1 **TITLE**

2 **Optical flow analysis reveals that Kinesin-mediated advection impacts on the orientation of**

3 **microtubules in the *Drosophila* oocyte**

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5 **RUNNING TITLE**

6 Microtubule orientation analyzed by optical flows

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29 **KEY WORDS**

30 *self-organization; active processes; optical flow; cytoplasmic streaming; cytoplasmic flows;*

31 *microtubules; plus ends; motor proteins*

ABSTRACT

The orientation of microtubule networks is exploited by motors to deliver cargoes to specific intracellular destinations, and is thus essential for cell polarity and function. Reconstituted *in vitro* systems have largely contributed to understanding the molecular framework regulating the behavior of microtubule filaments. In cells however, microtubules are exposed to various biomechanical forces that might impact on their orientation, but little is known about it. Oocytes, which display forceful cytoplasmic streaming, are excellent model systems to study the impact of motion forces on cytoskeletons *in vivo.* Here we implement variational optical flow analysis as a new approach to analyze the polarity of microtubules in the *Drosophila* oocyte, a cell that displays distinct Kinesin-dependent streaming. After validating the method as robust for describing microtubule orientation from confocal movies, we find that increasing the speed of flows results in aberrant plus end growth direction. Furthermore, we find that in oocytes where Kinesin is unable to induce cytoplasmic streaming, the growth direction of microtubule plus ends is also altered. These findings lead us to propose that cytoplasmic streaming - and thus motion by advection – contributes to the correct orientation of MTs *in vivo.* Finally, we propose a possible mechanism for a specialised cytoplasmic actin network (the actin mesh) to act as a regulator of flow speeds; to counteract the recruitment of Kinesin to microtubules.

HIGHLIGHT SUMMARY

Cytoskeletal networks do not exist in isolation, but experience crowded and dynamic intracellular environments. However, microtubule-environment interactions are not well understood, and such system-environment interactions are an unresolved question in biology that demands bridging across disciplines. Here we introduce an optical flow motion estimation approach to study microtubule orientation in the *Drosophila* oocyte, a cell displaying substantial cytoplasmic streaming. We show that microtubule polarity is affected by the regime of these flows, and furthermore, that the presence of flows is necessary for MTs to adopt their proper polarity. With these findings we are contributing to further understanding how microtubules organize in their impacting natural environment.

INTRODUCTION

Eukaryotic life depends on many dynamic processes, including for example cell division, cell migration, and cell polarization. These processes in turn strongly rely on highly organized microtubule (MT) arrays. All MT networks are polarized, with the minus end of each filament linked to a nucleating centre (MT organising centre or MTOC), and the plus end growing away from these centres. This polarity is exploited by specific motor proteins to transport cargoes along MTs in a defined direction, and is thus essential for the function of MT networks.

A number of biophysical studies, using reconstituted *in vitro* systems, have helped to understand the mechanical properties of MTs, setting the stage for deciphering the behavior of MTs *in vivo*. However, much needs to be learnt about the properties of MTs in their natural intracellular environment. For example, a rather new concept emanating from *in vivo* experiments is that controlling nucleation and the position of minus ends of single filaments alone is not always sufficient to establish the proper polarity of the entire MT network. MT plus ends must be controlled as well in order to allow motor proteins to deliver their cargoes to the correct destination. Plus ends can be regulated at various levels, including dynamic instability, capturing, and direction of growth. Dynamic instability describes a process in which MT polymerisation is interrupted by a rapid depolymerisation phase, followed by a 'rescue' process(Mitchison and Kirschner, 1984). Various MT-associated proteins, such as motors and MT plus end-tracking proteins (+TIPs), are known to regulate dynamic instability (Akhmanova and Steinmetz, 2015). MT plus ends can also be captured at the cell cortex, a process also involving +TIPs and motors, such as the Dynein/Dynactin complex (Nieuwburg *et al.*, 2017) (and reviewed in (Akhmanova and Steinmetz, 2015)). However, very little is known about how the direction of growth of plus ends, and therefore the orientation of MTs, is controlled in cells. In axons, adenomatous polyposis coli (APC) seems to regulate plus end direction (Purro *et al.*, 2008), while Fidgetin-like1, a MT-associated ATPase, controls both dynamics and plus ends direction (Fassier *et al.*, 2018). MT bending also impacts on the direction of plus tip growth, as the MT tip rotates due to local bend formation (Kent *et al.*, 2016). Furthermore, +TIPs that contain actin-binding motifs can influence MT growth direction by guiding dynamic plus ends along actin bundles (Kodama *et al.*, 2003; Jiang *et al.*, 2012).

In the mid-oogenesis stage 9 (st9) oocyte of *Drosophila melanogaster*, MTs are nucleated from the antero-lateral cortex in a gradient of diminishing abundance toward the posterior pole, where nucleation is absent. In addition, the growing plus ends exhibit a weak posterior orientation bias (Doerflinger *et al.*, 2006; Parton *et al.*, 2011; Nashchekin *et al.*, 2016). This weak polarization bias of plus ends towards the posterior pole of the oocyte is sufficient and necessary for the localization of body plan determinants to the very 'posterior tip', as well as the formation of the pole plasm (needed for germ cell development) in this region. For example, the plus end motor Kinesin-1 (Kin from here on) localizes *oskar* mRNA to the 'posterior tip' of the st9 oocyte, an essential step in the establishment of the anterior-posterior (A-P) axis and the formation of the pole plasm (Brendza *et al.*, 2000).

3

98   In addition to a polarized MT cytoskeleton, the oocyte displays a Kin-dependent bulk motion of the
99   cytoplasm called cytoplasmic streaming or flows (Palacios and St Johnston, 2002; Serbus *et al.*,
100   2005; Ganguly *et al.*, 2012; Drechsler *et al.*, 2017). Compared to st9, late stage oocytes (st11)
101   exhibit faster and larger scale flows that are induced not only by Kin-mediated viscous drag of
102   transported cargo (similar to st9), but also by Kin-mediated MT-sliding (Lu *et al.*, 2016; Monteith *et*
103   *al.*, 2016). At these later developmental stages, these faster flows are important for mixing the
104   cytoplasm of the large oocyte (Ganguly *et al.*, 2012), and aiding the asymmetric localization of
105   developmental determinants and mitochondria (Forrest and Gavis, 2003; Hurd *et al.*, 2016; Lu *et*
106   *al.*, 2018). In addition, mathematical modeling has suggested that faster flows can induce parallel
107   MT arrays when the MTs are near a barrier (such as the cortex)(Monteith *et al.*, 2016). However, in
108   st9 oocytes, when posterior cargoes such as *oskar* mRNA are first localized, MT sliding has been
109   excluded as the source of cytoplasmic streaming (Lu *et al.*, 2016). Cytoplasmic flows at st9 are
110   slowed down by a cytoplasmic actin mesh. When this actin mesh dissolves during mid-oogenesis,
111   the flows become faster and more organized, and the MT arrange into sub-cortical bundles
112   (Dahlgaard *et al.*, 2007; Quinlan, 2013). However, the mechanism by which the actin mesh
113   regulates flows is unknown. Furthermore, it is unclear whether cytoplasmic flows at st9 have an
114   impact on the organization of the MT cytoskeleton. Recently, we found that st9 flows constitute a
115   key force driving the persistent motion of vesicles and actin filaments (Drechsler *et al.*, 2017).
116   These observations prompted us to address the question of how advection (active transport
117   induced by fluid flows) impacts on the polarization of the MT network in st9 oocytes. In this way,
118   we aim to contribute to the insufficient knowledge on how the direction of MT growth is controlled
119   *in vivo*.

120   In order to assess the global MT orientation in st9 oocytes, and to investigate the growth direction
121   of MT plus ends *in vivo*, we used EB1::GFP (EB1 from here on). EB1 exclusively decorates the
122   growing plus end of MTs, resulting in dynamic 'comets' moving through the cytoplasm at a speed
123   of ~100-600 nm/sec across various cell types, including the oocyte (at an average and maximum
124   speeds of 230 nm/sec and 600 nm/s, respectively (Parton *et al.*, 2011; Nieuwburg *et al.*, 2017).
125   Analyzing the orientation of MTs in complex networks has proven technically challenging, and
126   requires suitable imaging and image analysis tools. Especially for the *Drosophila* oocyte, we found
127   the published method too demanding on the imaging level, requiring state of the art wide-field
128   deconvolution microscopy and elaborate image processing (Parton *et al.*, 2011), and thus
129   unfeasible for the various experimental conditions that our study required. Here, we developed an
130   image analysis strategy that allows an efficient characterisation of direction and distribution of EB1
131   comets from confocal image series by an optical flow (OF)-based motion estimation approach. In
132   general, OF allows to estimate the apparent motion of objects or other intensity variations in a
133   sequence of images (Horn and Schunck, 1981). In addition, variational OF methods constitute a
134   well-established framework for dense motion estimation, and do not require elaborate
135   segmentation or tracking of the studied structures. OF methods outperform popular methods, such

136 as particle image velocimetry (PIV), for motion analysis in certain settings and, in particular, in the
137 presence of noise (Ruhnau et al., 2005; Vig *et al.*, 2016). While variational OF methods have been
138 used predominantly to investigate the dynamics of entire cells (Amat *et al.*, 2013; Boric *et al.*, 2013;
139 Guo, 2014), recent works focused also on intracellular motility (Delpiano *et al.*, 2012; Frerking *et al.*, 2014; Vig *et al.*, 2016; Boquet-Pujadas *et al.*, 2017; Huang *et al.*, 2017).
140 *al.*, 2014; Vig *et al.*, 2016; Boquet-Pujadas *et al.*, 2017; Huang *et al.*, 2017).

141 In this study, we investigated how cytoplasmic streaming influences the polarization of the MT
142 cytoskeleton. For this, we introduce a two-step image analysis approach that is based on image
143 denoising and variational OF, and is able to estimate approximate velocities (speed and direction)
144 of EB1 motion in confocal image sequences. This approach will assist many researchers
145 interested in characterizing MT polarity from confocal images of their tissue of choice. Our findings
146 revealed that cytoplasmic streaming is necessary and sufficient to regulate the polarity of the MT
147 network in st9 oocytes. Furthermore, our data suggests that the actin mesh regulates the
148 recruitment of Kin to MTs, indicating a new mechanism by which the actin cytoskeleton influences
149 MT-based transport and advection. With this work, we further contribute to understanding the *in*
150 *vivo* properties of MTs, and the interactions of MTs with their natural environment.

151 **RESULTS**

152

153 **Quantification of MT plus-tip directionality by optical flow analysis**

154 To study the spatial orientation of MT filaments *in vivo*, we used oocytes expressing EB1 (Figures
155 1, 3, 4 and 5). EB1 constitutes a marker for growing MT plus-tips and has been used in fly oocytes
156 before (Parton *et al.*, 2011; Nieuwburg *et al.*, 2017). In these previous studies, wide-field
157 deconvolution microscopy was used to image EB1 dynamics in various areas of st9 oocytes.
158 However, wide-field microscopy has a limited focus depth, only allowing to image EB1 dynamics
159 close to the cortex of the relatively large oocyte, and requires a complex acquisition procedure. In
160 order to improve imaging depth and simplify the acquisition procedure, we implemented a strategy
161 that combines conventional confocal microscopy with image denoising and motion analysis by
162 variational OF analysis.

163 OF-based motion estimation relies on the assumption that particles approximately maintain their
164 pixel intensity as they move. In thick biological samples the intensity of fluorescent signals declines
165 with the depth of the imaging plane. To minimize the effects of tissue thickness on signal intensity,
166 we imaged within a single focal plane in the middle of the oocyte over the course of at least one
167 minute (for details see Material and Methods). With this approach, we avoided measurable
168 bleaching and ensured that EB1 comets would only be detected within a thin focal plane of ~1.3
169 µm. Thus, comets moving orthogonally to the focal plane would be lost instead of getting dimmer
170 or brighter. As a consequence, our analyzes capture the 2D motion of EB1 comets within a 3D
171 intracellular environment. However, the orientation of individual oocytes on the microscope slide is
172 random. Together with averaging across several cells, this results in 2D data obtained from various
173 angles (one precise orientation per cell), which indirectly allows to acquire information about the
174 3D behavior. As OF is formulated on a per-pixel basis, this method allows inferring a displacement
175 vector for each pixel and does not require sophisticated tracking of individual particles (Figure 1A
176 and B). Since MTs grow one order of magnitude faster compared to the speed of cytoplasmic flows
177 in st9 oocytes, we took one image every 650 ms, resulting in a loss of spatial resolution, and
178 causing the raw data to exhibit considerable high noise (Figure 1D). Due to this high noise level,
179 preceding denoising of the image data was required prior to applying the motion estimation. We
180 found that total variation-based (Rudin et al., 1992) image denoising with additional temporal
181 regularization was sufficient to improve the signal-to-noise ratio and the visibility of EB1 comets
182 (Figure 1E, Figure S1-S3 and Movie S1).

183 In the next step, displacement vector fields were computed with a variational OF method (Figure
184 S4). After accounting for the pixel size and the time interval between consecutive frames, these
185 displacements can be regarded as approximate velocities of EB1 motion (Figure 1F-H).
186 Importantly, the acquired velocities are a sum of all motion experienced by the imaged EB1 signal,
187 including filament growth and MT displacement by advection and transport. A typical image
188 sequence of 100 frames and a 512 x 256 pixel resolution results in a total number of approximately

6

189 13 million computed vectors that require appropriate interpretation. The computation time for
190 processing one typical sequence amounted to less than 25 minutes on average.

191 We visualized the estimated vector fields with the help of a standard colour-coding (Baker, 2011).
192 The vector at each pixel and at a certain frame is represented by a colour that is determined by the
193 direction of the signal's movement (see the colour-coding at the boundary of the images in Figure
194 1F-H). The colour's intensity is determined by the relative velocity of the signal. The stronger the
195 intensity, the higher the velocity. For our statistical analyzes, we considered only vectors located
196 within a hand-drawn segmentation mask that outlines the oocyte in each sequence (Figure 1H).
197 The direction of each vector could then be represented in polar coordinates. The distribution of the
198 angles obtained from each individual cell is visualized in a rose diagram (Figure 1O and P). In
199 addition, we show the histogram of the distributions averaged over all cells of a given genotype
200 (Figure 1Q).

201 We then used OF to describe the MT orientation in control oocytes (Figure 1O-S, Figure S5). Since
202 all cells were orientated with the posterior pole to the right during imaging, the angles given in the
203 rose diagram reflect the directional movement of EB1 comets. For a quantitative description of MT
204 orientation, we determined the mean angular direction of the estimated motion of EB1 signals (θ_{avg},
205 considering all recorded cells of one genotype, Figure 1R, Table 1) and the frequency of
206 movements directed towards the anterior (all within the region 270° to 90°, blue hemicircle in
207 Figure 1C) or the posterior (all within the region 90° to 270°, orange hemicircle in Figure 1C). As a
208 descriptor of how focused MTs grow towards the very 'posterior tip', we also determined the
209 relative frequency of posterior-directed EB1 signals that fall within a sector of 60° (from 30° to
210 330°), called 'posterior tip', inspired by the region where determinants localize and pole plasm
211 forms (light ogange sector in Figure 1C). This 'posterior tip' frequency only considers EB1 signals
212 that were previoulsy found to grow towards the posterior (orange hemicircle in Figure 1C), and
213 thus the 'posterior tip' value represents a sub-population of all posterior signals that grow with an
214 angle between 30° to 330°. In summary, we find that all growing MTs exhibit a global (over the
215 entire oocyte) posterior orientation bias, with 66% of all comets growing towards the posterior of
216 control cells (Figure 1R). These findings are in agreement with previous reports of biased MT
217 orientation in st9 oocytes (Parton et al., 2011), and provide a first level of validation to establish OF
218 as a reliable way to investigate the orientation of MTs. In addition, we find that 40% of the 66% of
219 comets that grow towards the posterior are oriented towards the 'posterior tip', where determinants
220 localize and the pole plasm forms (Figure 1S).

221

222 **OF is sufficient to detect changes in microtuble network orientation**

223 In order to test whether OF-based approaches are able to capture EB1 directionality in other cell
224 types, we investigated EB1 comets in the follicular epithelium surrounding the egg chamber. In
225 these epithelial cells, MTs are nucleated apically and grow predominantly towards the basal

226 membrane, parallel to the lateral membranes of the cell. Notably, single comets can easily be

227 identified and followed by eye, which allowed us to assess the accuracy of the OF results. OF

228 analysis captures the directionality of MTs in these follicle cells accurately, supporting the

229 suitability of the approach to estimate MT orientation *in vivo* (Movie S2 and Figure S6-S7).

230 We next tested whether OF can pick up changes in the architecture of MT neworks in the oocyte,

231 and analyzed cells harboring a mutation in *gurken (grk)*, which exhibit MT organization and cell

232 polarity defects (Gonzalez-Reyes *et al.*, 1995; Roth *et al.*, 1995; Januschke *et al.*, 2002). With our

233 OF analysis we were able to detect an aberrant MT organization in *grk* oocytes, with EB1 comets

234 showing a variable orientation from cell-to-cell (Movie S3 and Figure S6). Although, the number of

235 tested cells is low, and thus the actual biological meaning of these findings needs further

236 investigation, our results confirm that OF is able to detect aberrations in MT networks in complex

237 systems like the oocyte.

238

239 **Aberrantly fast cytoplasmic flows change MT motion, bundling and orientation**

240 In order to investigate the relationship between cytoplasmic advection and the organization of the

241 MT network, we monitored the bulk behavior of MTs in control cells and in oocytes with faster

242 flows. Oocytes mutant for *cappuccino (capu)* or *spire* display faster flows at st9 (Figure S9A, B)

243 and fail to localize posterior cargoes like the mRNA-binding protein Staufen (Figure 1I, K and M)

244 (Theurkauf, 1994; Dahlgaard *et al.*, 2007; Quinlan, 2013). Capu (a formin) and Spire are actin

245 nucleators that are required for the formation of a cytoplasmic actin mesh that regulates the

246 magnitude of flows by a yet unknown mechanism. It has been shown in fixed samples that the

247 removal of the actin mesh seems to cause bundeling of MTs in close proximity to the cell cortex.

248 However, dynamic MT behavior in living mutant oocytes has not been addressed in detail.

249 To monitor the bulk movement of MTs, we imaged cells expressing the MT associated protein

250 Jupiter::GFP (Jup), which labels the entire MT filament. Compared to control cells, we observed

251 that MTs in homozygous *capu^{EY12344}* or transheterozygous *spire^1/Df(2L)Exel6046* mutant oocytes

252 appear as thick bundles, which dramatically bend and buckle (Figure 1I-N' and Movie S5). Such

253 higher 'packing' of filaments does not necessarily indicate their crosslinking, but argues that the

254 high hydrostatic pressure in fast flowing oocytes is sufficient to bundle MT filaments. More

255 importantly, and in contrast to previous descriptions, our data demonstrate that in *capu* and *spire*

256 oocytes MTs cluster even when they are not in close proximity to the cortex, and thus that bundles

257 can form even when the MTs are not pushed against the cortex. Furthermore, the observed MT

258 bundles appear longer when compared to control cells, extending further into the posterior (red

259 arrows in Figure 1L and N). In summary, these results indicate that changing the regime of

260 cytoplasmic advection impacts on the bulk motion of MTs, as well as on their cytoplasmic

261 bundeling state.

262 In *capu* and *spire* mutant oocytes flows can reach speeds up to 200 nm/s, well within the regime of

263 plus tip growth. In such mutant situation, the plus tip of single MTs might not be able to 'outgrow'

264   the effect of cytoplasmic advection, and thus flows might become a dominant contributor of MT
265   orientation. To test whether faster flows alter the orientation of MTs, we quantified the directionality
266   of EB1 signals in *capu* mutant cells with our OF approach, and found substantial alterations
267   (Figure 1T-X). Compared to controls, *capu* mutants exhibit large amounts of comets in close
268   proximity to each other, which move into the same direction. This suggests that *capu* mutant
269   oocytes display arrays of parallel growing filaments (Figure 1T and Movie S4). The motion of such
270   EB1 'arrays' (which varies from cell to cell, Figure 1T-X), is likely to correlate with the bundling of
271   MT filaments described above, and could be interpreted as a direct result of faster flows.
272   Interestingly, the global posterior EB1 orientation bias was only mildly decreased in most *capu*
273   mutant oocytes (Figure 1V, W vs. Figure Q, R and Table 1). However, MTs are less focused
274   towards the posterior tip in *capu* mutants, with only 25% of the posterior-directed EB1 signals
275   growing with an angle between 30° to 330°. These findings are in a good agreement with the
276   determinant mis-localization defects in *capu* and *spire* mutant cells (Figure 1X vs. Figure 1K and
277   M).

278   In summary, these findings together demonstrate that the lack of the actin mesh, and the resulting
279   increase in flows speeds, impact on the orientation of MTs, and lead us to speculate that at mid-
280   oogenesis flows must be kept in a precise regime for molecular determinants to be localized
281   correctly. However, how the actin mesh actually regulates the magnitude of flows in the oocyte is
282   not known

283

The cytoplasmic actin mesh counteracts the recruitment of Kinesin to MTs *in vivo*

285   The MT plus-tip directed motor protein Kin constitutes the major driving force of cytoplasmic flows
286   in the *Drosophila* oocyte (Palacios and St Johnston, 2002). To start investigating possible
287   mechanisms by which the actin mesh regulates the magnitude of cytoplasmic flows, we monitored
288   the distribution of Kin (Kin1-700-GFP) in control and homozygous *capu*^{EY12344} mutant oocytes. This
289   truncated motor protein lacks all its tail domains, and is thus not autoinhibited and unable to
290   transport cargo (Williams *et al.*, 2014). In control oocytes, Kin1-700 predominantly localizes to the
291   posterior of the cell, in a gradient of diminishing abundance from anterior to posterior (Figure 2A
292   and D, and (Williams *et al.*, 2014)). In stark contrast, Kin1-700 fails to accumulate at the posterior
293   in *capu* mutant oocytes (Figure 2B and F, Movie S10), and is found instead on filamentous
294   structures closely resembling MTs (compare Figure 2B to Figure 1L and Movie S10 to Movie S5).
295   This result indicates that, in the absence of *capu*, Kin becomes strongly recruited to MTs. The
296   same Kin behavior can be induced acutely by *ex vivo* treatment with F-actin destabilising drugs like
297   Cytochalasin D (data now shown), indicating that the observed behavior of Kin is due to the lack of
298   the actin mesh and not to potential developmental defects that might arise from the lack of *capu*
299   during earlier stages of oogenesis. In fixed control cells, Kin1-700 accumulates to posterior regions
300   of the cell (Figure 2D), where MTs are less abundant (Figure 2C), while the motor strongly co-

301   localizes with MTs in fixed *capu* mutant oocytes (Figure 2E, F, and J- L). This observation

302   suggests that the actin mesh alters the ability of Kin to become recruited to MTs.

303   To further understand the relationship between the actin mesh and the interaction of Kin with MTs,

304   we monitored the localization of the motor in different *capu* mutant alleles. For this, we utilized

305   three mutant alleles that harbor distinct point mutations in the FH2 domain, and that affect the *in*

306   *vitro* actin assembly rate of Capu to different degress (*capu^{L219}*, *capu^{L201}* and *capu^{38}*, in increasing

307   severity) (Yoo *et al.*, 2015). In order to exclude effects from potential second site mutations, we

308   generated trans-heterozygous animals between these alleles and the strong hypomorphic mutation

309   *capu^{EY12344}* (Figure 2 and TableS1). In order to estimate the amount of cytoplasmic F-actin, we

310   measured relative signal intensities in phalloidin-stained control and mutant oocytes (Figure 2M-

311   Q). In agreement with the *in vitro* data (Yoo *et al.*, 2015), *capu^{L219}* oocytes displayed a mild

312   decrease in signal intensities, followed by *capu^{L201}* and *capu^{38}*, resulting in the strongest reduction

313   in fluorescence intensities (Figure 2Q). We next tested the localization of Kin1-700 in these three

314   allelic combinations of *capu*, and found that in *capu^{L219}* oocytes, Kin is found at the posterior region

315   of the cell (Figure 2R), but also at small MT bundles (arrow in Figure 2R). This recruitment of Kin to

316   MTs was further enhanced in *capu^{L201}* and *capu^{38}*, to levels similar to what we observed in the

317   homozygous *capu^{EY12344}* cells (Figure 2S and T, compare to Figure 2B). Thus, the strength of Kin

318   recruitment to MTs seems to follow the strength of the defects in the actin mesh. It is important to

319   note that the speed of cytoplasmic flows is equally increased in *capu^{L219}*, *capu^{L201}* and *capu^{38}*

320   oocytes ((Yoo *et al.*, 2015) and data not shown). Taken together, this indicates that the amount of

321   Kin recruited to MTs mainly depends on the amount of cytoplasmic F-actin. Together with our

322   previous finding that a higher number of active Kin results in faster flows (Ganguly *et al.*, 2012),

323   these findings point towards a model by which the actin mesh is regulating the magnitude of flows

324   by limiting the efficient recruitment of Kin to MTs.

325

326   **Cytoplasmic streaming is necessary for MTs to display a correct orientation**

327   Our results so far show that Kin-dependent cytoplasmic flows need to be maintained at low speeds

328   in st9 oocytes for MTs not to mis-orient. Consequently, we next asked whether cytoplasmic

329   streaming might actually be necessary to sustain a proper organization of the MT network. In other

330   words, is the motion direction of MT plus ends altered in st9 oocytes that lack Kin-dependent

331   cytoplasmic streaming?

332   To address this question, we first analyzed MTs in oocytes lacking *kinesin heavy chain* (*khc*). The

333   complete loss of the Kin motor unit (mutant allele *khc^{27}*, from hereon called *khc^{null}*) results in a fully

334   penetrant absence of cytoplasmic flows (Figure S8)(Palacios and St Johnston, 2002; Serbus *et al.*,

335   2005). Compared to control cells, MTs in *khc^{null}* mutant oocytes display very little overall motion

336   and appear rather 'stiff' (Figure 3A,A', compared to Figure 1J,J' and Movie S6). However, oocytes

337   without Kin do not only lack cytoplasmic streaming, but also lack cargo transport towards the

338   'posterior tip' and display an aberrant actin mesh (Figure 3D and Figure S9) (Brendza et al., 2000;

339　Drechsler et al., 2017). To test whether the altered bulk behavior of MTs is due to the lack of

340　streaming or other aberrations in Kin mutant oocytes, we monitored MTs in st9 oocytes carrying

341　distinct mutations in the Kin motor domain, resulting in a slower motor (two mutant alleles known

342　as khc^{23} and khc^{17}, hereafter summarized as khc^{slow}) (Brendza et al., 1999). St9 khc^{slow} oocytes

343　display a normal cytoplasmic actin mesh (Figure S9) and are able to transport a considerable

344　amount of cargo towards the posterior (Figure 3E and F) (Serbus *et al.*, 2005; Loiseau *et al.*,

345　2010). Importantly, and identical to khc^{null} cells, they lack any cytoplasmic advection at st9,

346　allowing us to study the impact of flows on MT behavior in the presence of transport (Figure

347　S8)(Serbus *et al.*, 2005). Similar to khc^{null} cells, MTs in khc^{slow} oocytes appear stiff and no motion

348　could be detected in kymographs (Figure 3B-C' and Movie S6). These observations indicate that

349　the altered motion of MTs in oocytes without Kin is indeed due to a lack of cytoplasmic streaming.

350　Thus, Kin-dependent cytoplasmic advection is necessary for MTs to display a wild-type bulk

351　motion.

352　We next analyzed EB1 directionality in khc^{slow} oocytes (Figure 3G-K). As in control cells, we

353　observed dynamic EB1 comets throughout the entire cytoplasm (Movie S7). However, in stark

354　contrast, the distribution of orientation angles displayed a more focused bias towards the posterior

355　of the cell (Figure 3I vs. Figure 1Q). Consequently, khc^{slow} mutant oocytes displayed an increased

356　posterior plus-tip bias, with 75% of EB1 signals directed towards posterior, compared to 66% in

357　control cells (Figure 3J vs. Figure 1R and Table 1). Furthermore, 54% of these posterior-directed

358　EB1 signals (the group consisting of 75% of all comets) displayed a 'posterior tip' orientation,

359　compared to 40% in controls (Figure 3K vs. Figure 1S and Table 1). These findings indicate that

360　the orientation of the growing MT plus ends in st9 oocytes does not only depend on nucleation or

361　anchoring of minus ends, but also on the presence of cytoplasmic flows. In other words, advection

362　is necessary for MTs to display a correct orientation.

363　To further investigate the impact of cytoplasmic flows and Kin activity on MT orientation, we

364　analyzed the regional organization of the MT network along the A-P axis in control, khc^{null} and

365　khc^{slow} oocytes. We divided each oocyte into an anterior and a posterior region (Figure 4A), and

366　analyzed EB1 directionality in each of these two regions (Table 1). As previously shown, the

367　posterior EB1 bias increases along the A-P axis of st9 oocytes (Figure 4B-D) (Parton et al., 2011).

368　In the anterior region, we found 62% of signals directed towards posterior (Figure 4C), while this

369　bias was increased to 74% in the posterior region (Figure 4D). As already demonstrated for the

370　global posterior EB1 bias (Figure 4B), khc^{slow} oocytes also showed a dramatic change of MT

371　orientation along the A-P axis, with a 74%, posterior bias in the anterior region (Figure 4E vs.

372　Figure 4C) and an even further increased 81% posterior bias in the posterior region (Figure 4F vs.

373　Figure 4D). Additionally, the ratio of signals directed towards the 'posterior tip' (Figure 1C) in both

374　anterior and posterior regions of the khc^{slow} cell was substantially increased, compared to controls

375　(Figure 4E and F, and Table 1). This clearly demonstrates that oocytes with a slower Kin, and thus

376　without cytoplasmic streaming, display a stronger polarization of the entire MT network towards the

377 posterior pole, a key region in the establishment of the embryonic body axis. While the lack of Kin

378 (khc^{null}) seemed to cause only minor defects in the global organization of the MT cytoskeleton

379 (Figure 3L-Pand Figure 4B), the regional analysis of EB1 directionality in khc^{null} oocytes revealed

380 major differences compared to control cells. In the anterior region of khc^{null} cells, we detected an

381 unexpected drop of the posterior bias (56% compared to 66% in controls), which indicates that the

382 complete lack of Kin does indeed affect MT network organization (Figure 4G vs. Figure 4C). It

383 needs to be mentioned here that oocytes lacking Kin also fail to localize the nucleus, which in turn

384 is associated with MT minus ends (Williams et al., 2014). Therefore, this observed MT behavior in

385 khc^{null} cells might primarily reflect the mis-localization of a certain subset of MTOCs in the cell.

386 However, MTs in the posterior region of khc^{null} cells displayed a slightly more focused growth

387 towards the key 'posterior tip' (Figure 1C), similar to khc^{slow} cells (Figure 4H vs. Figure 4D and F,

388 right rosette panels, and Table 1).

389 Taken together, our data allow us to draw certain conclusions about the relationship of Kin-activity,

390 cytoplasmic streaming, and the organization of MTs. Firstly, in st9 oocytes cytoplasmic flows need

391 to be in a defined regime to ensure proper MT orientation. Secondly, in the presence of Kin-

392 mediated transport, but in the absence of cytoplasmic flows (khc^{slow} condition), there are more plus

393 ends growing towards the posterior, suggesting that cytoplasmic streaming is necessary for MTs to

394 polarize in the precise pattern observed in control cells. And thirdly, together with cytoplasmic

395 flows, other Kin-mediated processes, such as cargo transport or nucleus anchoring affect the

396 organization of the MT network, supporting previously published findings (Nieuwburg et al., 2017;

397 Zimyanin et al., 2008).

398

399 Reconstitution of cytoplasmic flows in st9 khc^{slow} oocytes rescues MT orientation

400 Our data suggest that Kin-mediated cytoplasmic streaming is necessary for the MT network to

401 completely adopt its wild-type organization in the st9 oocyte. To further investigate the connection

402 between cytoplasmic advection and MT orientation, we analyzed EB1 directionality in $capu,khc^{slow}$

403 double mutant oocytes. With this experiment we aimed to test whether the re-establishment of

404 cytoplasmic streaming in a mutant background is sufficient to rescue MT orientation.

405 It has been suspected that faster cytoplasmic flows in $capu$ mutants can be slowed down again by

406 introducing a khc^{slow} mutation (Dahlgaard et al., 2007). Therefore, we generated a double mutant,

407 harboring the alleles $capu^{EY12344}$ and khc^{17}. To verify the functionality of this double mutant, we first

408 investigated posterior cargo localization in fixed cells. As expected, ~85% of $capu,khc^{slow}$/+ cells

409 (which are essentially $capu$ mutants, Table S1) failed to correctly localize Staufen to the posterior

410 pole of the cell (Figure 5A, n=20). In comparison, ~72% of $capu,khc^{slow}$ double mutant cells

411 localized Staufen into a posterior crescent (n=25). However, the majority of oocytes that displayed

412 Staufen in a crescent, also showed Staufen accumulation in posterior dots (n=11/18). This

413 constitutes a phenotype that is usually associated with the khc^{slow} alleles (Figure 5B vs. Figure 3E

414 and F). Together, these data confirmed that our double mutant is comparable to the previously

12

415 reported alleles (Dahlgaard et al., 2007), and that slow Kin is sufficient to rescue the major cargo
416 localization defects seen in *capu* mutants. However, since cargo transport was not rescued to wild-
417 type levels, and was instead found to be similar to *khc^slow* oocytes (Loiseau et al., 2010), it is
418 obvious that the decreased cargo transport efficiency of slow Kin cannot be rescued by re-
419 introducing cytoplasmic streaming by the lack of *capu*. It is furthermore unclear whether this is due
420 to a reduced translocation speed of slow Kin, or to a defect in cargo anchoring in *capu* mutant cells
421 (Tanaka *et al.*, 2011).

422 Next we tested whether premature fast cytoplasmic flows in *capu* mutants are indeed slowed down
423 again in *capu,khc^slow* oocytes. As expected, and similar to *capu* single mutants, *capu,khc^slow*/+
424 oocytes displayed aberrant fast flows (Figure 5C, Movie S8). In contrast, cytoplasmic flows in
425 *capu,khc^slow* double mutant oocytes were indeed slowed down again and appeared similar to those
426 observed in control cells (Figure S8). We then tested whether the rescue of cytoplasmic streaming
427 in the double mutant also affected MT bulk movement. Compared to *capu,khc^slow*/+ oocytes, which
428 displayed fast flows and consequently MT bundling, the reduction of flow speeds in the double
429 mutant caused a MT bulk movement similar to that observed in control cells (Figure 5C-D' and
430 Movie S8).

431 To finally test whether the re-establishment of cytoplasmic streaming in a *khc^slow* background (or
432 the reduction of fast flows in *capu* background) rescues the MT orientation defects observed in
433 either single mutant, we analyzed EB1 directionality in *capu,khc^slow*/+ (Figure 5E-I) and *capu,khc^slow*
434 double mutant oocytes (Figure 5J-N and Movie S9). EB1 comets in *capu,khc^slow*/+ oocytes
435 exhibited values that are similar to what we observed in *capu* mutants (Figure 1T-X), suggesting
436 that the heterozygous presence of a *khc^slow* mutant chromosome does not substantially affect the
437 *capu* mutant phenotype (Movie S9, 61% of EB1 signals pointing towards posterior, and 34% of this
438 61% are growing towards the 'posterior tip' in *capu,khc^slow*/+ cells, Figure 5F-I and Table 1).
439 Conversely, EB1 comets in *capu,khc^slow* double mutant cells displayed a posterior motion bias
440 indistinguishable from that of control cells (Movie S9, 66% posterior bias, Figure 5M vs. Figure 1R
441 and Table 1). Finally, also the fraction of all posterior comets pointing towards the 'posterior tip'
442 was found to be similar in *capu,khc^slow* double mutant cells (39%) and controls (40%) (Figure 5N
443 vs. Figure 1S and Table 1), further strengthening the idea that cytoplasmic streaming constitutes a
444 substantial contributor to a correct MT plus end focussing, and thus MT organization, in the oocyte.

445 In summary, our results now clearly demonstrate that in the absence of *capu* and the actin mesh, a
446 slower Kin motor protein is sufficient to restore the correct regime of cytoplasmic flows, resulting in
447 the correct polarization of the MT cytoskeleton, and an almost restored transport of cargo towards
448 the posterior. Therefore, it seems that the actin mesh is essential in oocytes that exhibit normal
449 Kin-mediated transport, likely by regulating the amount of Kin recruited to MTs, in order to ensure
450 the proper regulation of cytoplasmic streaming, which in turn is an important contributor to the
451 observed MT orientation.

DISCUSSION

Due to its large size (80-100 μm along the A-P axis) and the fact that MT minus ends are nucleated and anchored along the antero-lateral cortices of the cell, the *Drosophila* st9 oocyte is arguably one of the most challenging *in vivo* systems in which to analyze the dynamic behavior of MTs. The use of confocal microscopy, together with OF based analysis tool described in this study, allowed us to quantify the motion direction of plus ends within a 2D focal plane along the entire length of this cell. Together with others, the present study supports the existence of a complex mechanical and/or biochemical relationship between motion of cytoplasmic components (whether by advection or by transport), cytoplasmic F-actin and MTs in the *Drosophila* oocyte. This holds true for our model system, but is likely transferable to many other cell types. For example, experimental data and modeling of cytoplasmic motion in Characean algae (where cytoplasmic flows are acto-myosin dependent) suggests that paralle actin cables and ordered streaming patterns self-organize in an interdependent manner (Foissner and Wasteneys, 2000; Woodhouse and Goldstein, 2013).

Kin-mediated cargo transport through a highly viscous medium, such as the cytoplasm, inevitably induces bulk motion of such medium, which will cause lateral displacement forces on MT filaments and thus induce a feedback on their spatial orientation. As a consequence, cytoplasmic streaming needs to be kept in a defined speed regime and at a biased random pattern in order for the MT network to properly polarize. In the absence of the actin mesh (as in *capu* or *spire* mutants), there is an increased persistence and speed of flows, resulting in parallel alignment and bundling of MTs, as well as strong defects on MT orientation. The actin mesh - which itself requires Kin activity for its proper organization (Drechsler et al., 2017) - is required for the maintenance of this correct regime of advection.

With our work, we show that our image analysis approach - based on a rigorous image analysis framework - is able to infer and quantify directionality of EB1 motion from confocal image sequences. The use of an efficient iterative optimisation algorithm enabled us to analyze entire image sequences at once, as compared to a frame-by-frame analysis. In comparison to more commonly used techniques, such as particle tracking or PIV, OF allows us to perform motion estimation on pixel level in challenging circumstances, such as low signal-to-noise ratios and small particle size. Most importantly, this image analysis approach can handle standard confocal microscopy data and the method does not require demanding imaging techniques or costly computing hardware. Moreover, the image analysis depends only on a few parameters that can be easily adjusted. However, two limitations of the developed methodology need to be pointed out. Firstly, due to the use of a variational framework for the image analysis, both the denoised sequences and the estimated velocities exhibit a loss of contrast, leading to underestimated speeds of EB1 comets. This is particularly due to the temporal regularization required in both steps to overcome the above-mentioned challenges. Secondly, our statistical analyses of directions of EB1 comets are based on velocities computed for all pixels within each segmented oocyte. We are

14

490   aware that not every image pixel portrays exactly one EB1 comet. Furthermore, EB1 comets in the
491   oocyte are subjected to different forces. In summary however, our results demonstrate that our
492   approach is able to monitor general plus end direction in most, if not all, cell types and thus
493   constitutes an efficient and reliable analytical framework for MT polarity studies. Therefore, OF is
494   absolutely suitable to efficiently describe the orientation of MTs in crowded intracellular
495   environments.

496   It is unknown how the actin mesh slows down Kin-dependent cytoplasmic flows or how it might
497   affect MT organization. It has been proposed that the presence of a viscoelastic actin network can
498   increase the effective viscosity of the cytoplasm, and counteract the viscous drag of cargo
499   transport by Kin (Quinlan, 2016). Furthermore, actin-MT crosslinking proteins have been
500   demonstrated to allow a potent crosstalk between both filament species (Preciado Lopez *et al.*,
501   2014), presumably coordinating cytoskeletal organization *in vivo*. However, our data from
502   *capu,khc^{slow}* double mutant oocytes, which do not form an actin mesh, suggest that the mesh itself
503   is not essential to allow a correct MT orientation when Kin is slower. In this 'artificial' mutant
504   situation, the correct regime of flows seems sufficient to allow proper MT network organization. It is
505   thus possible that the actin mesh regulates the activity of Kin more directly, for example by
506   tethering the motor to its filaments (directly or indirectly through cargo). Such model is indeed
507   supported by our finding that Kin becomes efficiently recruited to MTs when the actin mesh is
508   absent (Movie S10 and Figure 2). This observation could explain how the mesh regulates not only
509   flows, but also the higher degree of MT bundling, as a larger number of Kin molecules recruited to
510   MTs is likely to result in faster flows (Ganguly *et al.*, 2012). Furthermore, this could also result in a
511   higher degree of effective crosslinking of adjacent filaments (Lu et al., 2016). However, the
512   recruitment of Kin to MTs depends on various regulatory mechanisms, including interactions with
513   cargo and MT-associated proteins like Ensconsin (Metivier *et al.*, 2019). For this reason we used
514   the truncated form Khc1-700 in our analyses. Whether, and how, the actin mesh influences the
515   interaction of full length Kin with MTs - in the presence of other MT-binding regulatory mechanisms
516   - needs to be further addressed in the future. Nevertheless, our findings indicate a novel potential
517   mechanism of how the actin and MT cytoskeletons can regulate each other.

518   Our data establish cytoplasmic streaming in st9 oocytes as a contributing factor for the correct
519   organization of the MT cytoskeleton. If - and how - a stronger posterior polarization of the MT
520   network may affect cargo delivery to the posterior is unknown. Tracking *oskar* mRNA particles in
521   *khc^{slow}* oocytes did not reveal a stronger orientation bias of cargo movement (Zimyanin *et al.*,
522   2008). However, it is unclear whether trajectories of *oskar* transport always reflect the organization
523   of the underlying MT network, and whether all MTs would be equally used by slow Kin motor/cargo
524   complexes.

525   Previous studies suggested that the pattern of nucleation and minus end anchoring along the
526   cortex are sufficient to organize the MT network and to allow correct cargo transport to define the

527 A-P axis of the animal. Consequently, mutant oocytes harboring defects in nucleation and/or
528 anchoring of minus ends also display polarity defects (Doerflinger et al., 2006; Nashchekin et al.,
529 2016). Furthermore, mathematical modeling suggested that cytoplasmic streaming at mid-
530 oogenesis is negligible to explain the correct localization of posterior cargoes like *oskar* mRNA
531 (Khuc Trong *et al.*, 2015). However, our study demonstrates that cytoplasmic streaming is involved
532 in the orientation of MT plus ends, and we conclude that the localization of the minus ends alone is
533 not sufficient to define the precise overall organization of the network. This was most obvious in
534 *khc^slow* oocytes, which in our hands lack cytoplasmic streaming entirely, and displayed an
535 increased posterior orientation MT bias (Figure 3). The advantage of analyzing slow Kin mutants is
536 that other Kin-dependent processes, like cargo transport and formation of the actin mesh, do take
537 place. Despite showing only mild defects in the distribution of developmental determinants, *khc^slow*
538 mutant oocytes fail to give rise to a healthy offspring, suggesting that oocyte polarity is affected
539 (Moua *et al.*, 2011). Consistently, we found an increased posterior bias of EB1 signals in *khc^slow*
540 mutant oocytes, strongly suggesting that the lack of cytoplasmic streaming was causative for this
541 observation.

542 In summary, the combination of various forces produced within living cells demands a complex set
543 of biochemical and biomechanical regulatory mechanisms for cytoskeletal networks to organize
544 correctly. Our results show that, in *Drosophila* st9 oocytes, advection by cytoplasmic streaming
545 contributes to the polarization of MTs by affecting the directional motion of MT plus ends. All these
546 observations further stress the need for a combination of different experimental approaches in
547 order to fully understand the dynamic organization of cytoskeletons, from simplified *in vitro*
548 systems to complex *in vivo* situations.

549 **METHODS**

550 **Fly stocks and genetics**

551 Flies were kept at standard corn meal agar and raised at room temperature (21°C). Detailed

552 genotypes of all fly stocks can be found in Table S1. Germ line clones for the analysis of *khc*

553 mutant alleles have been induced by the FLP/FRT ovoD system (Chou and Perrimon, 1996).

554 Germline clones in Figure 5 were identified by the absence of nuclear GFP in germline cells.

555

556 **Live imaging**

557 Female flies of the desired genotypes were collected and fattened on dry yeast for 12-16 h prior to

558 imaging. Ovaries were dissected in a small drop of halocarbon oil (Voltalef S10, VWR) on a glass

559 coverslip and single egg chambers were separated using fine tungsten needles. Images were

560 acquired on a Leica SP5 inverted confocal microscope, using a 40×/1.3 Oil DIC Plan-Neofluar

561 (Jup) or a 100×/1.4 Oil DIC objective (EB1). Signals were detected using a Leica HyD Hybrid

562 Detector. For MT bulk movement, a single plane from the middle of the oocyte was imaged at a

563 scan speed of 100 Hz and at an image resolution of 1,024 × 1,024 pixels (corresponding to one

564 image every 10.4 s). For EB1 imaging the oocyte was fitted and oriented within a 512 x 256 pixels

565 frame and a single plane image was taken every 0.65 s. Image sequences of at least 100 frames

566 (65 s) were taken, inspected visually, and bleach corrected using Fiji (Schindelin *et al.*, 2012). In all

567 experiments the middle of the oocyte was defined as the focal plane in which the oocyte had its

568 largest area.

569

570 **Immunostainings**

571 Egg chambers were dissected in PBS+0.1% Tween20 and fixed in 10% formaldehyde in

572 PBS+0.1% Tween20 for 10 min. Fixed ovaries were incubated with anti-αTubulin (MAB1864,

573 Sigma-Aldrich, cloneYL1/2, 1:100) or anti-Staufen (gift from. D. St Johnston, 1:3,000) in PBS+2%

574 Tween20 over night at 4°C. After four consecutive washes Alexa-coupled secondary antibodies

575 (1:100) were incubated for two hours at room temperature. Native fluorescence of GFP was

576 imaged without amplification. Images were acquired on a Leica SP5 inverted confocal microscope,

577 using a 40×/1.3 Oil DIC Plan-Neofluar objective. For phalloidin staining, egg chambers where

578 dissected and fixed in 10% methanol free formaldehyde in PBS, containing 0.1% Tween-20

579 (PBT0.1), for a maximum of 10 min. Fixed cells were washed 4x in PBT0.1 and incubated with

580 1 μM TRITC-coupled phalloidin (in PBT0.1, Sigma-Aldrich) over night at 4 °C. The stained

581 samples were washed 4x in PBT0.1, mounted, and imaged imediatly under identical conditions,

582 using a Zeiss LSM800 equipped with a 40×/1.3 Oil DIC Plan-Neofluar objective. Normalized

583 fluorescence intensities were acquired using FiJi.

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587 **Image denoising and optical flow based motion estimation**

588 Motion analysis of the recorded two-dimensional image sequences was performed using a two-

589 step procedure. The first step aimed to remove noise contamination from the unprocessed

590 sequences, while the goal of the second step was to estimate motion in terms of displacement

591 vector fields from the so-improved sequences. As noise may significantly disturb the motion

592 estimation (Ruhnau et al., 2005; Burger *et al.*, 2018), this two-step procedure is crucial for

593 obtaining robust estimation results. In the first step, we recovered from each recorded image

594 sequence u^δ an improved version u by solving a variational image denoising problem with spatio-

595 temporal regularization. It reads

$$u = \text{argmin}_u \frac{1}{2} \left\| u - u^\delta \right\|^2 + \alpha_1 \| \nabla u \|_{2,1} + \frac{\beta_1}{2} \| \partial_t u \|^2.$$

596 While the first two terms on the right-hand side resemble standard image denoising with total

597 variation regularization (Rudin L.I, 1992) in space, the third term connects subsequent frames by

598 penalising temporal changes within the recovered solution. As EB1 comets typically appear in

599 several subsequent frames at similar positions, it allows to effectively remove randomly distributed

600 noise from a sequence (see Figure 2D,E, Movie S2, and SI). Here, $\alpha, \beta > 0$ are regularization

601 parameters that balance the three terms and need to be chosen appropriately. Moreover, the

602 norms (as defined in equation S21) are taken over the entire image sequence.

603 The result u served as input to the motion estimation step, in which we estimated a displacement

604 vector field $\vec{v} = (v_1, v_2)^T$ by solving

$$\vec{v} = \text{argmin}_{\vec{v}} \frac{1}{2} \| \partial_t u + \langle \nabla u, \vec{v} \rangle \|^2 + \alpha_2 \| \nabla \vec{v} \|_{2,1} + \frac{\beta_2}{2} \| \partial_t \vec{v} \|_{2,2}^2.$$

605 Here, $\vec{v} = \vec{v}(x)$ is defined for every pixel x of the image u and is an estimate of the motion that u

606 experiences at x from one image frame to the subsequent one in time. That is, the vector $\vec{v}(x) =$

607 $(v_1(x), v_2(x))^T$ points in the direction of motion at x, and $|\vec{v}(x)|$ is an estimate of the speed of the

608 motion at x. The first term on the right-hand side in the above equation aims to approximately

609 solve the optical flow equation (Horn and Schunck, 1981), while the second and third terms

610 incorporate spatio-temporal regularization of the vector-valued unknown (for further details see SI).

611 The use of the vector-valued total variation allows for spatial discontinuities in the displacement

612 vector field. We found that in both steps the temporal regularization was key and the analysis of

613 individual frames did not yield satisfactory results. Both minimisation problems were approximately

614 solved using the primal-dual hybrid gradient method (Chambolle, 2011) and graphics-processing

615 unit (GPU) acceleration. An in-depth description of both models, their numerical solution, and

616 parameter choices as well as implementation details can be found in the SI.

617

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AUTHOR CONTRIBUTIONS

MD designed and performed research, analyzed data, and wrote the paper. IMP designed research, analyzed data, and wrote the paper. LAK performed research and analyzed data. LFL and CBS developed new analytic tools, analyzed the data, and wrote the paper. HD and MB contributed analytic tools.

COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

The source code of our implementation and of the data analysis is available online (https://doi.org/10.5281/zenodo.2573254). All relevant data and the computational results are available from the corresponding authors upon request.

842 **FIGURE LEGENDS**

843 **Figure 1 – MT orientation is aberrant in oocytes with faster flows.**

844 **A)** Schematic representation of MT orientation. EB1 specifically associates with the growing end
845 (plus-end) of MTs and therefore serves as read-out of their spatial orientation. The orientation of
846 MT growth is represented as an angle, deviating from an imaginary anterior (180°) to posterior axis
847 (0°). **B)** Simplified scheme, illustrating the underlying principle of OF-based motion estimation.
848 Shown are two consecutive frames of a 5 x 5 pixel wide image sequence that contains a
849 rectangular object of different pixel intensities - from light green (little signal) to dark green
850 (maximum signal). OF assumes that the intensities of a signal do not change along its trajectory.
851 Based on this assumption, variational OF allows to estimate a displacement vector for each pixel
852 (the yellow arrow shows the displacement vector of the centre pixel of the object). **C)** Definition of
853 growth directions based on OF-estimated velocities. Angles of velocities between 90°-270° are
854 regarded as anterior (A, blue hemicircle), the complementary set of angles as posterior (P, orange
855 hemicircle). The sub-population of all posterior signals (orange) that fall between 330°-30° (light
856 orange sector) are considered to grow towards the 'posterior tip'. **D)** Single frame of an
857 unprocessed image sequence (raw data) showing an oocyte expressing EB1. The magnified area
858 is indicated by a dashed box. **E)** Same frame as shown in (D), after applying the denoising step
859 (Movie S2). Scale bars are 10 μm. **F)** Shown is the OF (displacement vector) field between two
860 frames of the image sequence in D. **G)** Average OF (over the entire sequence). **H)** Hand-drawn
861 segmentation mask of the oocyte. For the analysis, only the displacement vectors within this
862 segmentation were considered. **I)** Staufen (green) localization in control oocytes (arrow). The
863 protein localizes in a tight posterior crescent by the end of st9. **J)** Living control oocyte expressing
864 the MT-binding protein Jup (Movie S5). **J')** Kymograph/space-time plot (along the indicated dotted
865 line in C), showing dynamic bending of MTs over time. **K and M)** Staufen fails to localize in *capu* or
866 *spire* mutants and distributes throughout the cytoplasm of the cell. **L and N)** Living *capu* (L) or
867 *spire* (N) mutant oocytes, expressing Jup. Fast cytoplasmic flows lead to the formation of dense
868 and long MT bundles (red arrows), as well as increased MT bending (Movie S5). **L' and N')**
869 Kymograph (along dashed line in L and N) indicate a stronger displacement of MT bundles over
870 time in *capu* (L') or *spire* (N') mutants. **O)** Standard deviation (temporal) projection of EB1 comets
871 in a control image sequence (in total 65 s). **P)** Rose diagram (angular histogram) with 50 bins
872 depicting the distribution of EB1 directions in individual control cells within the corresponding
873 segmented oocyte. Each colour represents the angular histogram of the directions from one
874 oocyte. **Q)** Same data as shown in (P) with angular histograms averaged over all cells (n=8). Error
875 bars (in black) indicate the standard deviation for each bin (in blue). **R)** Mean angular direction θ_{avg}
876 of the histogram shown in (Q) (also indicated by a red line in (Q)) and the length r (between 0 and
877 1) of the mean resultant vector (length of black line in (Q) originating from the centre), which
878 relates to the circular variance $S = 1 - r$ of the distribution shown in (Q). Anterior-posterior bias of
879 all EB1 growth directions. **S)** Rose diagram similar to (P) for control cells but with growth directions

24

880   binned into four bins (30°-90°, 90°-270°, 270°-330°, and 330°-30°). Moreover, the fraction of

881   posterior-growing EB1 comets pointing towards the 'posterior tip' (330°-30°) is indicated. **T)**

882   Standard deviation (temporal) projection of EB1 comets in an image sequence of a *capu* mutant

883   oocyte (in total 65 s). **F-I)** Quantified EB1 directions in *capu* cells, following the same experimental

884   pipeline described for the control cell.

885

886   **Figure 2 – The cytoplasmic actin mesh counteracts the recruitment of Kinesin to MTs**

887   **A,B)** Living control (*capu*^{EY12344}/*CyO* in A) and *capu* mutant oocyte (*capu*^{EY12344}/*capu*^{EY12344} in B),

888   expressing Khc::GFP. While the fusion protein mainly localizes posteriorly in control cells (A), it

889   strongly decorates MTs in *capu* mutant cells (B and Movie S10). **C-F)** Fixed control

890   (*capu*^{EY12344}/*CyO*) oocytes (C,D) and *capu* mutant cells (*capu*^{EY12344}/*capu*^{EY12344})(E,F), expressing

891   Khc::GFP. Cells were stained against αTubulin (magenta) and the Kin1-700-GFP fusion protein

892   (green). In controls, Tubulin and Khc::GFP localize to opposed gradients and show little overlap

893   (C,D). Conversely, in *capu* mutants, Khc::GFP strongly co-localizes with Tubulin (red arrows in

894   E,F). Insets in D and F show merged channels. **G-L)** Higher resolution images of fixed control

895   (*capu*^{EY12344}/*CyO*) (G-I) and *capu* mutant (J-L) cells, stained against αTubulin (magenta) and GFP

896   (green). While Khc::GFP localizes diffusely around MTs in control cells (G-I), it strongly co-

897   localizes to MTs in *capu* mutants (red arrows in J-L). **M-P)** The cytoplasmic actin mesh in control

898   (M, *capu*^{EY12344}/*CyO*) and transheterozygous *capu*^{EY12344}/*capu*^{L219} (N), *capu*^{EY12344}/*capu*^{L201} (O)*,* and

899   *capu*^{EY12344}/*capu*^{38} (P). **Q)** Quantification of signal intesities of the actin mesh as shown in (M-P). All

900   cells harbor the strong hypomorphic allele *capu*^{EY12344} over either CyO (n=15), *capu*^{L219} (n=21),

901   *capu*^{L201} (n=20) or *capu*^{38} (n=21). **R-T)** Khc::GFP expressed in living *capu*^{EY12344}/*capu*^{L219} (R),

902   *capu*^{EY12344}/*capu*^{L201} (S)*,* and *capu*^{EY12344}/*capu*^{38} (T) oocytes. Red arrows indicate Khc::GFP positive

903   MT bundles.

904

905   **Figure 3 – Kin activity impacts on the spatial orientation of MTs in the oocyte.**

906   **A-C)** Still frames and kymographs of live oocytes expressing Jup. Cells harbor a null mutation

907   (*khc*^{null} *(27)*), or single point mutations in the motor domain (*khc*^{slow} *(23)* and *khc*^{slow} *(17))*, rendering

908   the motor slower. Compared to controls, all *khc* mutant cells exhibit no cytoplasmic flows and thus

909   no MT bulk motion (Movie S6). **D-F)** Posterior cargo localization in different *khc* mutant alleles.

910   Staufen (green) is not transported to the posterior in cells lacking Kin (*khc*^{null} *(27)*) and is found in

911   the anterior corners of the cell (arrows). In contrast, in both of the slow Kin alleles (*khc*^{slow} *(23)* and

912   *khc*^{slow} *(17)*) a considerable amount of Staufen becomes transported to the posterior. However,

913   compared to controls (Figure 2A) Staufen is not only localized in a posterior crescent, but also in

914   dots within the posterior cytoplasm (arrows). **G-P)** OF analysis of EB1 growth directionality in

915   *khc*^{sllow} (G-K, n=10) and *khc*^{null} (L-P, n=10) oocytes, following the same experimental pipeline

916   described in Figure1.

917

918

919 **Figure 4 – Anterior and posterior regional impact of Kin activity on MT orientation.**

920 **A)** Single frame of an oocyte expressing EB1. Dashed lines represent the anterior and posterior

921 regions of the oocyte used to extract orientation data. **B)** Global EB1 signal directions within the

922 entire oocyte (as shown in Figure 1 and 3). **C-H)** In each case, from left to right: distribution of EB1

923 growth orientation visualized in a rose diagram, the anterior-posterior orientation, and the 'posterior

924 tip' orientation. **C,D)** Data for control cells (n=8). **E,F)** Data for khc^{slow} mutant cells (n=10). **G,H)**

925 Data for khc^{null} mutant cells (n=10).

926

927 **Figure 5 – Cytoplasmic streaming constitutes a major contributor to MT orientation.**

928 **A,B)** Posterior cargo localization in $capu,khc^{slow}$/+ (A) and $capu,khc^{slow}$ double (B) mutant cells.

929 While Staufen (green) partially localizes to the posterior of the cell in $capu,khc^{slow}$/+ oocytes (right

930 arrow in A), the majority of the protein localizes in cytoplasmic clouds (left arrow in A). In

931 $capu,khc^{slow}$ double mutant cells, Staufen localizes in a posterior crescent but also accumulates in

932 dots close to the posterior (arrow in B). **C,D)** Still frames and kymographs of live $capu,khc^{slow}$/+

933 (C,C') or $capu,khc^{slow}$ double (D,D') mutant oocytes, expressing Jup. **E-N)** OF analysis of EB1

934 growth directionality in $capu,khc^{slow}$/+ (E-I, n=10) and $capu,khc^{slow}$ (J-N, n=8) oocytes.

TABLES

Table 1 – Summary of EB1 orientation data, extracted from confocal time series by variational OF analysis.

genotype	n	global EB1::GFP					EB1::GFP in anterior oocyte			EB1::GFP in posterior oocyte		
		% ant.	% post.	% post. tip	θ_{avg}	r	% ant.	% post.	% post. tip	% ant.	% post.	% post. tip
control	8	34 ± 6	66 ± 6	40 ± 4	353.08±0.05	0.260	38 ± 5	62 ± 5	40 ± 5	26 ± 11	74 ± 11	41 ± 8
capu	10	39 ± 14	61 ± 14	25 ± 8	318.92±0.06	0.182						
khc^{null}	10	38 ± 6	62 ± 6	41 ± 7	357.35±0.07	0.202	44 ± 4	56 ± 4	36 ± 3	28 ± 13	72 ± 13	46 ± 13
khc^{slow}	10	25 ± 10	75 ± 10	54 ± 15	1.62±0.02	0.412	26 ± 11	74 ± 11	52 ± 14	19 ± 10	81 ± 10	57 ± 17
capu,khc^{slow}\+	10	39 ± 13	61 ± 13	34 ± 5	335.08±0.07	0.167						
capu,khc^{slow}	8	34 ± 15	66 ± 15	39 ± 5	19.67±0.05	0.285						
Tracking data from Parton et al. 2011												
control		42	58				46	54		37	63	
par-1		49	51									

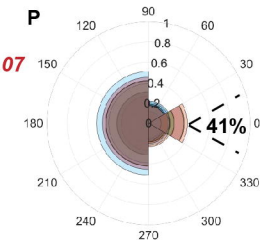- similar to control
- stronger posterior bias
- weaker posterior bias

Anterior-posterior orientation bias of microtubule growth in per cent (± 95% confidence interval). *n* represents the number of cells analyzed. θ_{avg} gives the average angle of all EB1 orientations of all cells of a given genotype. *r* constitutes a measure of variance and lies between 0 and 1. The larger *r*, the less variable the data set.

27

A plus tip growth

MT — angle of growth — t_1 t_2 30° 0°

● - EB1 comet

B 5 px / 5 px / time

C 90° / 30° / posterior tip / 330° / 270° / A / P

D raw images — EB1::GFP

E denoised images — EB1::GFP

F one image pair

G time average

H time average — oocyte

I control — Staufen DNA

J Jupiter::GFP

J' time

K *capu^EY12344* — Staufen DNA

L Jupiter::GFP

L' time

M *spire^1/Df* — Staufen DNA

N Jupiter::GFP

N' time

O control — EB1::GFP
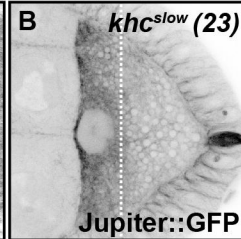
P

Q

R $\theta_{avg} = 353.08° \pm 0.05$ / $r = 0.260$ / A 34% | P 66%

S < 40%

T *capu^EY12344* — EB1::GFP

U

V

W $\theta_{avg} = 318.92° \pm 0.06$ / $r = 0.182$ / A 39% | P 61%

X < 25%

A khc^null (27) **A'** Jupiter::GFP time →
B khc^slow (23) **B'** Jupiter::GFP time →
C khc^slow (17) **C'** Jupiter::GFP time →
D khc^null (27) Staufen DNA
E khc^slow (17) Staufen DNA
F khc^slow (23) Staufen DNA

G khc^slow EB1::GFP

H
I
J θ_avg = 1.62° ± 0.02 r = 0.412 A 25% | P 75%
K < 54%

L khc^null EB1::GFP
M
N
O θ_avg = 357.35° ± 0.07 r = 0.202 A 38% | P 62%
P < 41%

anterior region of the cell

posterior region of the cell

A *capu,khc^{slow}/+* — Staufen DNA

B *capu,khc^{slow}* — Staufen DNA

C *capu,khc^{slow}/+* — Jupiter::GFP

C' time

D *capu,khc^{slow}* — Jupiter::GFP

D' time

E *capu,khc^{slow}/+* — EB1::GFP

F

G

H $\theta_{avg} = 335.08° \pm 0.07$
$r = 0.167$

A 39% | P 61%

I <34%

J *capu,khc^{slow}* — EB1::GFP

K

L

M $\theta_{avg} = 19.67° \pm 0.05$
$r = 0.285$

A 34% | P 66%

N <39%