected with an automated pipeline of EPU software.

1082 30 frames were collected per movie in the linear mode at a dose rate of 1.87 electrons/pixel/s
1083 and a total dose of 44.17 electrons/Å². Images were collected at a magnification of 59,000×,
1084 resulting in 1.38 Å pixel size and a defocus range between -1.8 to -3.0 µm.

1085

1086 **Image processing and 3D reconstruction**

1087 Images were manually inspected for ice contaminants and filament quality, and unwanted
1088 micrographs discarded. EML4 and MACF1_C1024 datasets were not used for further
1089 analyses; micrographs in Fig. 6b are representative images. Movie frames of EML1 datasets
1090 were motion-corrected using the inbuilt feature of MotionCor2⁸⁹ in RELION 3.0^{90,91}.

1091 Contrast transfer function (CTF) estimation was done by GCTF⁹² on dose-weighted and
1092 motion-corrected summed images. Particles were manually boxed using the helical picking
1093 option of RELION 3.0⁹³ (box size 600 Å, overlapping inter-box distance 82 Å). Processing
1094 was carried out with RELION 3.0 and customised scripts to reconstruct MTs with a seam
1095 (pseudo-helical symmetry)⁹⁴. 2× binned particles were used during the initial stages of
1096 processing. Particles were segregated into classes based on protofilament numbers by one

1097 round of 3D classification using low-passed synthetic references of MTs made of different
1098 protofilament numbers (11, 12, 13, 14, 15 and 16). The dominant class contained
1099 14-protofilament MTs was further processed. A few cycles of refinement were used to
1100 improve the alignment of all the particles. A 14-protofilament MT with pixels doubled for
1101 loop regions of S9-S10 and H1-S2 (different in α - and β -tubulin), and low-pass filtered to
1102 6 Å, was used as the reference in these stages⁵⁸. The phi angle was determined for the
1103 particles, and the modal value was assigned to all particles in a single MT. This was further
1104 checked by 3D classification using low-pass-filtered references of 14-protofilament MTs with
1105 enhanced pixels for S9-S10 and H1-S2 loop regions rotated and shifted (by one monomer
1106 repeat of 4 nm) to accommodate all possible 28 positions for a 14-protofilament MT with a
1107 seam. The segments were flipped to the modal positions and corresponding phi angles were
1108 assigned to all segments in a given MT. Finally, refinement was carried out on unbinned
1109 aligned particles using a low-pass filtered 14-protofilament MT reference with and without
1110 applying helical symmetry. An atomic poly-alanine model of the HeLa cell tubulin dimer
1111 (PDB ID:6i2i) was fitted into the symmetrised map in UCSF Chimera⁹⁵ and refined manually
1112 by model building in Coot⁹⁶ followed by real-space refinement in PHENIX^{97, 98}. The refined
1113 model is available upon request. An atomic model for EML1-GFP could not be generated due
1114 to the low-resolution density corresponding to the EML1 region bound to the microtubule
1115 lattice. All images and structural superposition with the reconstruction of EML4_N207-bound
1116 MT (EMDB 1331; PDB ID 6i2i) were generated in UCSF Chimera⁹⁵. For data collection and
1117 processing details, see Supplementary Table 2.

1118

1119 **Material availability**

1120 Plasmids generated in this study are available upon request.

1121

1122 **Data availability**

1123 All commercial resources used here are detailed in Supplementary Table 3; primers for
1124 molecular cloning are listed in Supplementary Table 1; antibodies are listed in Supplementary
1125 Table 4. Source data are provided with this study. All data supporting the findings of this
1126 study are available from the corresponding author upon reasonable request.

1127

1128 **Code availability**

1129 The cryo-EM map for EML1-GFP bound to a MT is deposited in the Electron Microscopy
1130 Data Bank (EMDB) under the accession code EMD-32033. Gene sequences of newly
1131 identified MAPs (DCX_L, MAP11; see Supplementary Table 1) are deposited at GenBank
1132 under the accession codes OK539808 (DCX_L) and OK539809 (MAP11). Deposited data are
1133 listed in Supplementary Table 5.

1134 We used mostly free software for the analyses, details of the paid software are included in
1135 Supplementary Table 3. Home-made macros used in the analyses (Fig. 7d,g) have been
1136 deposited at Zenodo (<https://doi.org/10.5281/zenodo.5648066>).

1137

1138 **Statistics and reproducibility**

1139 Experiments in this work were not randomised and no blinding was used during the data
1140 analyses, as the experiments were designed and carried out by same group of people. Data
1141 were tabulated in Microsoft Excel and statistical analyses were performed using GraphPad
1142 Prism software. Whenever statistics is performed, data was pooled from at least three
1143 independent experiments. For the comparison of three or more groups, one-way ANOVA was
1144 used. P values ≤ 0.05 were considered significant. No statistical method was used to
1145 predetermine sample size, and no data were excluded from the analyses.

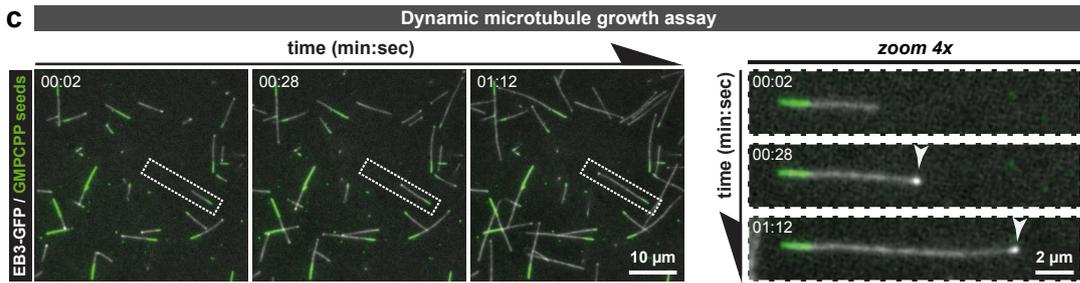
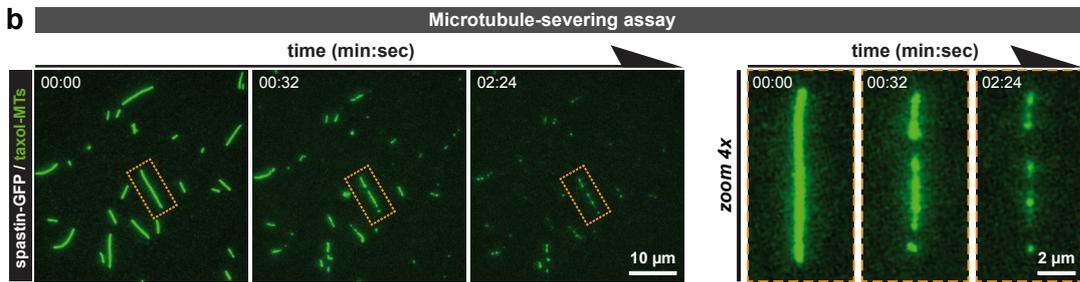
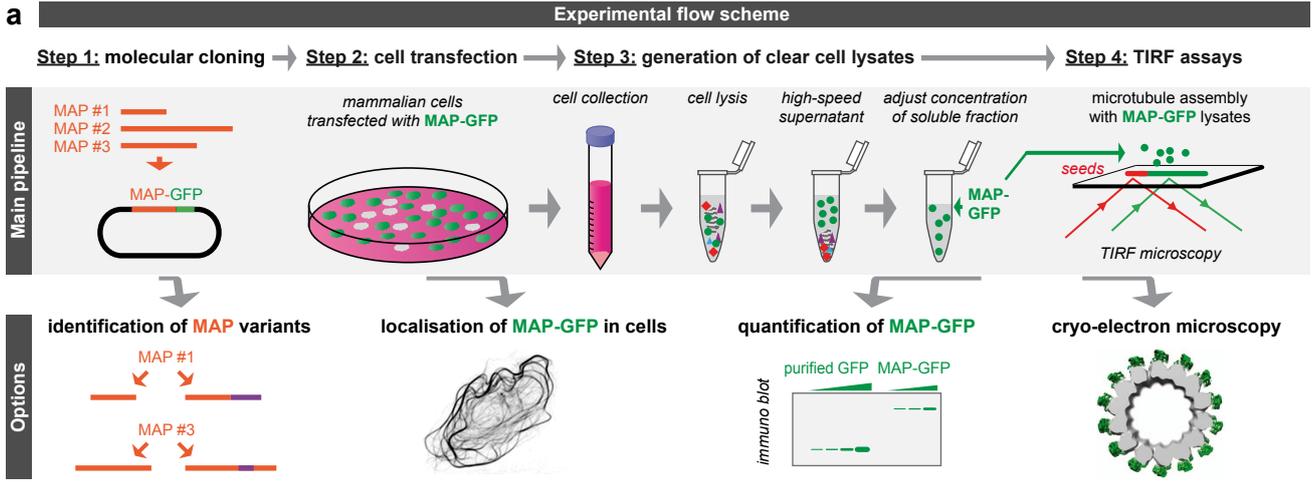
1146 The exact number of cells, independent TIRF, or cryo-EM experiments, as well as the number
1147 of cell lysate preparation used for each experiment are indicated in respective figure legends,
1148 and all numerical values used for the quantification are detailed in the source data file
1149 (Fig. 1b,c; Fig. 2-4; Fig. 5b; Fig. 7a,b,c,f; Fig. 8b,c; Extended Data Fig. 2-10; Supplementary
1150 information Fig. 1, 2).

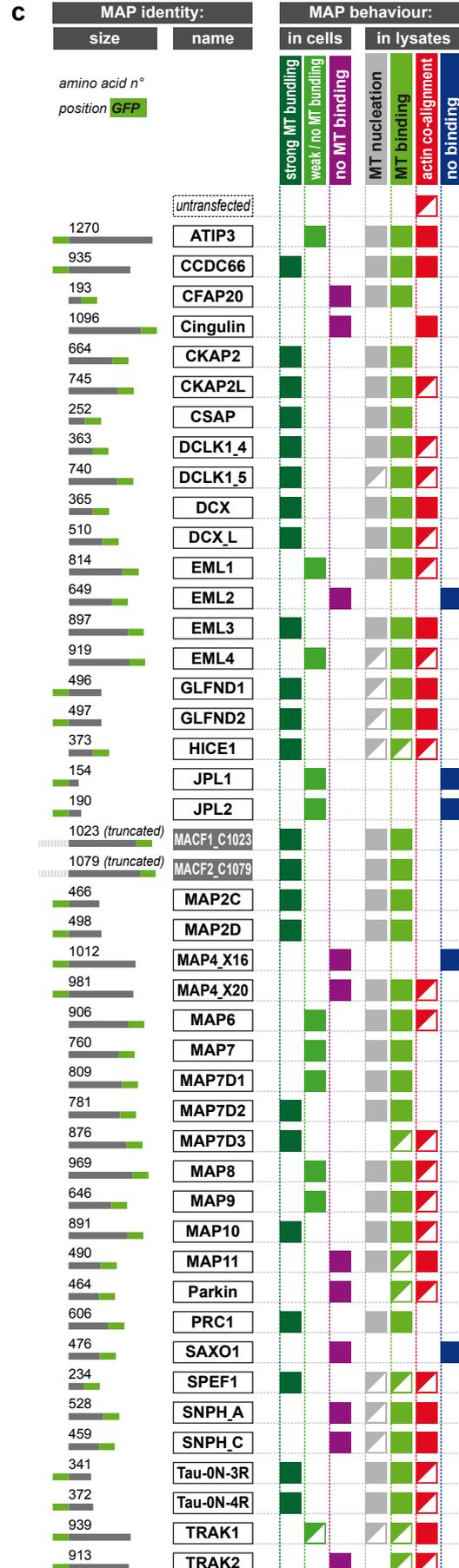
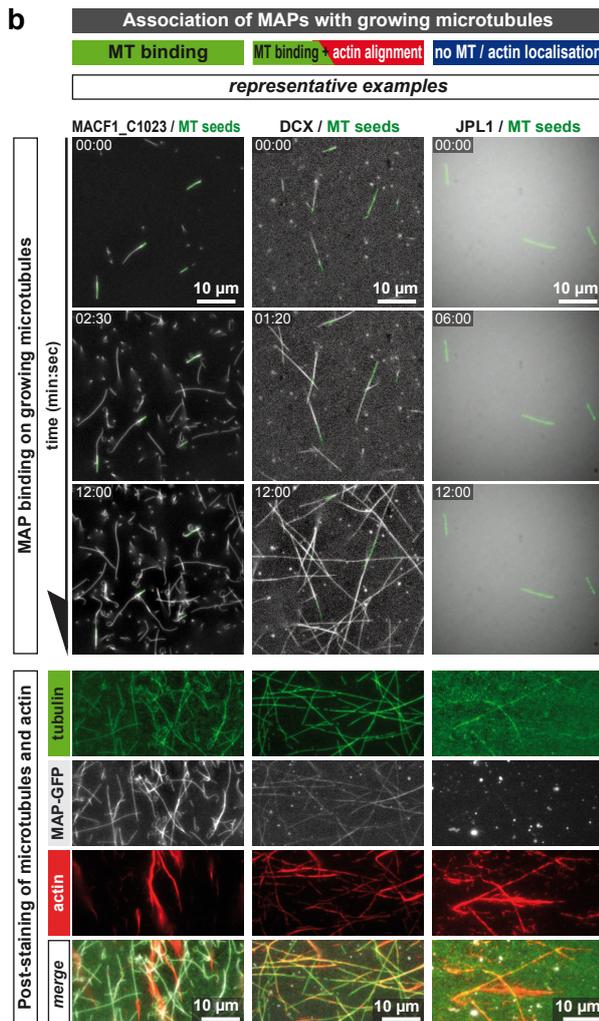
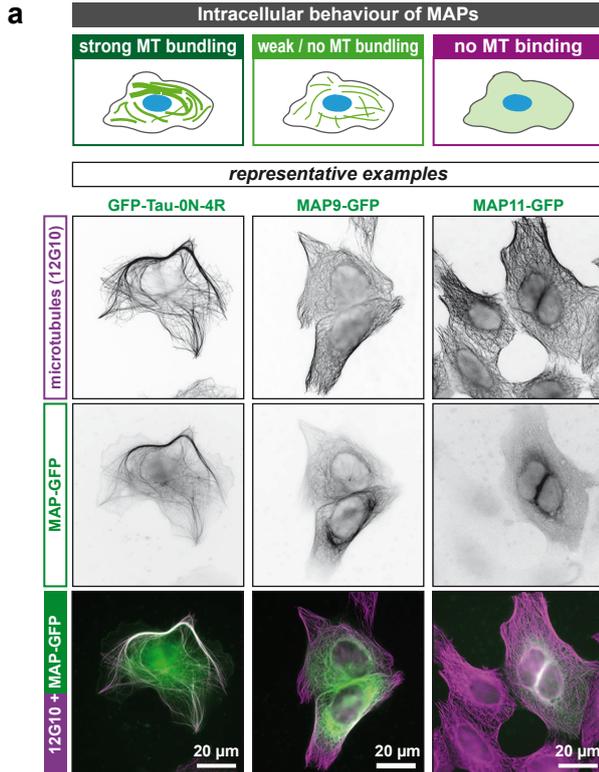
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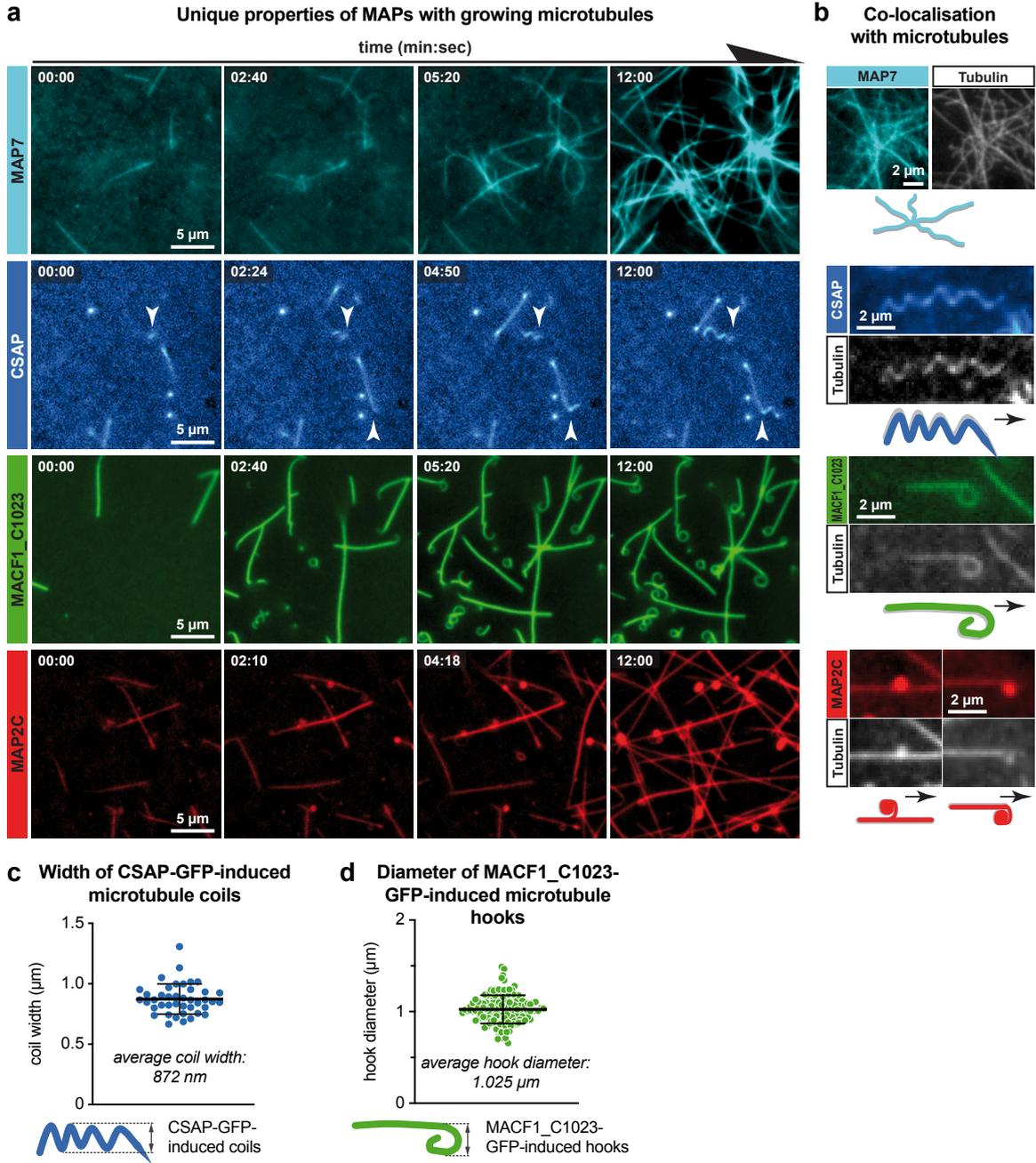
1152 **Method references**

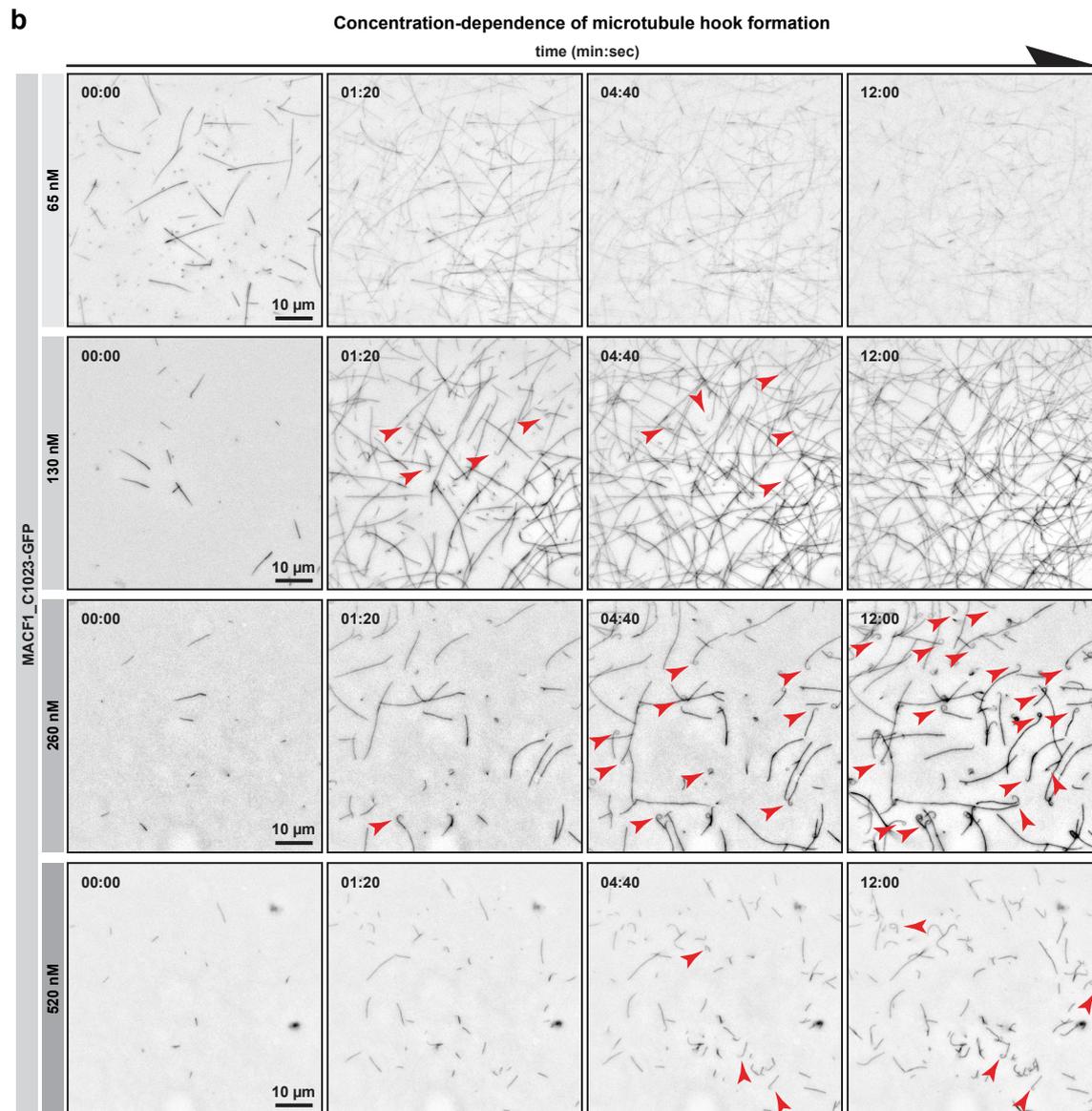
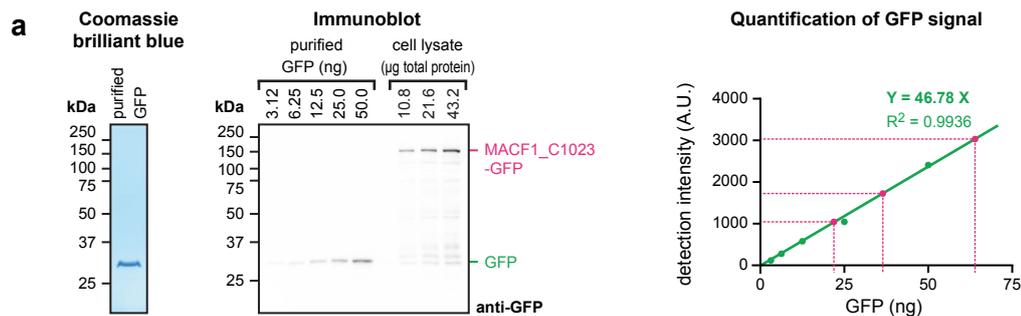
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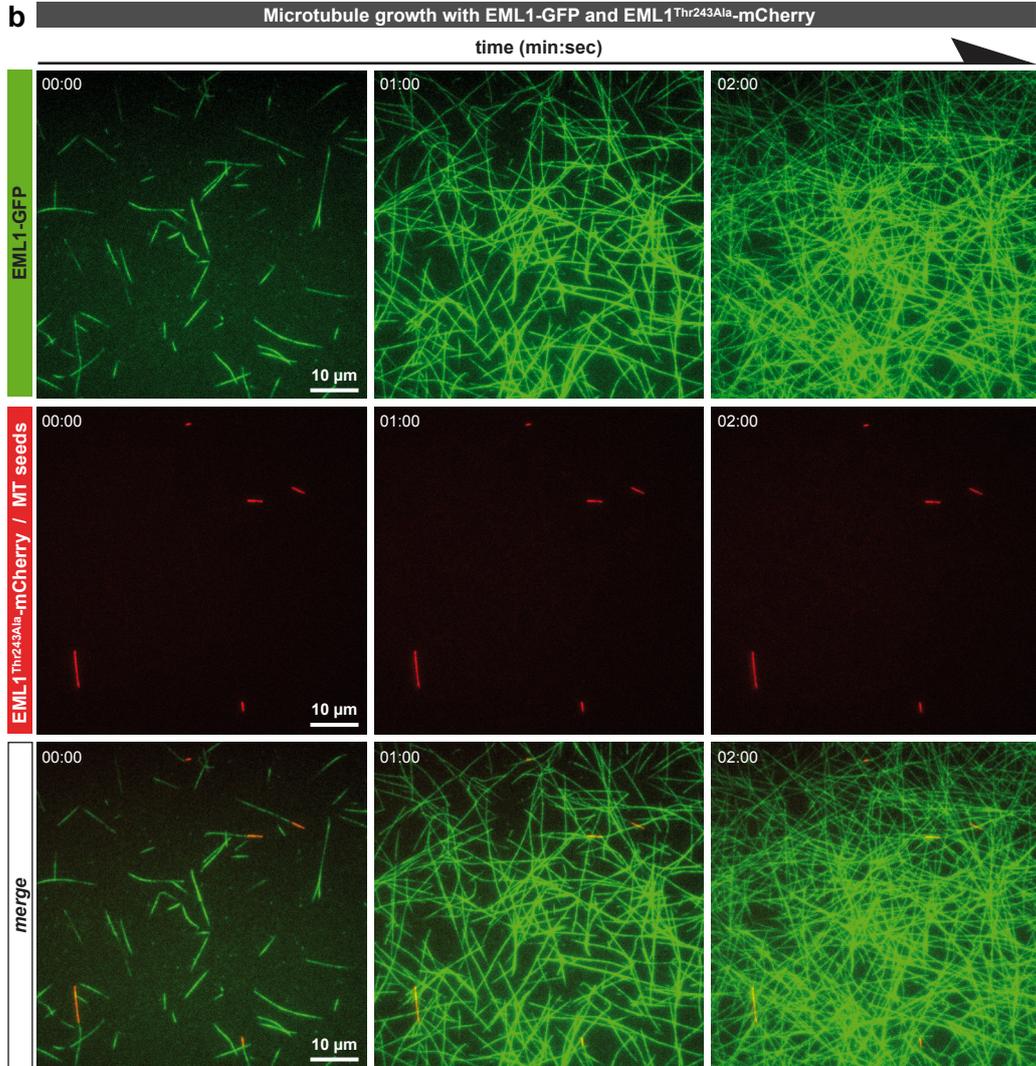
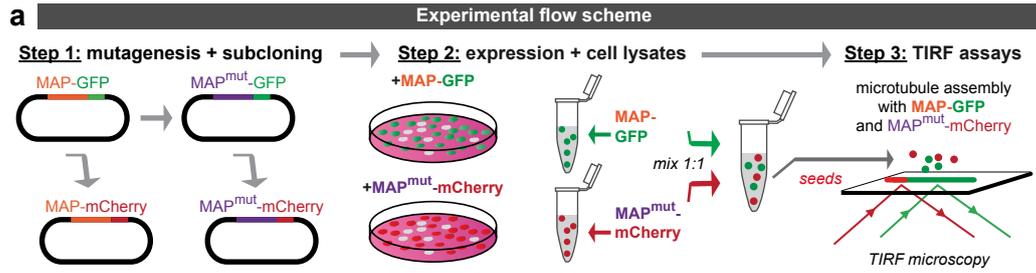
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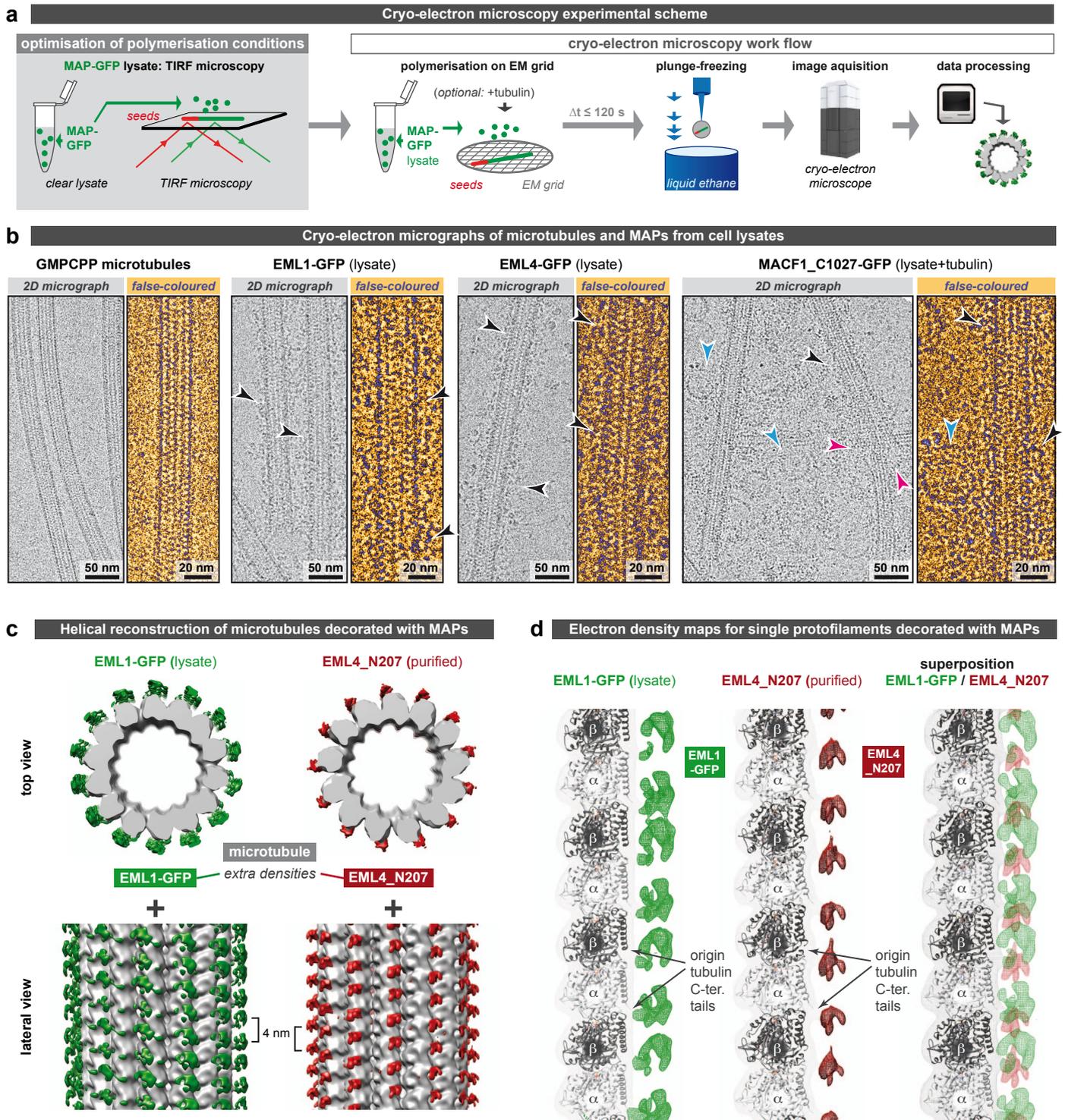




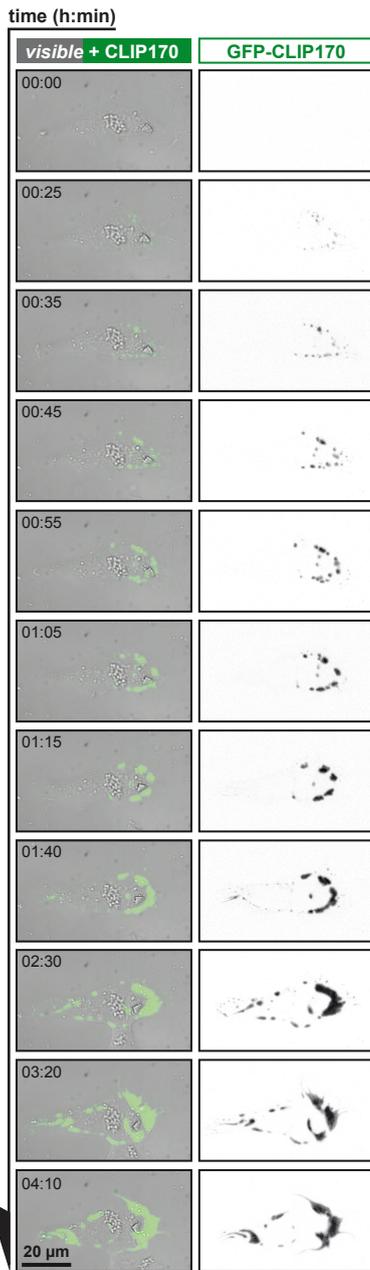




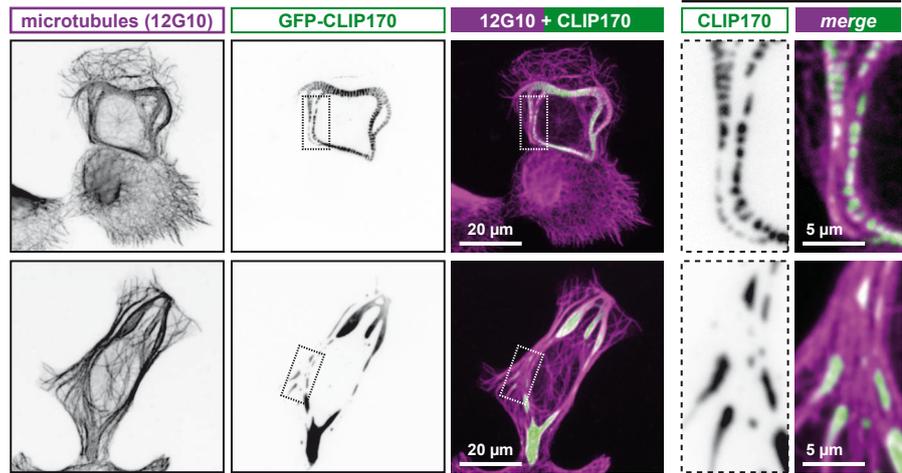




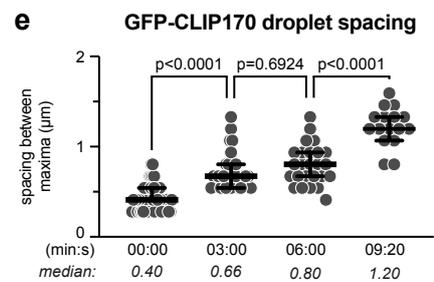
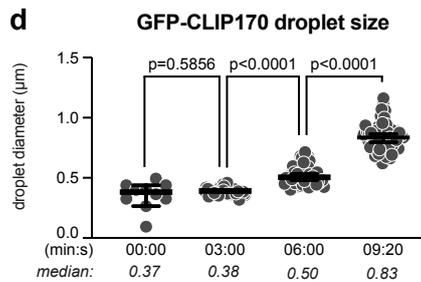
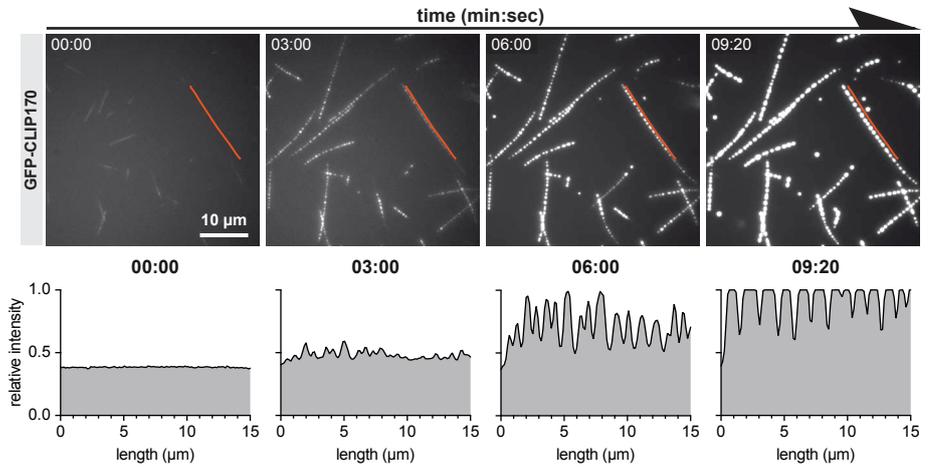
a Overexpression of GFP-CLIP170



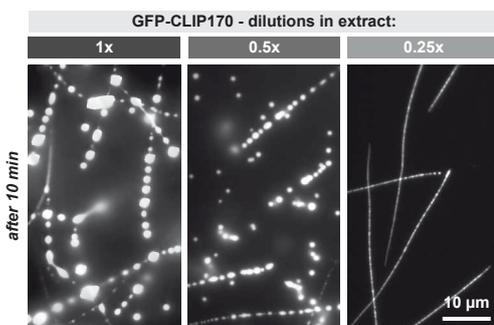
b Overexpression of GFP-CLIP170



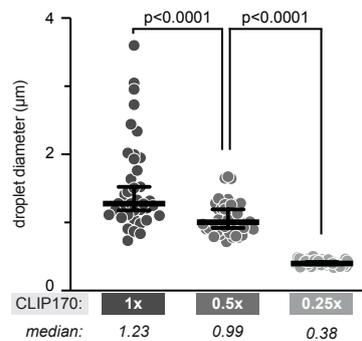
c GFP-CLIP170 time-dependent droplet formation



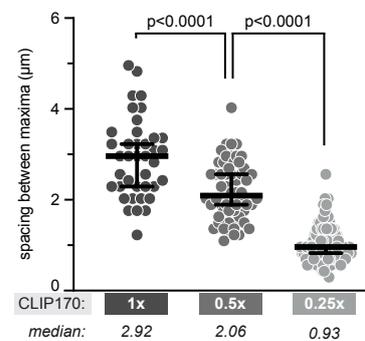
f GFP-CLIP170 concentration-dependent droplet formation

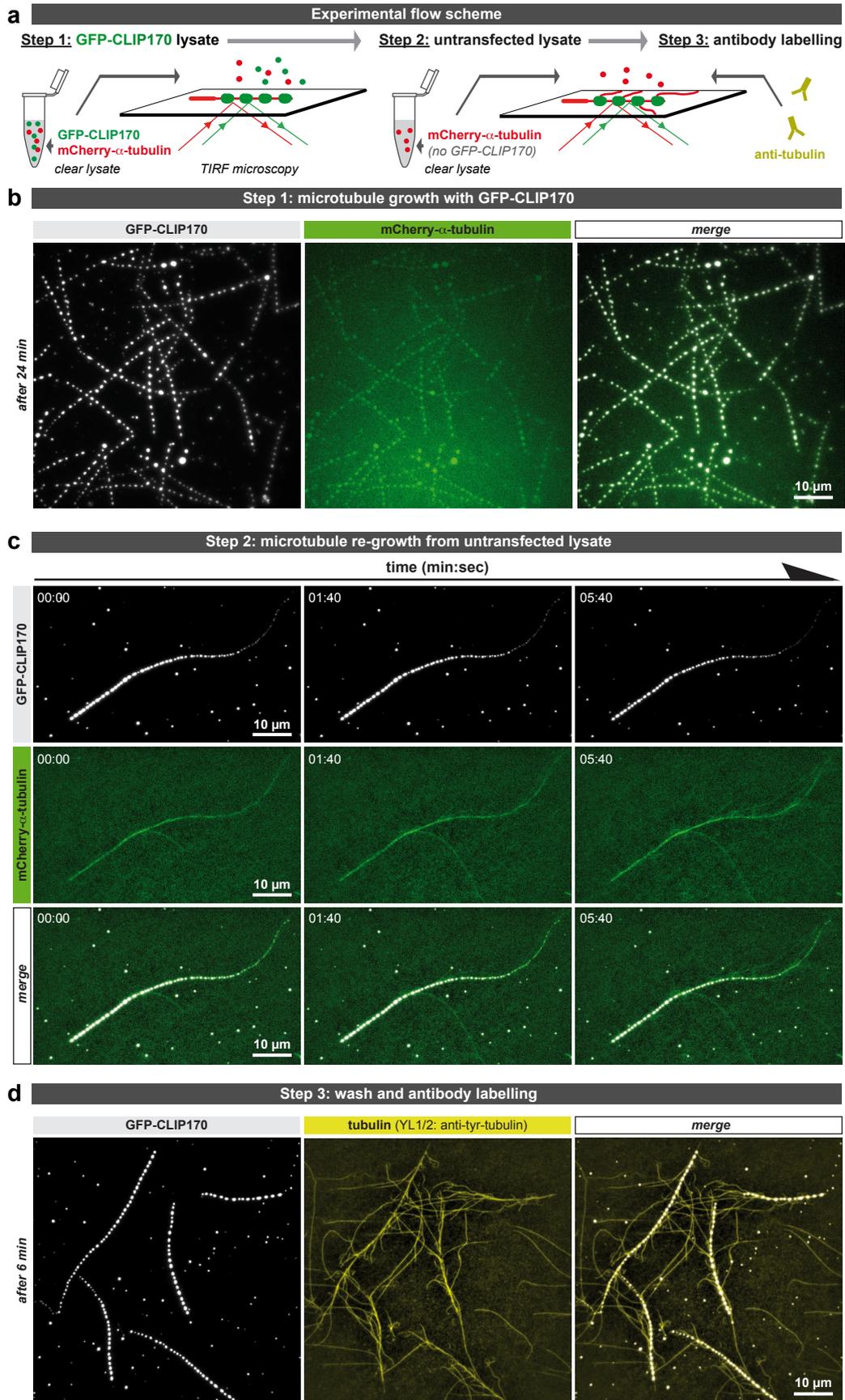


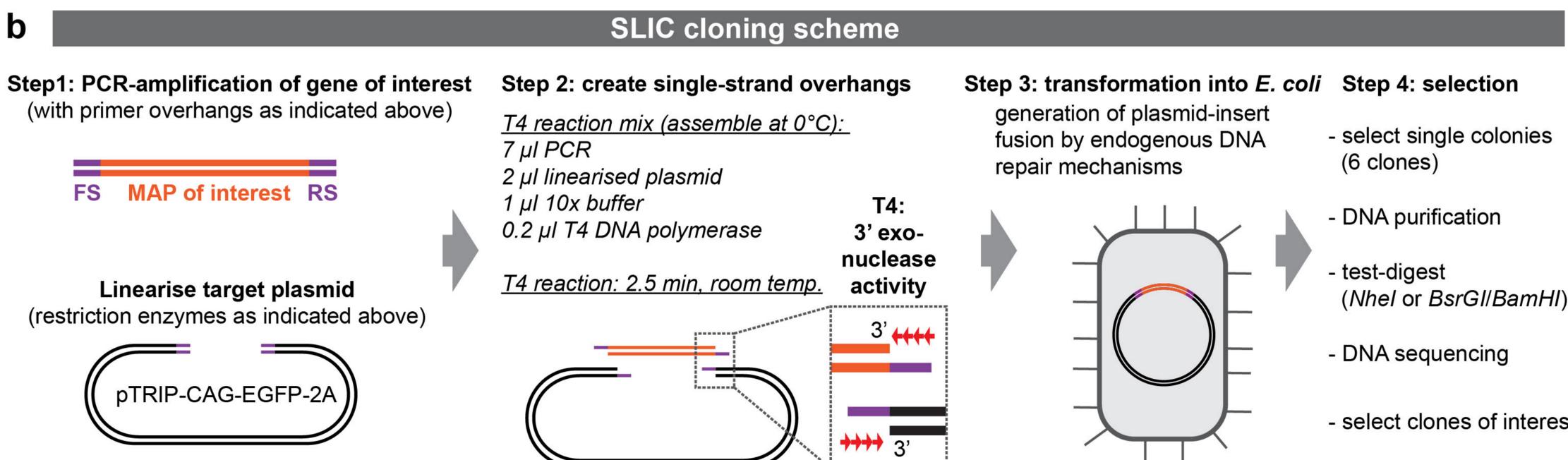
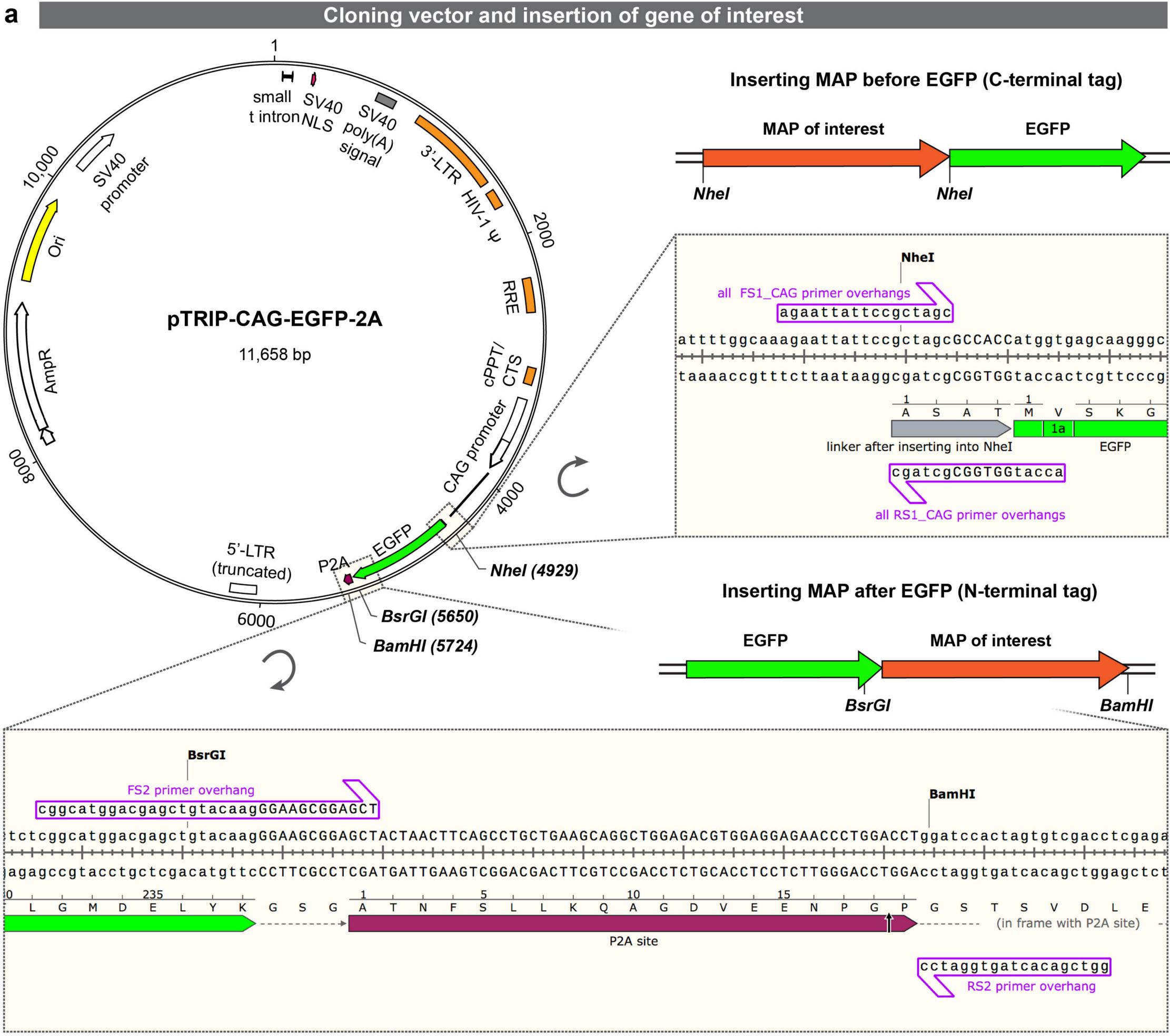
g GFP-CLIP170 droplet size



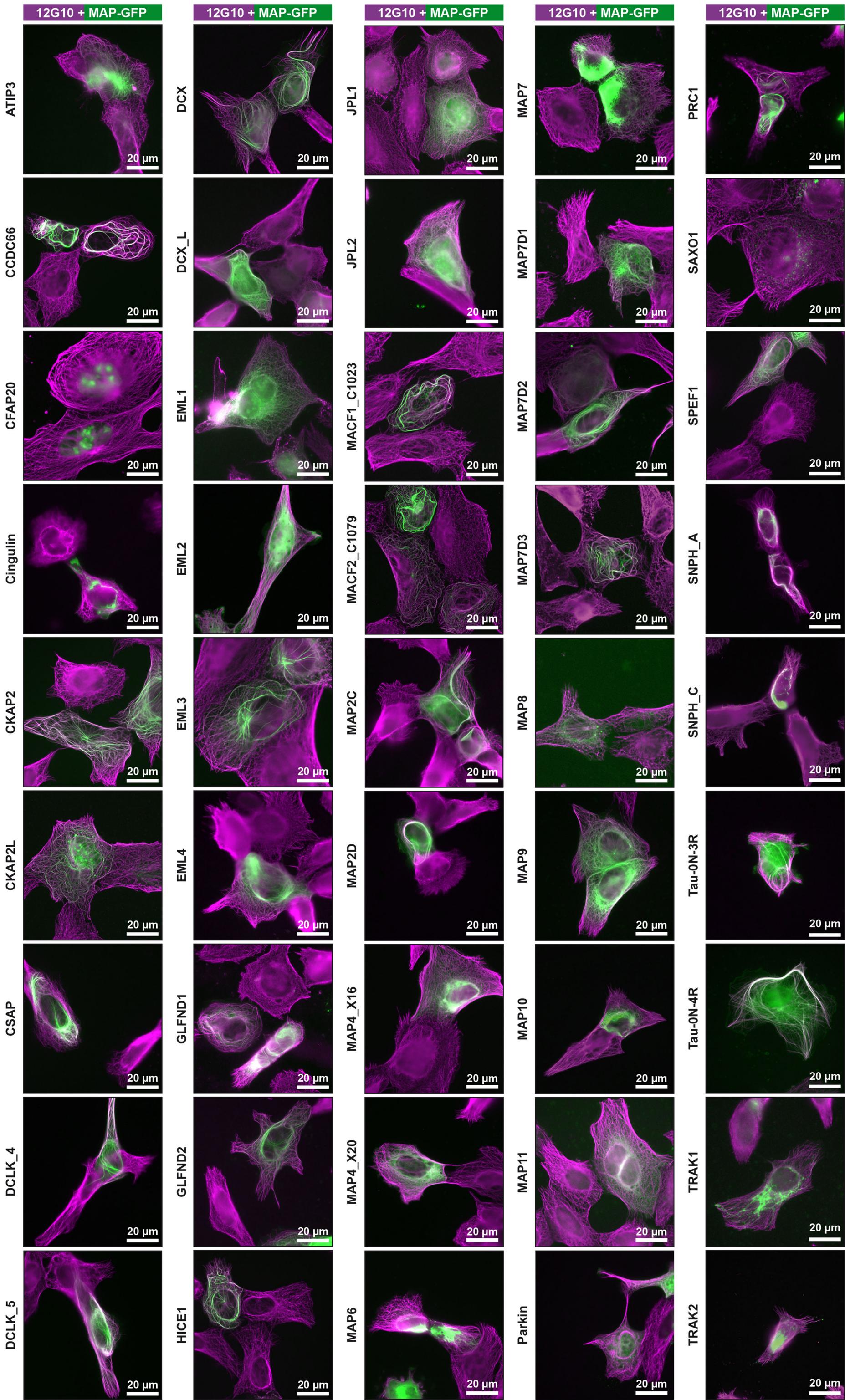
h GFP-CLIP170 droplet spacing



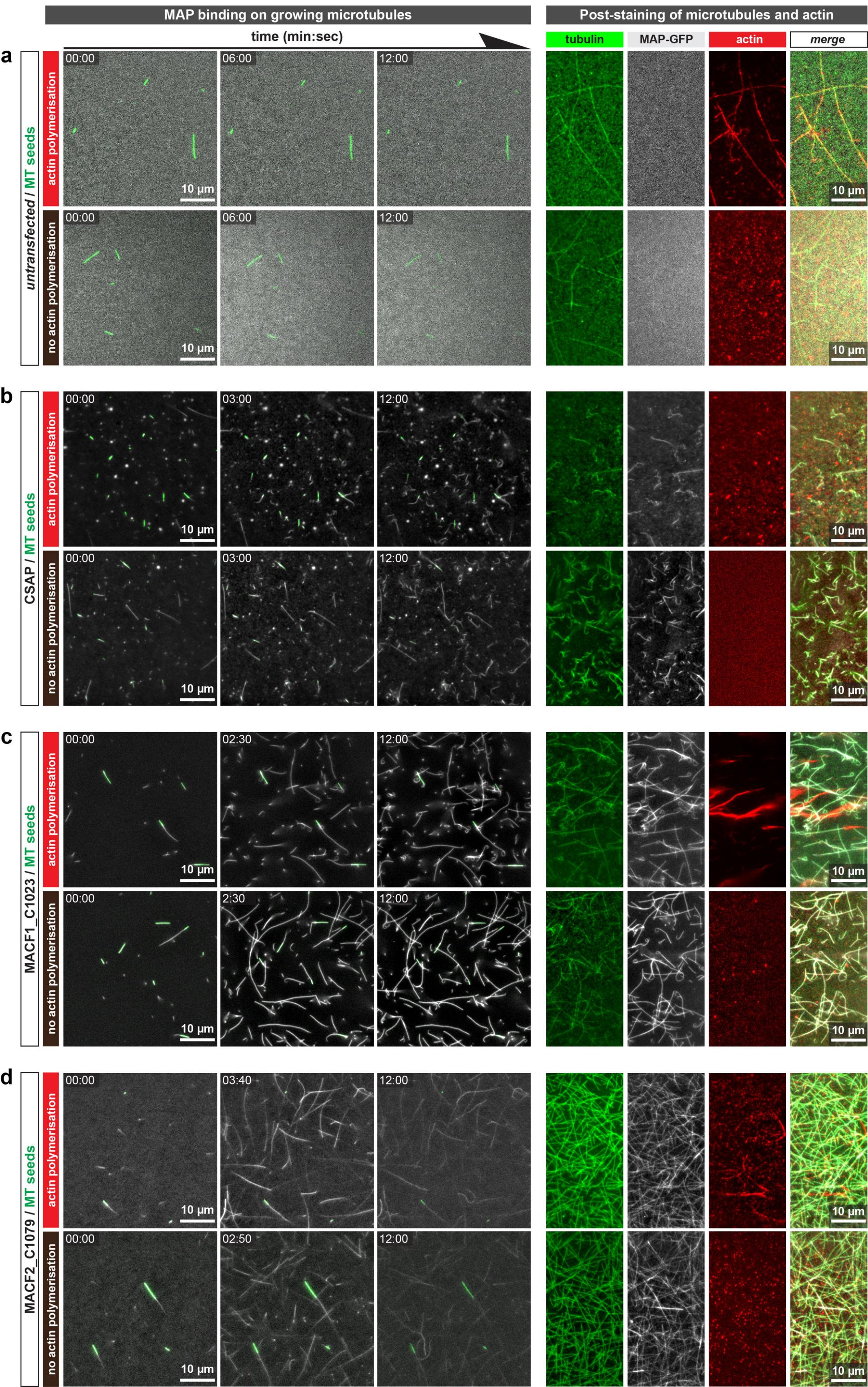




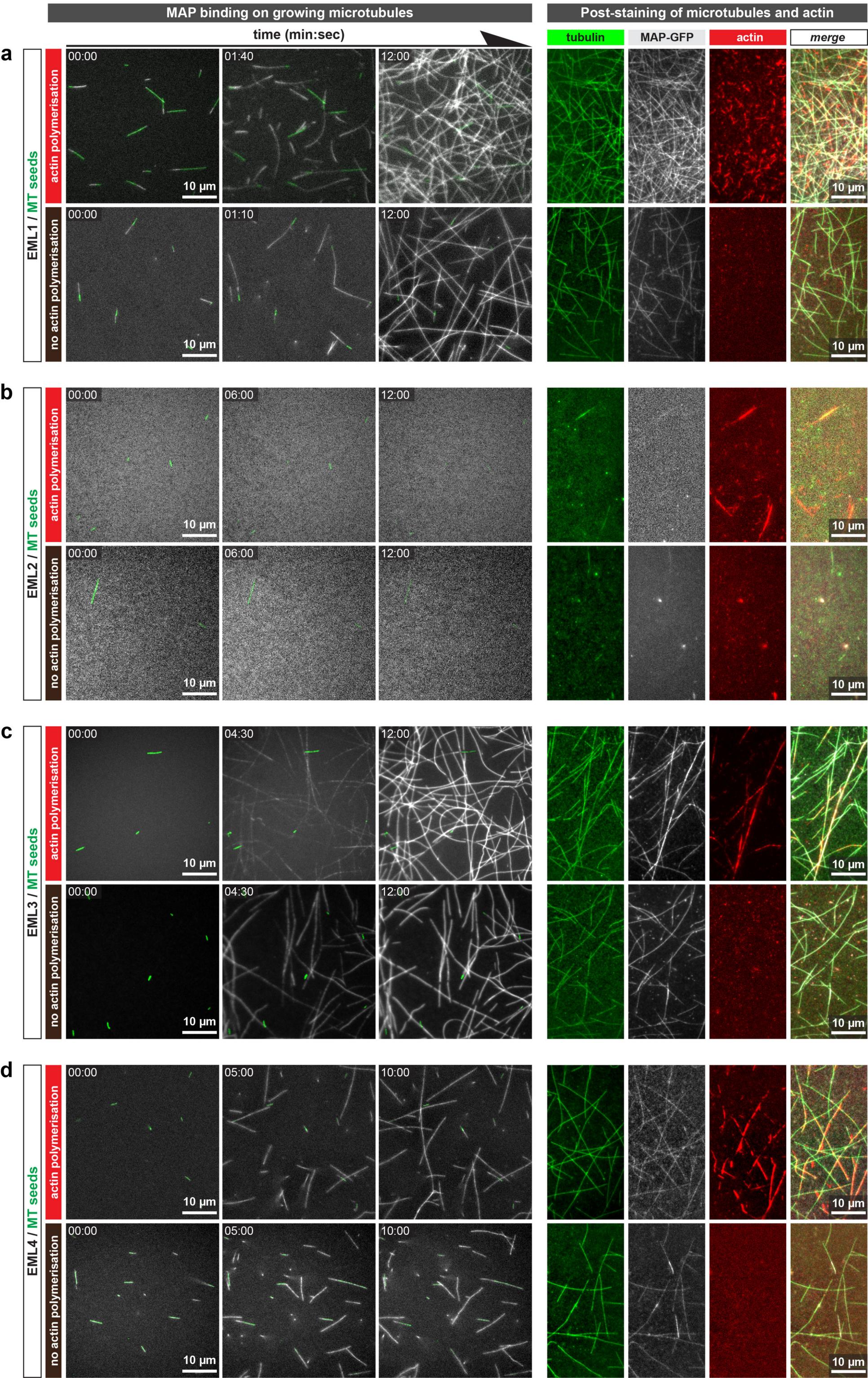
Organisation of microtubule cytoskeleton in cells with overexpressed GFP-MAPs



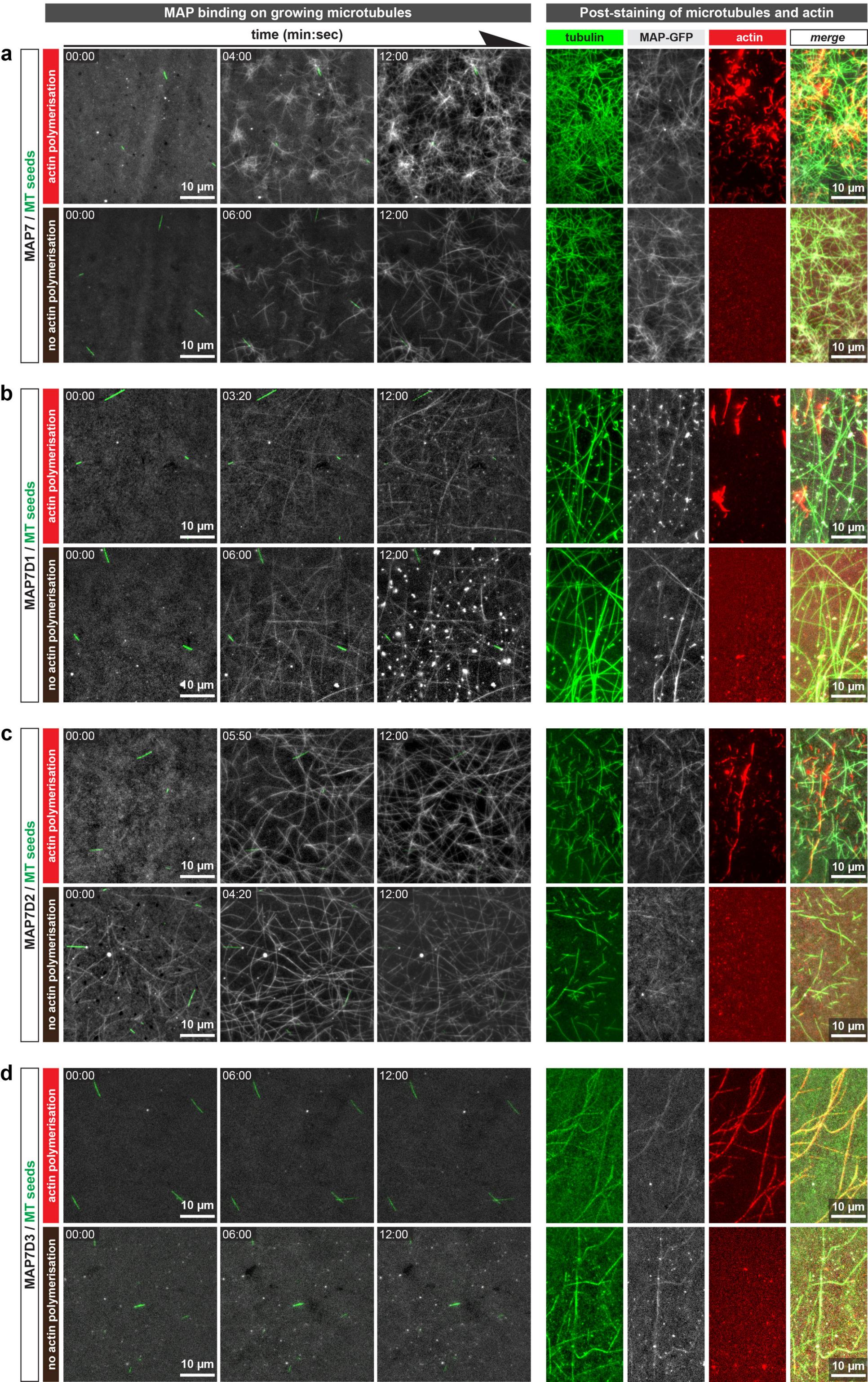
Association of MAPs with growing microtubules



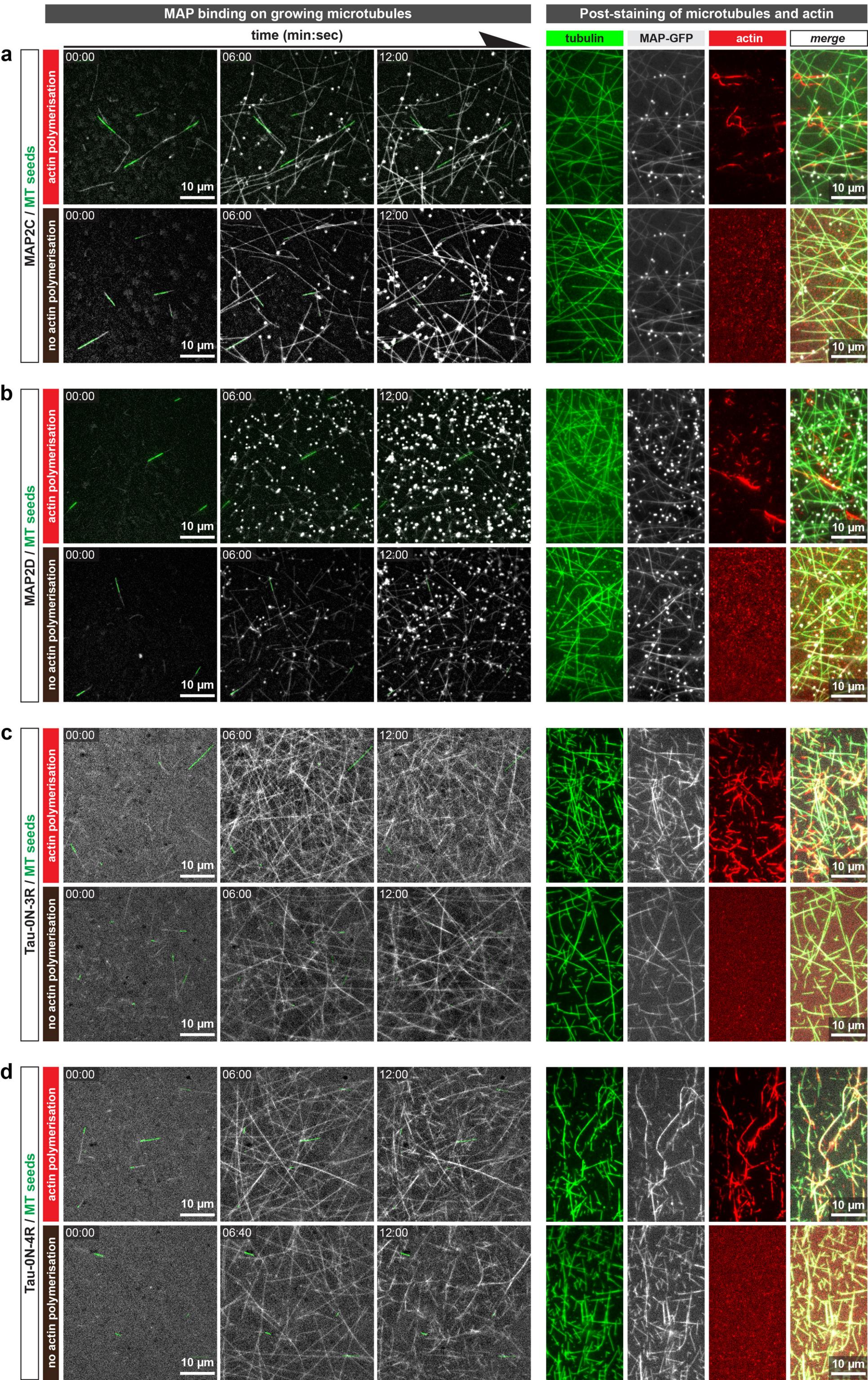
Association of MAPs with growing microtubules



Association of MAPs with growing microtubules

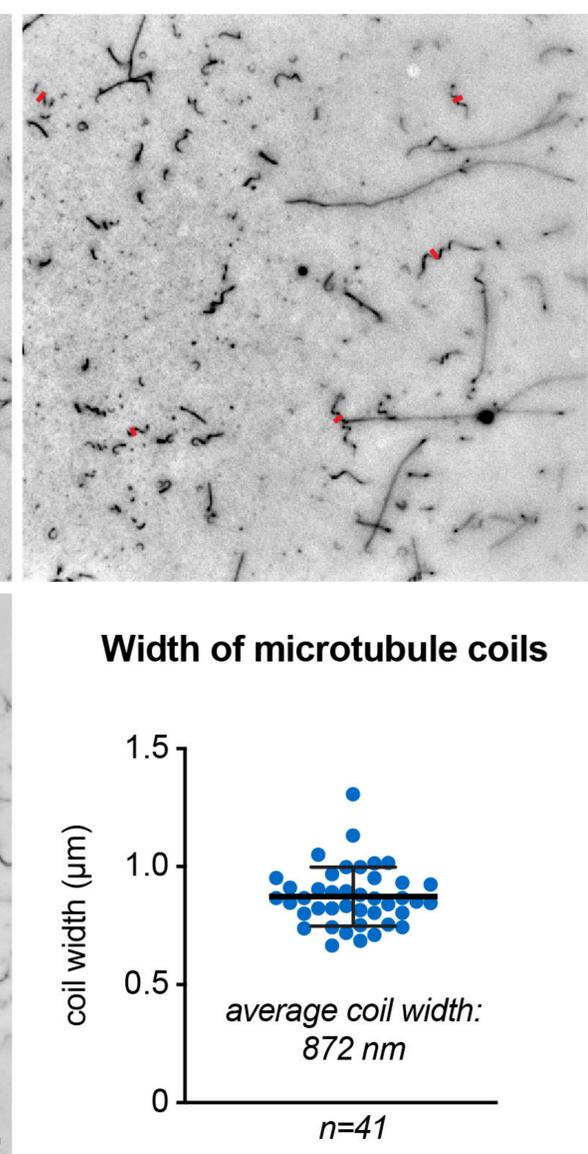
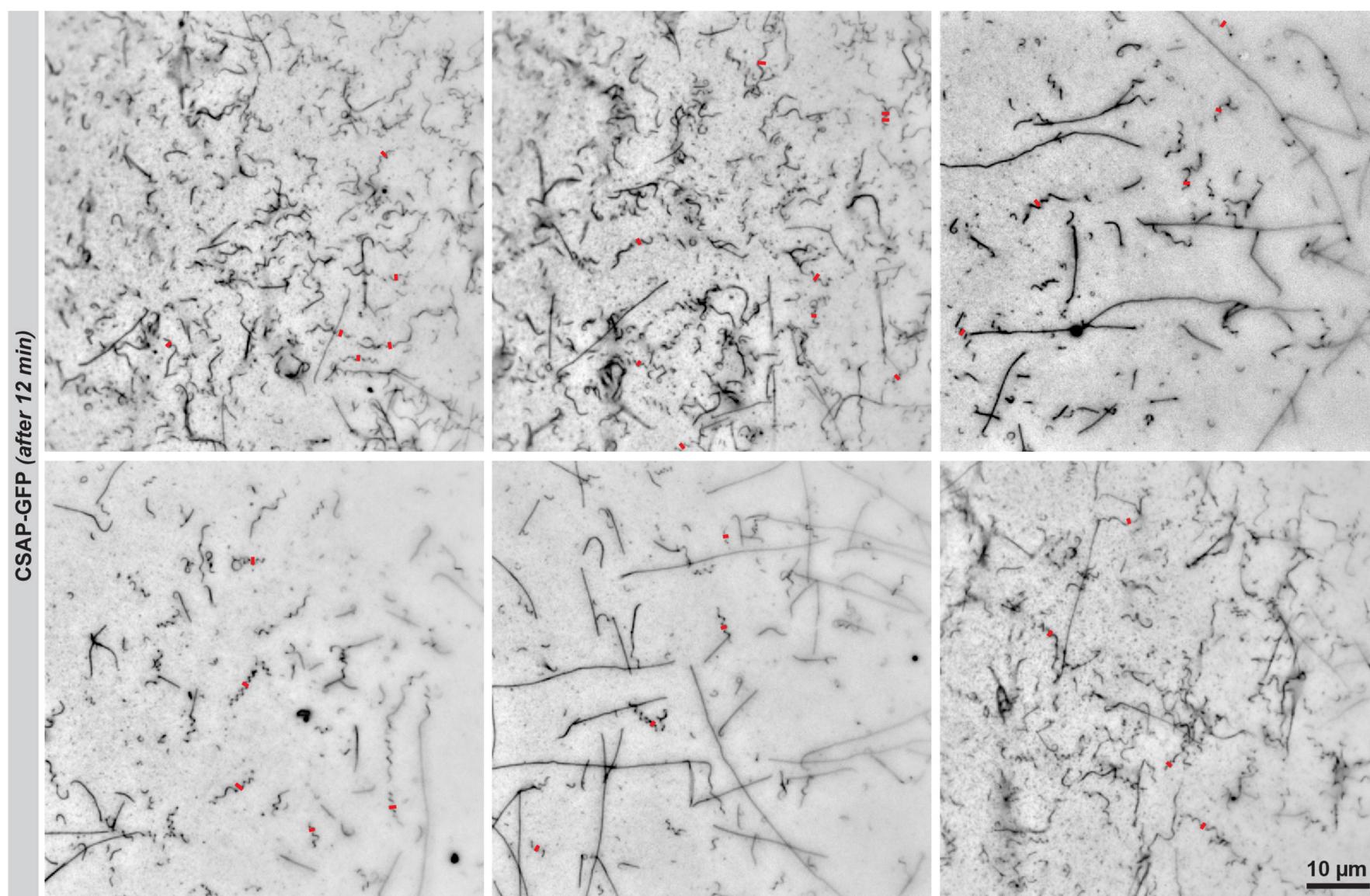


Association of MAPs with growing microtubules



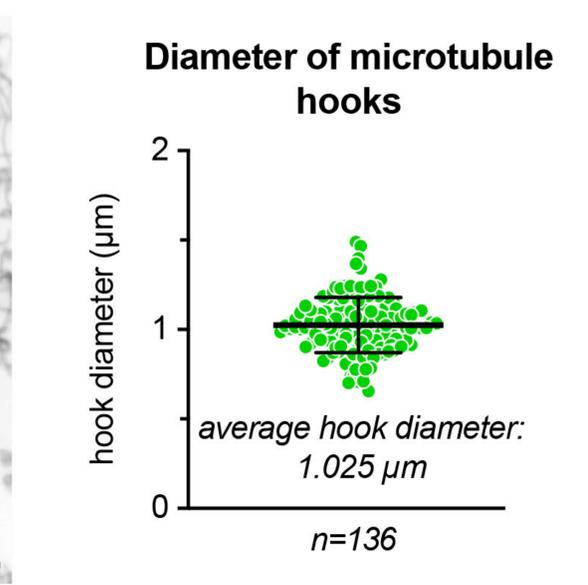
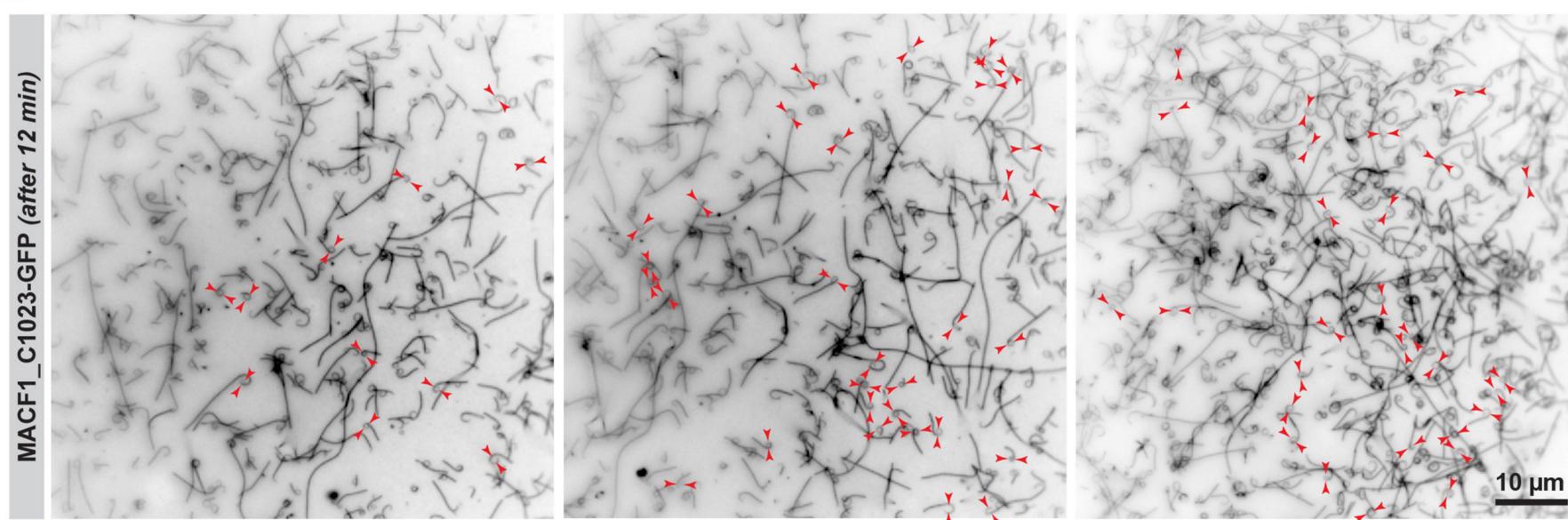
a

Measurement of CSAP-GFP-induced coil width

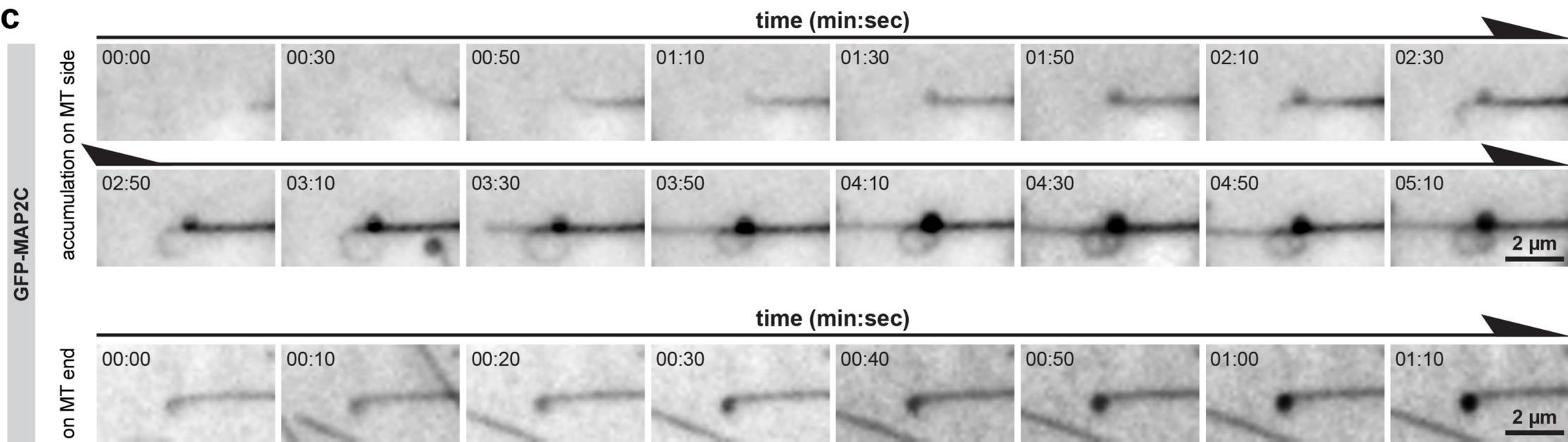


b

Measurement of MACF1_C1023-GFP-induced hook diameter

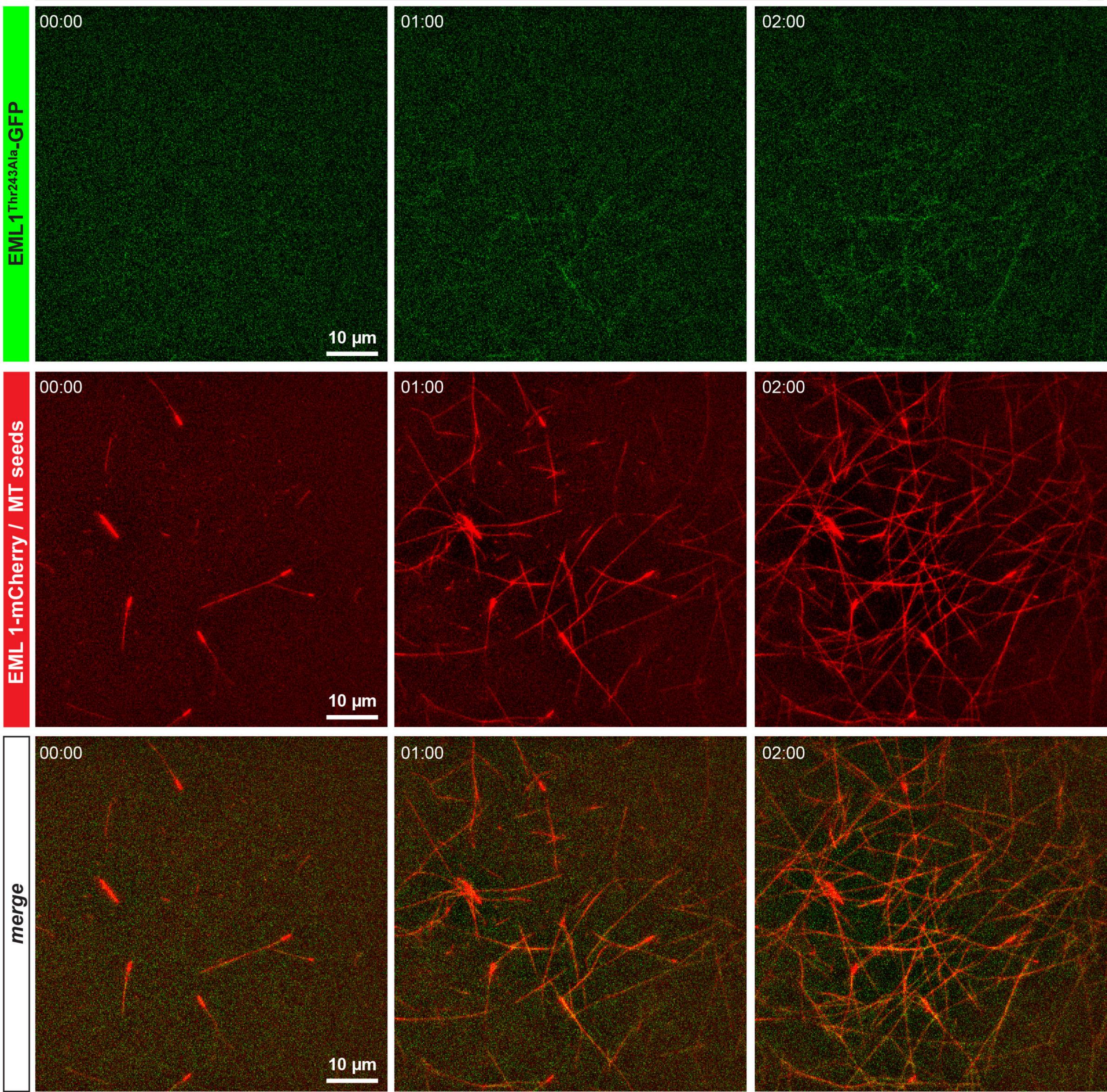


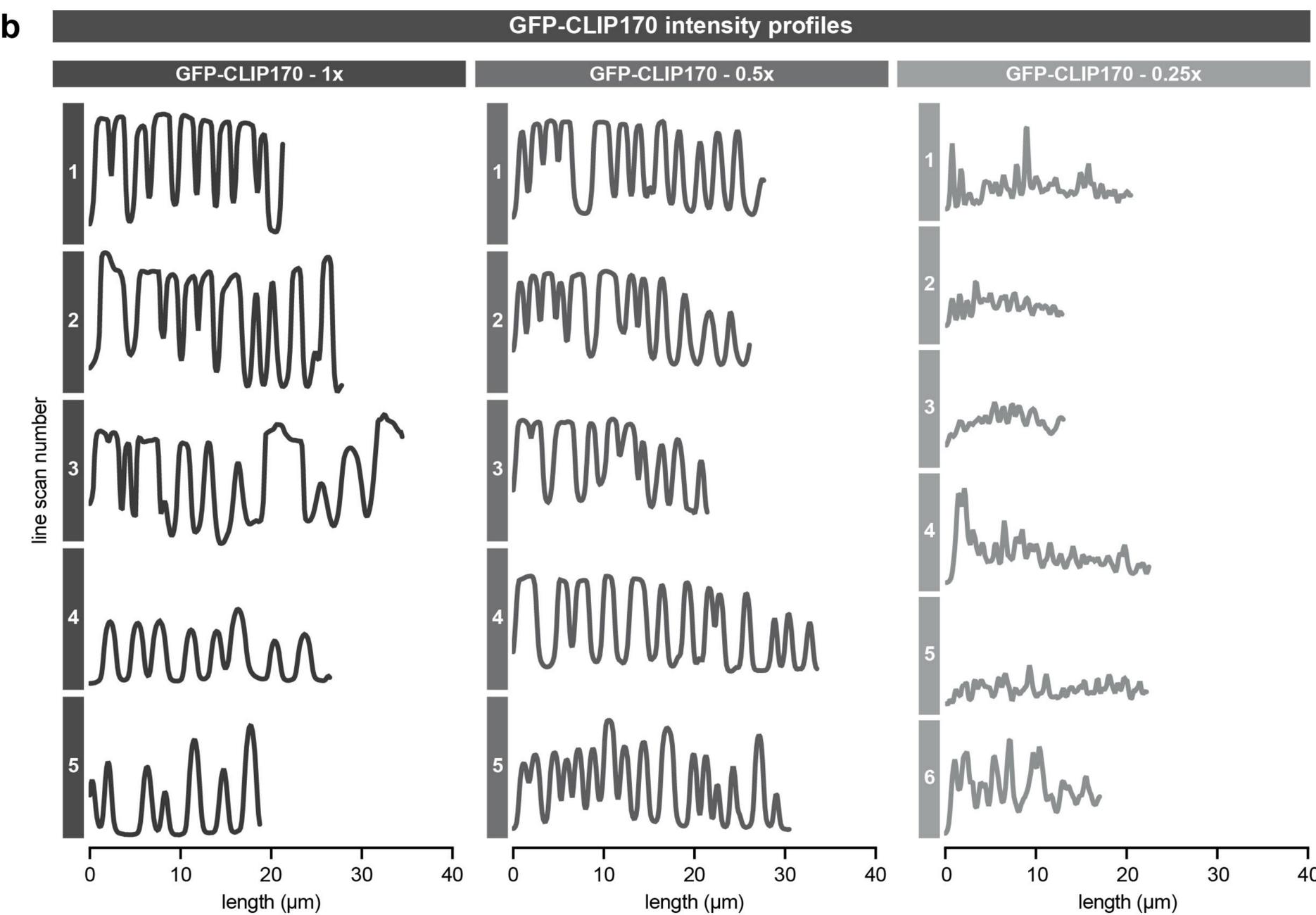
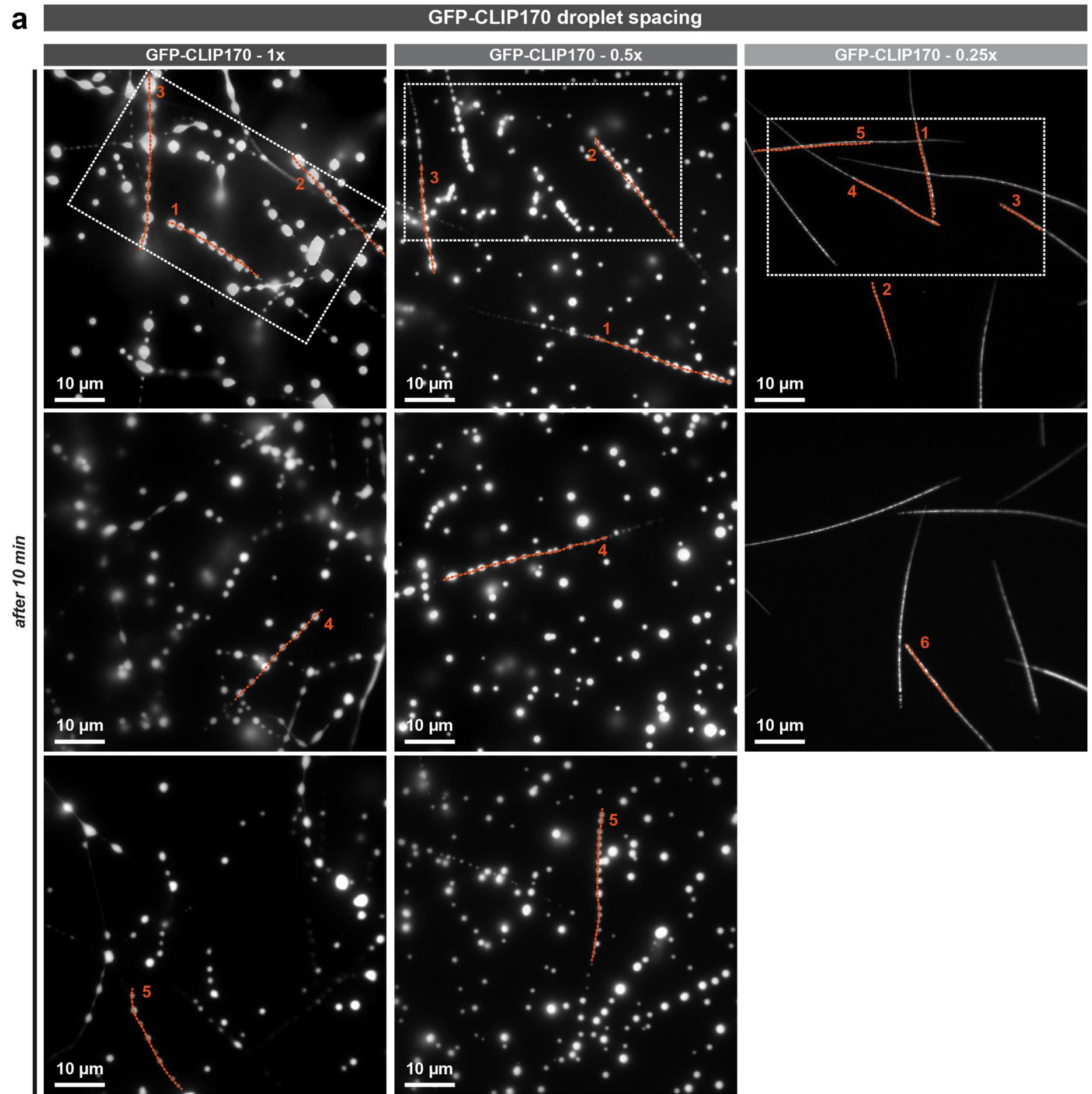
c



Microtubule growth with EML1-mCherry and EML1^{Thr243Ala}-GFP

time (min:sec)



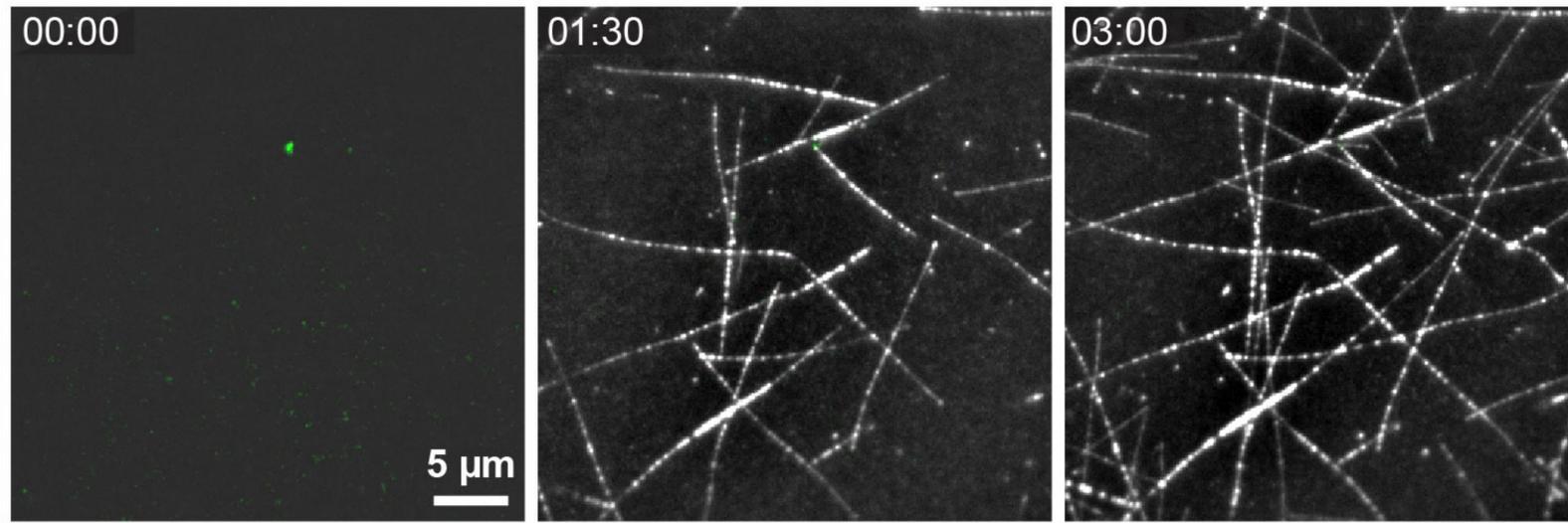
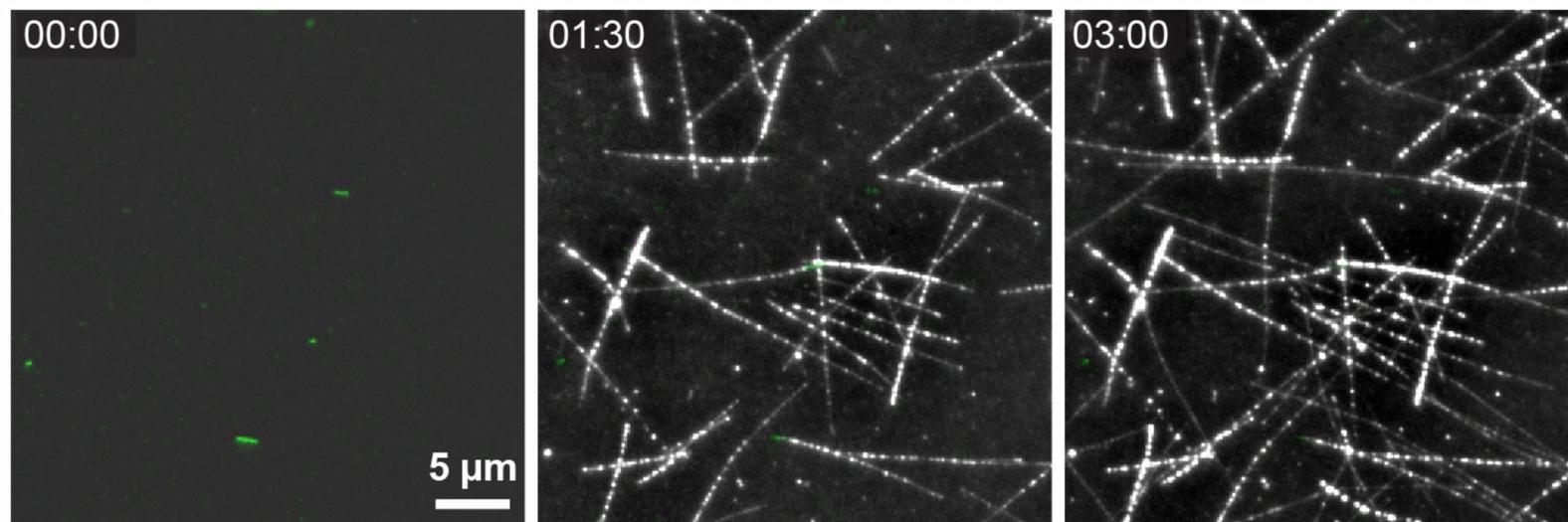
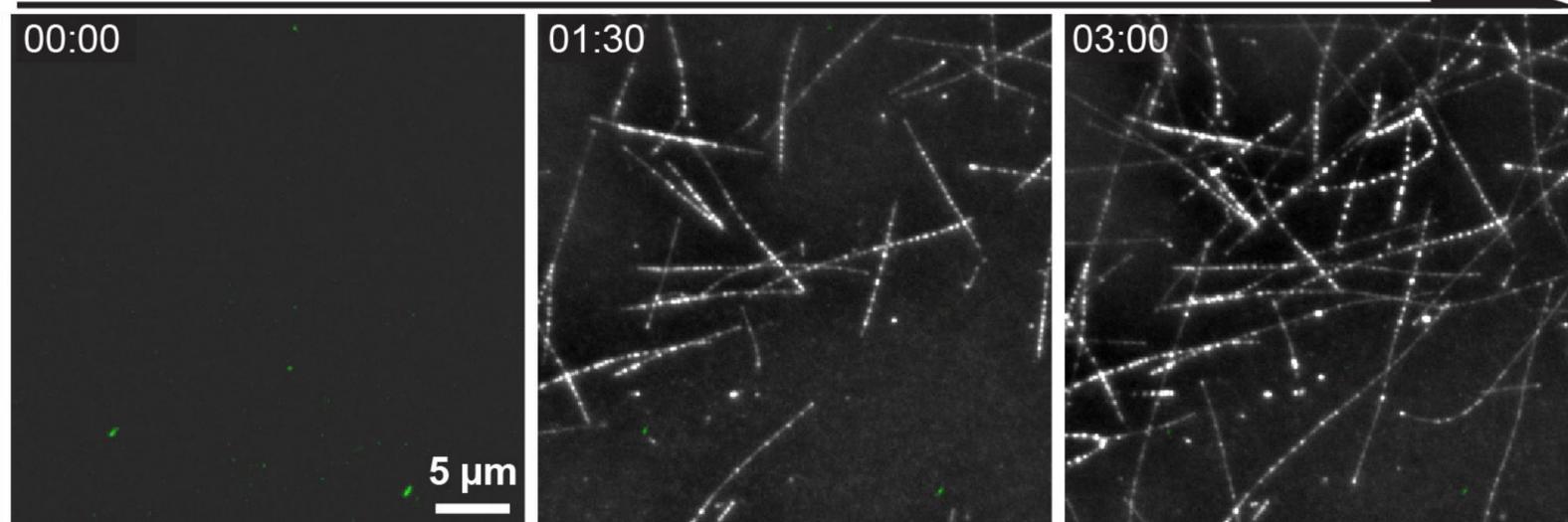


a

control conditions

time (min:sec)

GFP-CLIP170 / MT seeds



b

+ 1,6-Hexanediol

time from addition (min:sec)

