

1 Volumetric morphometry reveals mitotic spindle width as the best predictor of spindle 2 scaling

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10

11 Abstract

12 The function of cellular structures at the mesoscale is dependent on their geometry and
13 proportionality to cell size. The mitotic spindle is a good example why length and shape
14 of intracellular organelles matter. Spindle length determines the distance over which
15 chromosomes will segregate and spindle shape ensures bipolarity. While we still lack a
16 systematic and quantitative understanding of subcellular morphometrics, new imaging
17 techniques and volumetric data analysis promise novel insights into scaling relations
18 across different species. Here, we introduce Spindle3D, an open-source plug-in that
19 allows for the quantitative, unbiased, and automated analysis of 3D fluorescent data of
20 spindles and chromatin. We systematically analyse different cell types, including somatic
21 cells, stem cells and one-cell embryos across different phyla to derive volumetric relations
22 of spindle, chromatin, and cell volume. Taken together, our data indicate that mitotic
23 spindle width is a robust indicator of spindle volume, which correlates linearly with
24 chromatin and cell volume both within single cell types and across metazoan phyla.

25

26 Introduction

27 Size and shape in general are important biological features. Classically, morphometrics
28 have been studied on the level of organisms, tissues, and cells. Now, the continuous
29 improvement of imaging techniques and data analysis allows for the accurate
30 measurement of organelle geometry at the μm -scale and thus enables the development
31 of quantitative scaling laws at the mesoscale.

32

33 Anecdotal evidence suggested spindle length to scale with cell size. More recent studies,
34 however, show that the nature of spindle scaling and size control is more complex. In
35 different cells, spindle length spans over an order of magnitude and various molecular
36 scaling mechanisms are likely to contribute to different degrees to cover the entire length
37 regime (Rieckhoff et al., 2020). For example, the length of mitotic spindles increases with
38 cell length in small cells, but in very large cells spindle length approaches an upper limit
39 (Wuhr et al., 2008; Lacroix et al., 2018; Rieckhoff et al., 2020). More precisely, spindle
40 length scales linearly with cytoplasmic volume (Hazel et al., 2013; Good et al., 2013).
41 Further, spindle size needs to be coordinated with chromosome length, a fact that is
42 established (Mora-Bermúdez et al., 2007; Lipp et al., 2007; Dinarina et al., 2009;
43 Kieserman and Heald, 2011) but poorly understood. All the above observations point to
44 an important, in some cases still open, question: What are the relevant morphometric
45 measures to precisely formulate spindle scaling phenomena?

46

47 While most experimental studies still measure spindle length and cell diameter or collapse
48 them into area information, theoretical arguments regularly use volumetric data (Good et
49 al., 2013; Reber et al., 2013; Rieckhoff et al., 2020). However, most tools available for
50 analysing spindle size and geometry only allow for 2D analysis (Crowder et al., 2015;
51 Grenfell et al., 2016). Here, we show that spindle parameters differ significantly when
52 measured in 2D as compared to 3D, they are thus error-prone and might lead to incorrect
53 mechanistic conclusions. We argue that quantitative measurements from 3D datasets
54 are essential to allow for accurate mechanistic interpretation and to derive conceptual
55 scaling laws. Therefore, we use quantitative microscopy together with a newly developed
56 analysis tool “Spindle3D” and the segmentation software Ilastik (Berg et al., 2019) to
57 derive quantitative 3D morphometry data on spindle, chromatin, and cell volume. Our
58 data imply that mitotic spindle width is a more robust predictor of spindle scaling than
59 spindle length. Spindle3D is available free and open source as plug-in
60 (<https://sites.imagej.net/Spindle3D>) in Fiji (Schindelin et al., 2012) and allows for the
61 quantitative, unbiased, and automated analysis of 3D fluorescent data of spindles and
62 chromatin. Our analysis of a variety of cell types and phyla proves the robustness of the
63 Spindle3D plug-in, which thus can be broadly used by researchers to inform future

64 biological and physical concepts of spindle scaling and size control across many
65 experimental systems.

66

67 Results

68 3D-analysis of fluorescent spindle and chromatin data allows for the accurate extraction 69 of quantitative morphometric parameters

70 To extract quantitative parameters of spindle and chromatin morphometries, our plug-in
71 requires a two-channel Z-series with fluorescent tubulin and chromatin labelings (Figure
72 1A and S1) as a minimum input, which allows for object detection and spindle axis
73 localization (Material and Methods). Classically, spindle length is defined as the pole-to-
74 pole distance. Consistently, we first define the spindle axis, along which spindle length is
75 specified as the distance between the two spindle poles (Figure 1B). Next, spindle width
76 is measured orthogonally to the spindle axis and reflects the diameter of the spindle
77 ellipsoid at its equator. The volumes of all voxels within the segmented spindle mask add
78 up to yield a spindle's volume. As the orientation of the mitotic spindle can determine
79 both, the relative size and the position of the daughter cells (as reviewed in McNally,
80 2013), we provide a measure for the spindle angle, which describes the tilt of the spindle
81 axis relative to the reference plane. Chromatin can induce spindle assembly (Heald et al.,
82 1996) and influences the spindle's geometry (Dinarina et al., 2009). Hence, we use the
83 intensity profile of the chromatin to measure the metaphase plate, which expands
84 orthogonally to the determined spindle axis (Figure S1A). We define the metaphase plate
85 width as the extent of chromatin along its shortest axis and the metaphase plate length
86 as the mean chromatin diameter. Again, the volumes of all voxels within the segmented
87 chromatin mask add up to yield chromatin volume. In some cell types, we consistently
88 find the chromosomes aligned with a central opening in the metaphase plate, a
89 phenomenon we termed chromatin dilation, which is measured using a radial intensity
90 profile (Figure 1B and S1C).

91

92 By projecting our 3D microscopic images into 2D planes (Figure 1C), we identified and
93 quantified potential sources of error when spindle parameters are only analysed in two
94 dimensions. Collapsing 3D into 2D information resulted in the distortion of morphometric
95 parameters. The error is particularly evident when spindles are not perfectly parallel to

96 the substrate but tilted in Z (Figure 1D). As a consequence, axial extents such as spindle
97 length and metaphase plate width will be under- or overestimated, respectively. Even
98 moderate spindle angles of 25° produce measurement errors of approximately 12.5%
99 (Figure 1D). Furthermore, because spindle morphologies are not always perfectly
100 symmetrical, information on spindle width and metaphase plate length are lost in 2D
101 projections. Taken together, we recommend the acquisition and analysis of 3D datasets,
102 which is essential to derive accurate quantitative measurements, in particular if they shall
103 inform mathematical models.

104

105 [Spindle3D robustly derives morphometric parameters across a variety of cell types and](#) 106 [phyla](#)

107 To allow for an automated and quantitative analysis of spindle and chromatin parameters,
108 we developed a 3D morphometric analysis workflow (Figure S1). In order to demonstrate
109 its applicability and robustness, we subjected confocal images of live metaphase spindles
110 from wild-type HEK293 cells (*Homo sapiens*), a HeLa Kyoto cell line (*H. sapiens*), Ptk₂
111 cells (*Potorous tridactylis*), bovine zygotes (*Bos taurus*), and murine embryonic stem cells
112 (mESCs, *Mus musculus*) to our 3D morphometric analysis (Figure 2A). The workflow
113 produced segmented outputs with spindle axis-aligned voxel coordinates (Figure 2B).
114 Analysed spindle lengths ranged from 6.8 - 26.5 μm (Figure 2C). Together with mESCs,
115 the two human cell lines (HEK293 and HeLa Kyoto) showed spindles of comparable
116 lengths ($11.5 \pm 1.2 \mu\text{m}$ (mean \pm standard deviation), $11.5 \pm 1.4 \mu\text{m}$, $13.6 \pm 2.0 \mu\text{m}$,
117 respectively), contrasted by the considerably longer spindles of the bovine zygotes and
118 Ptk₂ cells with an average length of $16.9 \pm 2.9 \mu\text{m}$ and $16.4 \pm 2.9 \mu\text{m}$, respectively.
119 However, together with mESCs, Ptk₂ cells displayed narrower spindles (Figure 2D),
120 producing markedly increased spindle aspect ratios (Figure 2E), a shape descriptor
121 defined as the ratio of spindle length and width. We found that small aspect ratios often
122 coincided with flat spindle poles, while spindles with large aspect ratios had visibly
123 pointed poles. Of the data sets tested, bovine zygotes harboured spindles with the
124 largest volumes (Figure 2F) of $2069.4 \pm 661.1 \mu\text{m}^3$ (mean \pm standard deviation), consistent
125 with spindles reaching an upper limit in early development (Wühr et al., 2008). In contrast,
126 spindles in murine embryonic stem cells occupied only a fraction of this volume
127 ($408.8 \pm 101.6 \mu\text{m}^3$). Independent of cell type, the majority of spindles showed tilted

128 angles (Figure 2G) that fall within a range of 0 - 77°, highlighting the importance of 3D
129 analysis. Intriguingly, the volume and length of the metaphase plate (Figure 2H-I), did
130 neither reflect cell-type specific genome sizes nor chromosome numbers (Figure S3A-D),
131 hinting towards different levels of chromatin compaction (as reviewed in Levy and Heald,
132 2012). In addition, our analysis quantified various levels of chromatin dilation. Especially
133 in mESCs spindles, but also in a large fraction of the HeLa Kyoto population, chromatin
134 plates were frequently and considerably dilated (chromatin dilation > 0.5) (Figure 2J).
135 Taken together, the 3D analysis workflow provided by our plug-in robustly revealed cell-
136 type specific spindle and chromatin morphology across different cell lines.
137

138 [Fixation and sample preparation alter spindle and chromatin morphology](#)

139 In our explorations, we frequently observed a discrepancy in spindle sizes of live cells
140 compared to cells that were fixed and mounted on cover slides. To systematically test
141 the influence of fixation and mounting, we used HeLa Kyoto cells and mESCs stably
142 expressing tubulin-GFP, allowing us to directly benchmark the mounted cells to their live
143 counterparts (Figure 3A). Additionally, we measured cells that were chemically fixed, but
144 not mounted. Using our plug-in, morphometric analysis revealed a marked decrease in
145 spindle volumes in cells that were fixed and mounted in mounting media (Figure 3B). In
146 addition, we frequently observed deformed spindle shapes in these samples (Figure S3)
147 with shifted aspect ratios (Figure 3C). When samples were fixed but not mounted, spindle
148 volumes were comparable to live spindles (Figure 3B). In these samples, as well as in the
149 fixed + mounted samples, we observed a distorted volumetric relationship between
150 spindle and chromatin (Figure 3D). Taken together, we show that sample preparation
151 may induce artefacts in spindle and chromatin morphology and should be considered
152 with great care. Importantly, as the introduced errors are not isotropic, such analyses
153 may distort geometrical relationships and thus lead to error-prone scaling relations.
154 Therefore, we recommend using live cells where possible, and for assays with fixed
155 samples (e.g. immunofluorescence), we suggest refraining from mounting samples.
156

157 [Spindle width, rather than spindle length, reflects spindle volume](#)

158 Several lines of evidence imply that there is a correlation between chromatin dimensions,
159 spindle geometry, and steady-state microtubule polymer mass (as reviewed in Levy and

160 Heald, 2012). From our analyses, we can now establish such simple scaling relations.
161 We first explored the relationships between the length of the spindle and its volume
162 (Figure 4A) and between the width of the spindle and its volume (Figure 4B). We found
163 that, in both cases, the relationship can be described in terms of simple power laws.
164 However, when calculating the spearman's correlation coefficients r_s , we found spindle
165 width to correlate more strongly with spindle volume ($r_s = 0.91$, $p = 2 \times 10^{-160}$, Figure 4B)
166 than we observed for the relationship between spindle length and volume ($r_s = 0.77$, $p =$
167 2×10^{-83} , Figure 4A). This also held true when looking at the individual cell types (Figure
168 4A-B). As this was unexpected, we first verified manually that spindle length and width
169 were represented reliably by our automated measurements (Figure S4A-D). Because the
170 width of the spindle is in fact a three-dimensional parameter, we can measure spindle
171 width as the average extent of the spindle mask projected along the spindle axis (Figure
172 4B) or as the maximum or minimum spindle width (Figure S4E-G). In either case, spindle
173 width yielded strong correlations with spindle volume (Figure S4E-G). Chromatin has
174 been shown to affect both spindle length and shape (Dinarina et al., 2009; Hara and
175 Kimura, 2013). We thus plotted spindle volume as a function of chromatin volume (Figure
176 4C), which we find to correlate linearly ($r_s = 0.72$, $p = 3 \times 10^{-69}$). This is surprising because
177 in embryonic systems, varying chromatin content only had a weak effect on spindle size
178 while varying chromatin geometry influenced spindle assembly more drastically (Brown
179 et al., 2007; Wühr et al., 2008; Dinarina et al., 2009). We therefore plotted spindle length
180 and width against chromatin volume, and again found spindle width to correlate more
181 strongly ($r_s = 0.54$, $p = 2 \times 10^{-33}$ for spindle length, Figure 4D and $r_s = 0.73$, $p = 4 \times 10^{-72}$
182 for spindle width, Figure 4E) with chromatin volume. Previous data implied that symmetric
183 and thus functional spindles only self-organize around specific chromatin dimensions
184 (Dinarina et al., 2009). Indeed, we find an almost perfect linear relation between spindle
185 width and the length of the metaphase plate ($r_s = 0.87$, $p = 1 \times 10^{-128}$, Figure 4F) while
186 spindle length and metaphase plate length only showed moderate dependencies ($r_s =$
187 0.44 , $p = 9 \times 10^{-22}$). Taken together, we show that - contrary to common expectation -
188 steady-state spindle width, rather than spindle length, is a reliable predictor of overall
189 spindle volume.

190

191 [Spindle volume and chromatin volume scale linearly with cell volume](#)

192 In many systems, cell or cytoplasmic volume is a major determinant of spindle size (Good
193 et al., 2013; Hazel et al., 2013; Farhadifar et al., 2015; Wang et al., 2016; Lacroix et al.,
194 2018; Rieckhoff et al., 2020). So, to reliably measure cell volume, we took advantage of
195 the fact that mitotic cells expressing fluorescently-tagged tubulin show, next to the
196 prominent spindle signal, distinctive fluorescence of soluble tubulin throughout the
197 cytoplasm (Figure 5A). We thus used pixel classification-based 3D segmentation (Berg
198 et al., 2019) of mitotic cells expressing fluorescent tubulin as a read-out of cell volume
199 and cell sphericity to complement the morphometric data generated by our plug-in.
200 Based on this, we trained random forest classifiers to distinguish and predict mitotic cell
201 volumes (Figure 5B) and verified that this approach was as accurate as manual volumetric
202 segmentations guided by cell-membrane labellings (Figure S5A-C). We live-imaged and
203 analysed HeLa Kyoto, Ptk₂ and mESCs expressing fluorescently-tagged tubulin. With an
204 average cell volume of $\approx 6100 \mu\text{m}^3$ (HeLa Kyoto, Figure 5C), $7450 \mu\text{m}^3$ (Ptk₂) and 2850
205 μm^3 (mESCs), all cell types are expected to fall into the linear scaling regime (cell volume,
206 $V_c < 10^6 \mu\text{m}^3$ (Rieckhoff et al., 2020), cell diameter, $d_c < 140 \mu\text{m}$, (Crowder et al., 2015)).
207 In contrast to the other two cell lines, Ptk₂ cells did not round up during mitosis (Figure
208 5D). Nevertheless, all three cell types displayed volumetric scaling between the cell and
209 the respective spindle ($r_s = 0.86$, $p = 3 \times 10^{-57}$, Figure 5E). Interestingly, cells exerted a
210 cell type-specific spindle size specification: While spindle volumes in both HeLa cells and
211 mESCs occupied approximately 14% of total cell volumes, it was only 10% in Ptk₂ cells
212 (Figure 5F).

213
214 So far, we measured spindle volume as the total volume of all voxels within the
215 segmented spindle mask. Spindle volume, however, might considerably vary from
216 spindle mass (defined as the total steady-state microtubule polymer mass; Reber et al.,
217 2013) depending on microtubule density and spindle architecture. We therefore
218 quantified steady-state spindle mass using Spindle3D in conjunction with the cell volume
219 masks (Figure 5G). For all three cell types examined, spindle mass was directly
220 proportional to spindle volume ($r_s = 0.94$, $p = 6 \times 10^{-88}$, Figure S6A) and displayed a
221 comparable scaling relation with cell volume ($r_s = 0.93$, $p = 2 \times 10^{-84}$, Figure 5H).
222 Recurrently, spindle width, rather than spindle length, tightly correlated with spindle mass
223 (Figure S6B-C). When evaluating dimensional scaling, spindle length displayed a

224 moderate association with cell volume ($r_s = 0.75$, $p = 6 \times 10^{-35}$, Figure 5I), with notably
225 poorer correlation in mESCs ($r_s = 0.18$, $p = 0.16$), while spindle width robustly scaled with
226 cell volume ($r_s = 0.80$, $p = 2 \times 10^{-44}$, Figure 5J) in all three cell lines. Further, we found
227 chromatin volume to linearly scale with cell volume ($r_s = 0.88$, $p = 1 \times 10^{-62}$, Figure 5K).
228 Our current understanding of mitotic chromosome scaling with cell volume is limited to
229 so far a single candidate mechanism, i.e. chromatin packing density (as reviewed in Heald
230 and Gibeaux 2018). While studies in *Xenopus* and *C. elegans* show that mitotic
231 chromosome size decreases throughout embryogenesis (Hara et al. 2013; Ladouceur et
232 al. 2015; Kieserman and Heald 2011), systematic and quantitative data from somatic
233 cells are missing. Taken together, our 3D spindle morphometry revealed that spindle
234 volume and mass scale linearly with chromatin volume (but not chromosome number or
235 genome size) and cell volume. Intriguingly, in terms of the spindle's spatial dimensions, it
236 was not spindle length but rather spindle width that revealed a robust correlation with
237 chromatin and cell volume. Future work shall build on our size scaling analyses to
238 decipher the molecular mechanisms that drive spindle scaling and size control in different
239 species and during development.

240

241 Discussion

242 So far, early embryonic development, in particular of frogs, fish and worms (Wuhr et al.,
243 2008; Hara and Kimura, 2009; Wilbur and Heald, 2013), has provided experimental
244 models to study spindle scaling and size control. One advantage of early embryonic
245 development is the rapid and dramatic decrease in cell volume over several orders of
246 magnitude. In contrast, somatic cells only show a small variation in cell volume for a given
247 cell type, which makes it harder to discover potential scaling regimes (Marshall 2020).
248 Here, we use volumetric fluorescent microscopy data from somatic cells, stem cells as
249 well as one-cell embryos or zygotes, cells that round up during mitosis or are naturally
250 flat to systematically study scaling relations of spindle, chromatin, and cell geometry. We
251 thereby confirm data from early embryogenesis that within a similar cell size range ($V_c <$
252 $10^6 \mu\text{m}^3$, $d_c < 140 \mu\text{m}$) spindle volume and spindle mass scale linearly with cell volume.
253 However, whereas it is commonly assumed that spindle length must be tightly tailored to
254 the cell's dimensions to safeguard fidelity of chromosome segregation and cytokinesis
255 (Goshima and Scholey, 2010), we observed that spindle length was loosely correlated

256 with spindle, chromatin, and cell geometry. Instead, we found the width of the spindle to
257 be a robust predictor of both spindle volume and mass, and concerted with cell and
258 chromatin dimensions.

259

260 While it is undebatable that chromatin is sufficient to induce spindle assembly (Heald et
261 al 1996), it remains unclear how its volume, its surface area, its dimensions or a
262 combination of all these factors influence spindle assembly and geometry. In embryonic
263 systems, chromatin content has been shown to only have a minor effect on spindle size.
264 Therefore, it has been suggested that chromatin surface rather than chromatin volume
265 or mass influences spindle size (Brown et al., 2007; Wühr et al. 2008; Dinarina et al.,
266 2009). This is because chromatin triggers spindle self-organization via a diffusion-limited
267 RanGTP gradient, which promotes microtubule nucleation and growth (Gruss et al.,
268 2001). The spatial regulation of microtubule nucleation has recently been shown to
269 determine the upper limit of spindle length (Decker et al., 2018) and particularly in large
270 cells, spindle scaling has been suggested to be governed by microtubule nucleation
271 (Rieckhoff et al., 2020). Chromosomal nucleation, however, might be more relevant in
272 early embryonic systems than in somatic cells (Bird et al., 2008). Furthermore, a
273 combination of modeling and perturbation studies has shown that spindle length is
274 insensitive to the length scale of the Ran gradient in human tissue culture cells (Oh et al.
275 2016). Thus, how chromatin and the Ran gradient influence microtubule nucleation and
276 dynamics in different scaling regimes remains an exciting open question for future
277 research.

278

279 [Material and Methods](#)

280

281 [Antibodies](#)

282 Anti- γ -tubulin (mouse, Sigma T6557), anti-mouse AlexaFluor568 (rabbit, Thermo, A-
283 11061).

284

285 [Plasmids](#)

286 pEGFP-C1-mCherry-CaaX (gift from Paul Markus Müller, Berlin)

287

288 Cell lines

289 HeLa Kyoto and R1/E mESCs (gifts from Hyman lab, Dresden) and HEK293 (gift from
290 Beckman lab, Berlin), Ptk₂ cell lines (gift from Simons lab, Dresden).

291

292 Mammalian tissue culture

293 R1/E mouse embryonic stem cells (mESCs) were cultured in DMEM (high glucose,
294 pyruvate, Gibco) supplemented with 16 % FBS (Gibco), antibiotic-antimycotic
295 (Invitrogen), non-essential amino acids (Gibco), beta-mercaptoethanol (Gibco) and
296 recombinant mouse leukemia inhibitory factor (mLIF, ESGRO). For routine culturing, cells
297 were passaged every 48 hours and seeded at a density of 35,000 cells per cm² onto
298 gelatin-coated dishes. HeLa Kyoto, HEK293 and Ptk₂ cell lines were cultured in DMEM
299 (high glucose, pyruvate) supplemented with 10 % FBS and antibiotic-antimycotic
300 (Invitrogen) and passaged routinely. Prior to imaging, cells were seeded onto wells of 4-
301 well imaging dishes (Ibidi) or 24-well imaging dishes (Ibidi). To support growth in adherent
302 monolayer, mESCs for seeded onto wells coated with 5 µg/mL laminin-511 (BioLamina)
303 in 1xPBS (supplemented with Ca²⁺, Mg²⁺). All cells were maintained at 37 °C and 5 %
304 CO₂.

305 Transfection of HeLa Kyoto cells was performed using Lipofectamine 3000 (Thermo)
306 according to the manufacturer's instructions.

307

308 Chemical fixation and immunostaining of tissue culture cells

309 For chemical fixation, R1/E mESCs and HeLa Kyoto cell lines stably expressing tubulin-
310 GFP were seeded 24 h before at a plating density of 100,000 cells per cm², either directly
311 on 24-well imaging slides (Ibidi) or on coverslips. For optimal adherence and monolayer
312 growth, mESCs-designated wells and coverslips were coated with 5 µg/mL laminin 511
313 in 1x PBS (supplemented with Ca²⁺, Mg²⁺). Media were taken off and replaced with
314 microtubule-optimized fixation buffer (3.2 % paraformaldehyde (EM Sciences), 0.1 %
315 glutaraldehyde (EM Sciences) in 1x BRB80: 80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA,
316 pH 6.8 with KOH) pre-warmed to 37°C and incubated for 10 min at 37°C. Cells were
317 washed three times in 1x PBS before quenching with 0.1 M glycine (Roth) in 1x PBS for
318 10 min at RT and 0.1 % NaBH₄ (Sigma) in 1x PBS for 7 min at RT. DNA was stained with
319 SiR-DNA at a final concentration of 250 nM. Cells were imaged directly in 1x PBS ("fixed

320 only”) or embedded in mounting media (ProLong anti-fade, Invitrogen) and mounted on
321 cover slides (“fixed + mounted”).

322

323 For benchmarking of the Spindle3D analysis (see section “Image analysis reliability”,
324 Figure S4A), spindle poles were immunostained using anti- γ -tubulin antibodies (Sigma
325 T6557). Cells grown on 24-well imaging slides (ibidi) were fixed as described above. After
326 quenching, cells were immersed in blocking buffer (3 % BSA, 0.1 % Triton-x 100 in 1x
327 PBS) for 1 h at RT. Primary antibodies were diluted 1:100 in blocking buffer. Incubation
328 with primary antibodies was performed for 1 h at RT under gentle agitation. After three
329 1x PBS washes for 5 min each, cells were treated with 2 μ g/mL anti-mouse
330 AlexaFluor568-labelled secondary antibodies (Thermo, A-11061) in blocking buffer for 45
331 min at RT and constant agitation. After three final washes with 1x PBS (5 min each), DNA
332 was stained with Hoechst 33343 (Thermo, 62249) at a final concentration of 2 μ M and
333 imaged in 1x PBS.

334

335 [Image acquisition](#)

336 For imaging, HeLa Kyoto, Ptk₂ and HEK293 cells were incubated in imaging medium
337 (FluoroBrite DMEM (Gibco) supplemented with 10 % FBS (Gibco), 4 mM L-glutamine
338 (Invitrogen) and antibiotic-antimycotic (Invitrogen)). mESCs were incubated in stem cell
339 imaging medium (FluoroBrite DMEM (Gibco) supplemented with 16% FBS (Gibco), non-
340 essential amino acids (Gibco), beta-mercaptoethanol (Gibco), sodium pyruvate (Gibco),
341 antibiotic-antimycotic (Invitrogen) and mLIF (ESGRO)). To visualize chromosomes, cells
342 were treated with a final concentration of 250 nM SiR-DNA (Spirochrome). Since Ptk₂
343 cells did not show any incorporation of SiR-DNA, we instead incubated the cells for 5
344 min with Hoechst 33343 (Thermo, 62249) at a final concentration of 2 μ M in 1x PBS and
345 replaced the staining solution with imaging medium. Live-cell imaging was carried out
346 using stabilized incubation systems at 37 °C and 5 % CO₂.

347

348 Imaging was performed on multiple setups.

349 R1/E mESCs were imaged on a Zeiss LSM 800 system (Carl Zeiss Microscopy, Jena,
350 Germany) (sampling in xy: 0.27 μ m, z step size: 0.75 μ m, total number of slices: 32,
351 pinhole 48.9 μ m, unidirectional scan speed: 10, averaging: 2) using a C-Apochromat 40x

352 water objective (1.2 numerical aperture (NA)), the 488 nm (0.1 % power) and 640 nm (0.1
353 % power) laser lines and detection ranges of 410 - 558 nm and 586 - 700 nm,
354 respectively. While imaging, cells were incubated using a custom-built incubation
355 chamber (EMBL workshop).

356
357 HEK293 cells were imaged on a Nikon spinning disk (CSU-X) confocal system (Nikon
358 Corporation, Tokyo, Japan) equipped with an EMCCD camera (iXon3 DU-888 Ultra,
359 1024x1024 pixels, 13 μm pixel size) using a 60x Plan Apo oil (1.4 NA) objective (sampling
360 in xy: 0.22 μm , z step size: 0.3 μm , total number of slices: 150), 405 nm (9 % power) and
361 640 nm (10 % power) laser lines and an excitation time of 200 ms.

362 Ptk₂ cells were imaged on the same system using a 40x Plan Fluor oil (1.3 NA) objective
363 (sampling in xy: 0.34 μm , z step size: 0.3 μm , total number of slices: 100 - 150) and 405
364 nm (10 % power, 100 ms excitation) and 488 nm (18 % power, 300 ms excitation) laser
365 lines.

366
367 Again on the same system, HeLa Kyoto cells were imaged using a 60x Plan Apo oil (1.4
368 NA) objective (see above) or a 100x Plan Apo oil (1.45 NA) objective (sampling in xy: 0.14
369 μm , z step size: 0.2 μm , z ranges were selected individually per region of interest), using
370 the 488 nm (20 % power, 100 ms excitation) and 640 nm (12 % power, 100 ms
371 excitation) laser lines. Fixed samples of mESCs and HeLa Kyoto cells were recorded on
372 a Nikon spinning disk (CSU-X) confocal system (see above) using a 60x Plan Apo oil (1.4
373 NA) objective (see above).

374
375 Bovine zygotes were generated as previously described (Cavazza et al., 2020). Before
376 fertilization, bovine eggs were injected with 4 pl of mRNAs for mClover3-MAP4-MTBD at
377 200 ng/ μl and of H2B-mScarlet at 60 ng/ μl . Bovine zygotes were imaged in 20 μl of BO-
378 IVC (IVF Biosciences) at 38.8°C, 5% CO₂, 6% O₂ under paraffin oil in a 35 mm dish with
379 a #1.0 coverslip. Images were acquired with LSM800 confocal laser scanning
380 microscopes (Zeiss) equipped with an environmental incubator box and a 40x C-
381 Apochromat (1.2 NA) water-immersion objective. A volume of 65 μm \times 65 μm \times 60 μm
382 centered on the chromosomes was typically recorded. The optical slice thickness was
383 3.00 μm at a z-step size of 2.5 μm . Each zygote was typically imaged every 5 or 10

384 minutes, using the lowest possible laser intensity (>0.2% for the 488nm laser; >0.2% for
385 the 561 nm laser). mClover3 was excited with a 488 nm laser line and detected at 493 -
386 571 nm. mScarlet was excited with a 561 nm laser line and detected at 571 - 638 nm.

387

388 [Image processing and analysis](#)

389 After imaging, the only pre-processing step required for downstream analysis is a manual
390 crop of the mitotic cells of interest from the raw files. We suggest using the rectangular
391 selection tool in Fiji (Schindelin et al., 2012). Please note that the morphometric analysis
392 only works on spindles and chromatin that were fully captured in z. Furthermore, the
393 analysis requires fluorescent information of spindle microtubules and chromatin in
394 separate channels that are specified by the user. Other channels will be ignored, but will
395 be displayed in the output image.

396

397 The image processing and analysis are implemented in ImgLib2 (Pietzsch et al., 2012).
398 Spindle3D (<https://github.com/tischi/spindle3d>) is distributed as a Fiji plugin and can be
399 installed by enabling the Spindle3D update site (<https://sites.imagej.net/Spindle3D>). The
400 image processing and analysis pipeline runs fully automated and consists of the below
401 steps. Parameters are shown in quotation marks, with the default parameter values
402 indicated after a colon.

403

404 Isotropic resampling: To facilitate implementation of the image analysis algorithms all
405 channels of the input image are resampled to an isotropic voxel size ("voxel size for
406 analysis": 0.25 μm).

407

408 Metaphase plate initial segmentation: In order to find the location of the metaphase plate
409 in the image, we rely on the fact that the DNA signal in condensed chromosomes is
410 brighter than interphase DNA. To find an intensity threshold above which voxels belong
411 to condensed (metaphase) chromosomes, we first downsample the DNA image such
412 that the width of one voxel resembles the typical width of the metaphase plate ("voxel
413 size for initial DNA threshold": 1.5 μm) (see Figure S1A, left). The intensity values in the
414 downsampled image are computed by averaging with a gaussian blur with a sigma of
415 half the "voxel size for initial DNA threshold". In this downsampled image we find the

416 maximal (max) and minimal (min) intensity. We empirically determined that $(\text{max}+\text{min})/2$
417 serves as a reliable threshold. We then apply this threshold to the DNA image to create
418 a binary mask and perform a connected component labelling (Figure S1A, center). We
419 remove all connected components that touch the lateral (xy) image boundary and of the
420 remaining ones only keep the largest one, which we define to be the initial metaphase
421 plate object.

422

423 Metaphase plate center and orientation: To determine the orientation of the metaphase
424 plate, we use an algorithm from ImageJ's 3D Image Suite (Ollion et al., 2013) to fit a 3D
425 ellipsoid to the initial metaphase plate object, resulting in three vectors along the shortest,
426 middle, and longest axes as well as the coordinates of the metaphase plate center. To
427 facilitate the implementation of the subsequent algorithms, e.g. in terms of specifying
428 ranges to be included in certain computations, and also to facilitate visual inspection of
429 the images, we use these vectors to compute a transformation that puts all images into
430 a new coordinate system such that the new z-axis corresponds to the shortest axis of
431 the metaphase plate, roughly corresponding to the spindle pole-to-pole axis, and such
432 that the origin of the coordinate system coincides with the center of the metaphase plate.
433 We will refer to these transformed images as metaphase plate aligned images (Figure
434 S1A, right).

435

436 Metaphase plate width: Using the metaphase plate aligned DNA image, we compute an
437 average intensity profile along the shortest DNA axis (z-axis of the aligned image), limiting
438 the computations to a maximum width that is based on the extent of the shortest axis of
439 the initial ellipsoid fit times 2. We then compute the derivative of this profile at a resolution
440 of "metaphase plate derivative delta": $3\ \mu\text{m}$. We define the metaphase plate width as the
441 distance between the locations with the highest absolute values in the derivative (see
442 Figure S1B, left). This procedure is motivated by the fact that, due to the various
443 chromosomes and the diffraction limit of the microscope, the metaphase plate has an
444 overall irregular appearance. For example, our analysis is robust to an individual
445 chromosome "sticking out" of the metaphase plate as this will not shift above maxima of
446 the derivatives (see Figure S1B, left). In other words, our approach measures an average
447 width, determined by the average position of all chromosomes.

448

449 Metaphase plate length: Using the metaphase plate aligned DNA image, we compute an
450 average lateral radial intensity profile (see Figure S1B, center left), limiting the
451 computations to a maximum length determined by the extent of the longest axis in the
452 initial ellipsoid fit times 2. We define the metaphase plate length as 2 times the distance
453 between the origin to the position of the minimum in the derivative of the intensity profile.
454 Again, this approach reports an average measurement that is robust to any details that
455 the various arrangements of the chromosomes in the metaphase plate may have. In
456 addition, both the measurements of the metaphase width and length have the advantage
457 of not depending on the choice of any intensity threshold.

458

459 Chromatin dilation: We further utilize the average lateral radial DNA intensity profile (s.a.)
460 to calculate the ratio of the intensity in the center of the metaphase plate (position zero
461 along the radial profile) and the brightest part along the profile (see Figure S1B, center
462 right). This ratio is subtracted from 1 to report on the magnitude of the dilation in the
463 center of the metaphase plate, higher values corresponding to a more pronounced
464 opening, while small values reflect homogeneously closed metaphase plates.

465

466 Chromatin volume: In order to facilitate the comparison with previously published
467 measurements, we decided to adopt the method published in Hériché et al., (2014),
468 where the Otsu algorithm (Otsu, 1979) is used to determine an intensity threshold and
469 the chromatin volume is determined as the sum of the volume of all voxels above this
470 threshold. The Otsu algorithm relies on a bimodal (foreground and background) intensity
471 distribution. We therefore apply the Otsu algorithm to a region of interest determined by
472 the previously measured metaphase plate width and length (see Figure S1B, right) where
473 the intensity values only comprise the metaphase plate (foreground) and parts of the cell
474 devoid of DNA signal (background), but exclude (unwanted) DNA signal from surrounding
475 cells. We apply the determined threshold to the whole image, perform a connected
476 component analysis, remove regions touching the image borders and keep the largest
477 region, which we call segmented metaphase plate. The volume of this region is the
478 chromatin volume.

479

480 Spindle segmentation: We explored various methods of reliably determining an intensity
481 threshold for assigning pixels to the mitotic spindle and developed an algorithm relying
482 on the observation that, in all data we analysed, the metaphase plate length was always
483 substantially (on average 25%) larger than the spindle width. The direct vicinity of the
484 metaphase plate therefore contains a substantial fraction of pixels inside as well as
485 outside of the spindle. Thus, this region is well suited for determining an automated
486 threshold using the Otsu algorithm (see section “Chromatin volume”). Technically, we
487 apply the Otsu algorithm to all tubulin intensity values in a rim of a thickness of 1 pixel
488 around the segmented metaphase plate (see Figure S1C, center left). We then apply this
489 threshold to the whole tubulin image and perform a connected component analysis. As
490 there can be other cells with relatively bright tubulin intensities in the same image, we
491 filter the regions, only keeping regions where at least one of their pixels is within a defined
492 distance to the center of the metaphase plate (“spindle fragment inclusion zone”: 3 μm).
493 We will refer to the union of those regions as the spindle mask.

494

495 Spindle volume: The spindle volume is computed as the volume of all voxels in the spindle
496 mask (see Figure S1C, center left).

497

498 Spindle average intensity: The average gray value of all tubulin voxels within the spindle
499 volume mask.

500

501 Spindle intensity variation: Spindles have different degrees of homogeneity in terms of
502 their distribution of polymerised tubulin. We measure this by computing the coefficient of
503 variation of the (threshold subtracted) tubulin intensities within the spindle mask.

504

505 Spindle poles locations: In our algorithm, both spindle length and spindle orientation are
506 determined by the vector that connects the two spindle poles. We locate the spindle
507 poles in two steps. First, we draw a line profile through the spindle mask along the
508 shortest metaphase plate axis and through the metaphase plate center. The two
509 locations along the line profile where the spindle mask intensity drops from 1 to 0 (i.e. the
510 spindle mask ends) are the two initial spindle poles (see Figure S1C, center right). As the
511 spindle axis is often not completely aligned with the shortest metaphase plate axis, the

512 initial spindle poles need to be refined. To do so, we determine the locations of the pixels
513 with the maximum intensity in the tubulin image in a small neighborhood around the initial
514 spindle poles. The extent of this neighborhood is controlled by the “axial pole refinement
515 radius”: 1.0 μm and the “lateral pole refinement radius”: 2.0 μm , where “axial” refers to
516 along the shortest metaphase plate axis and “lateral” refers to the perpendicular
517 directions (see Figure S1C, center right).

518

519 Spindle center location: We define the middle between the two spindle poles as the
520 spindle center location.

521

522 Spindle center to metaphase plate center distance: The distance of the metaphase plate
523 center (s.a.) to the spindle center.

524

525 Spindle length: We define the spindle length as the distance between the two spindle
526 poles (see Figure S1C, center right).

527

528 Spindle angle: The two spindle poles allow to define a spindle axis vector that points from
529 one pole to the other. We apply below formula (in the coordinate system of the input data
530 where we assume the coverslip plane to be perpendicular to the z-axis) to compute the
531 angle of the spindle axis and the coverslip plane as follows: $90.0 - \text{abs}(\text{angle_degrees}(z\text{-}$
532 $\text{axis, spindle-axis}))$. For the computation of an angle between two axes there are always
533 two solutions. Here, the computations within the function `angle_degrees` are done such
534 that the smaller one, i.e. with a value between 0 and 90 degrees, is picked (see Figure
535 S1C, right).

536

537 Spindle coordinate system: We define a new coordinate system in which the spindle
538 center is at the origin and the spindle poles are aligned along the z-axis. This coordinate
539 system simplifies the following measurement.

540

541 Spindle widths: To measure the spindle width we perform a maximum projection of the
542 spindle mask along the spindle axis (the z-axis in the spindle coordinate system). We
543 smoothen the 2D projected spindle mask by a morphological opening operation with a

544 radius of 2 pixels. We then compute the width of the binary mask in steps of 10 degrees
545 (see Figure S1C, center right). We define the mean of the resulting widths as the “average
546 spindle width”. To capture potential anisotropies in the spindle shape, we fetch both the
547 minimum and maximum of the width at all measured angles, resulting in the outputs:
548 “minimal spindle width” and “maximal spindle width”.

549
550 Spindle aspect ratio: As a measure for spindle shape, we define the ratio of spindle length
551 and the average spindle width as the spindle aspect ratio.

552
553 Tabular output: The plugin outputs all measured values in a table, the column names
554 corresponding to the respective measurements.

555
556 Image output: The plugin also outputs a multichannel image, composed of the DNA and
557 tubulin signal, the DNA mask, the spindle mask, and another image containing three
558 points corresponding to the spindle poles and the spindle centre (see Figure 2B). All
559 images are sampled isotropically at the voxel size for analysis. For ease of inspection, all
560 images are aligned such that the x-axis corresponds to the measured spindle axis and
561 such that the center of the image corresponds to the spindle center.

562
563 Cell volume quantification

564 Cells expressing tubulin genetically fused to a fluorescent protein show characteristic
565 cytoplasmic background fluorescence (see Figure 5A). We used the machine-learning
566 based segmentation software Ilastik (Berg et al., 2019) to train pixel-classification models
567 to distinguish between true mitotic cytoplasm and all other voxels. Before training and
568 prediction, all images were rescaled to an isotropic voxel size of 0.25 μm to be consistent
569 with the analysis voxel size applied in the Spindle3D analyses. For training, training set
570 images were annotated in the auto-context module in Ilastik, a two-step workflow, where
571 the second stage receives the prediction results from the initial stage. In the first stage,
572 the default random forest algorithm was trained with the classes “spindle microtubules”,
573 “mitotic cytoplasm”, “interphase microtubules”, and “background”. Brightness features
574 were excluded, to avoid bias in fluorescence signal strength, i.e. varying expression levels
575 of fluorescent tubulin. We used all available texture and edge filters for training. In the

576 second stage, we trained another random forest to distinguish between the two classes
577 “true mitotic” and “other”, while again all brightness features were ignored and all texture
578 and edge features were included. After successful training, batch processing was
579 performed using the ilastik integration in Fiji. The second-stage probability masks (see
580 Figure 5A, center) were de-noised with a 3D Gaussian filter ($\sigma = 2$ pixels) and
581 thresholded at the cutoff value 0.5, reflecting the binary prediction approach to
582 distinguish between “true mitotic” and “other”. The volumes of the resulting segmentation
583 masks (see Figure 5A, right) were quantified using the “3D analyse regions” function in
584 the MorphoLibJ package (Legland et al., 2016).

585

586 Spindle mass quantification

587 In cells expressing fluorescently-tagged tubulin, we can define the average voxel gray
588 value within the cell mask (see section “Cell volume quantification”) as the average
589 concentration of tubulin in the whole cell $[T]_c$. Analogously, the average concentration of
590 tubulin in the spindle $[T]_s$ is reflected by the average voxel value within the spindle mask.
591 To account for system-internal noise of the imaging setup, we calculated the median
592 voxel values in the bottommost slices of the image stack. We then subtracted this value
593 from both $[T]_c$ and $[T]_s$. We define spindle mass as the sum of tubulin (free and polymer)
594 within the spindle volume V_s . To correct for cell-specific tubulin-GFP expression levels,
595 we normalised spindle masses by the cell-specific fluorescence correction factor $Fl_{corr} \sim$
596 $[T]_c$: Spindle mass = $[T]_s * V_s / Fl_{corr}$.

597

598 [Image analysis reliability and accuracy](#)

599 In order to benchmark the measurements of the plug-in, we labelled centrosomes of
600 tubulin-GFP expressing R1/E mouse embryonic stem cells with anti- γ -tubulin antibodies
601 (see section “Chemical fixation and immunostaining of tissue culture cells”). Having
602 imaged the spindles using confocal microscopy, in Fiji we located the outward-facing
603 edges of the γ -tubulin signals and defined their 3D coordinates as the ground-truth
604 spindle poles (see Figure S6A). The euclidean distance between the two poles was
605 defined as the ground-truth spindle length. In parallel, we located the 3D coordinates of
606 the poles exclusively by looking at the tubulin-GFP signals. The euclidean distance
607 between this pair of poles was defined as the manual spindle length. Ultimately, we

608 compared both the ground-truth spindle length and the manual spindle length
609 measurements to measurements derived via the Spindle3D plug-in.

610

611 Opposed to spindle length, we lack proper ground-truth references for spindle width.
612 Nevertheless, we benchmarked the plug-in's performance against human measurement.
613 To this end, we made use of the spindle axis registration of the Spindle3D analysis and
614 verified the orientation with the centrosomal γ -tubulin signals (see above). In Fiji, we used
615 Image > Stack > Reslice to match the direction of the spindle axis with the z axis and
616 performed a maximum projection. Using only the tubulin-GFP and γ -tubulin signals, we
617 manually determined 4 extents of the spindle in the projected image (see Figure S6B)
618 and calculated their mean to serve as the manual spindle width reference measurement.

619

620 To verify the performance of the pixel classification-based mitotic cell volume
621 quantification, we transfected tubulin-GFP expressing HeLa Kyoto cells with plasmids
622 encoding mCherry tagged with the CaaX motif (Clarke, 1992) for cell membrane
623 localisation. We acquired confocal images of mCherry-positive mitotic cells (see Figure
624 S6E) and performed our tubulin-GFP based cell volume quantification as described
625 above. In parallel, we manually segmented cell volumes using the mCherry-CaaX
626 landmark channel and the volume manager in the SCF MPI-CBG Fiji package
627 (<https://sites.imagej.net/SCF-MPI-CBG/>) (see Figure S6F) to generate a binary cell mask,
628 the volume of which was quantified via the "3D analyse regions" function in the
629 MorphoLibJ package (Legland et al., 2016).

630

631 For quality control, 3D image stacks were rendered using the multichannel visualisation
632 package ClearVolume (Royer et al., 2015) in Fiji.

633

634 [Data analysis and visualization](#)

635 After image analysis, we used the pandas (The pandas development team, 2020), SciPy
636 (Virtanen et al., 2020) and NumPy (Harris et al., 2020) libraries in Python to further analyse
637 the data. Statistical tests (Wilcoxon signed-rank test) were carried out using the
638 scipy.stats package. ANOVA and post-hoc testing was performed using the Python
639 statsmodel package (Seabold et al., 2010). Power-law fitting was performed in the

640 scipy.optimize library. Linear regression was performed in the scikit-learn package
641 (Pedregosa et al., 2011). All data visualization was carried out in the Python Altair library
642 (VanderPlas et al., 2018) and assembled in Adobe Illustrator 2021 (Adobe inc. 2020).

643

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660

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663

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666 the plugin, analyzed images, and performed data analysis; Writing–Original Draft, S.
667 Reber with input from T. Kletter and C. Tischer. S. Reusch and N. Dempewolf acquired
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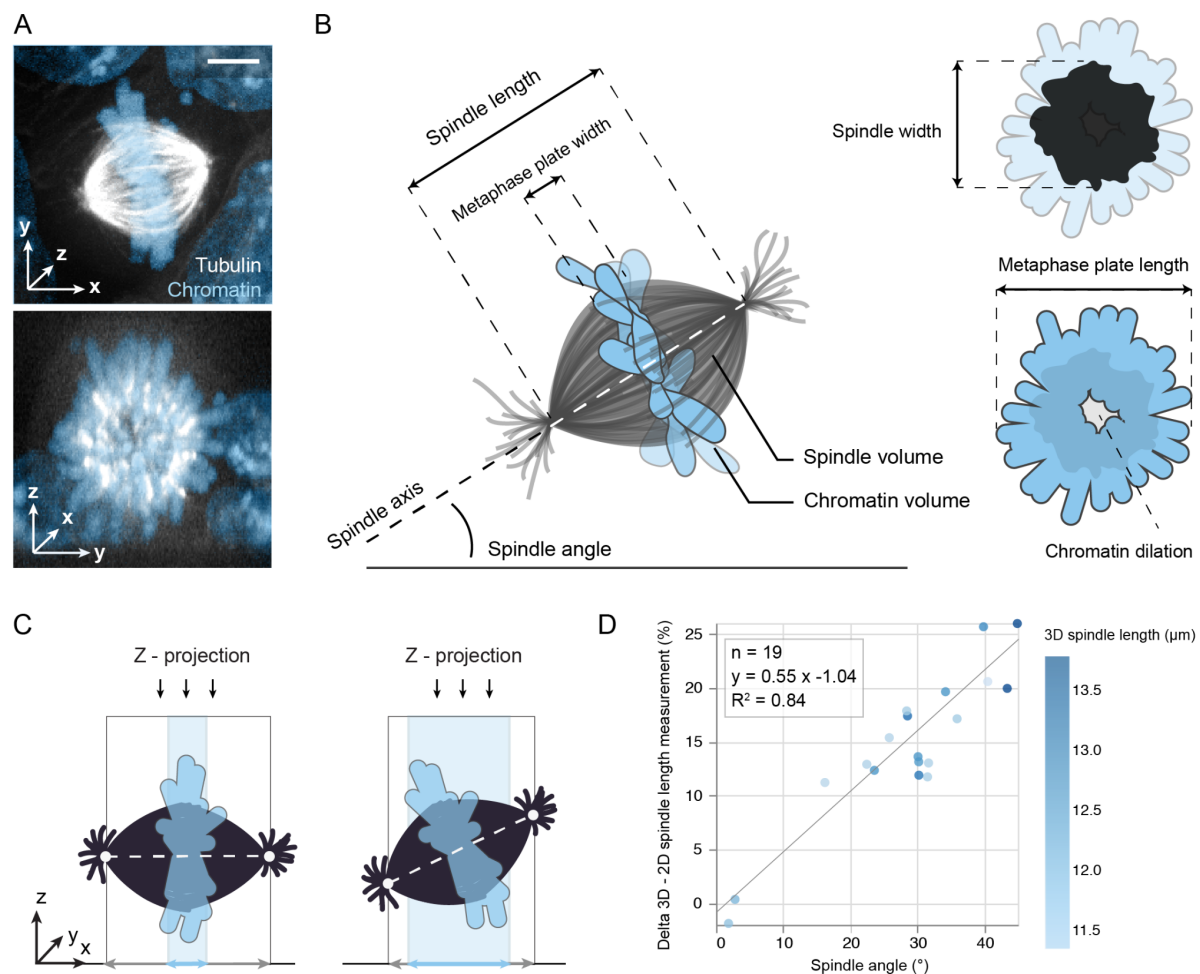
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857 **Figure 1: 3D analysis of fluorescent spindle and chromatin data allows for the**

858 **accurate extraction of morphometric parameters. (A)** Top: projected micrograph of

859 a mitotic mouse embryonic stem cell expressing tubulin-GFP (white), DNA stained with

860 Hoechst (blue). Bottom: same image resliced to display the equatorial section of the

861 spindle. Scale bar: 5 μm **(B)** Schematic of a mitotic spindle and its relevant morphometric

862 parameters extracted by Spindle3D. Along the spindle axis, Spindle3D measures spindle

863 length and metaphase plate width, and in the lateral direction spindle width and

864 metaphase plate length. Chromatin dilation quantifies the central signal strength of the

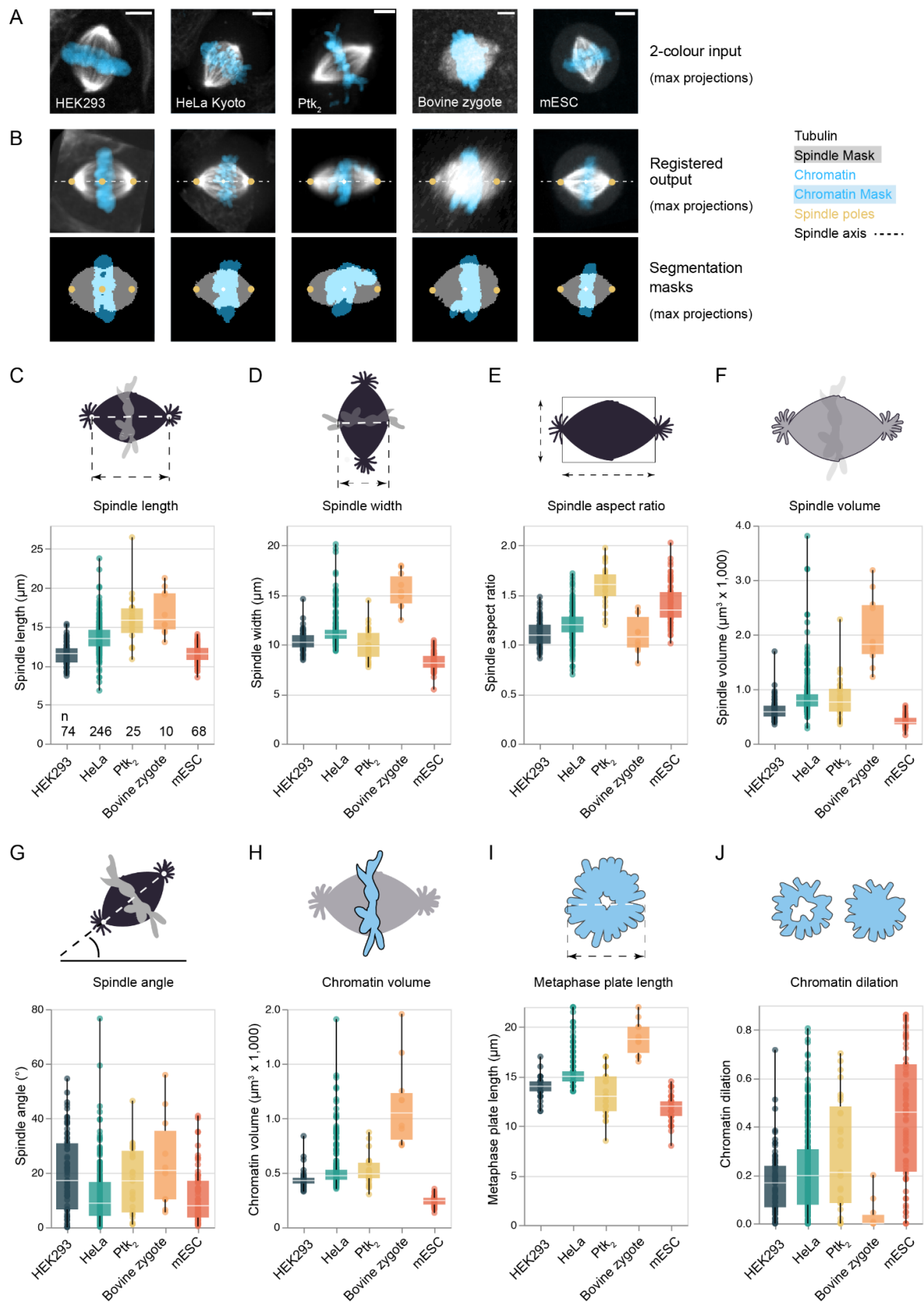
865 metaphase plate. **(C)** Morphometry on projected spindles distorts measurements, if

866 spindle axes are tilted. **(D)** The relationship between the spindle angle and the

867 percentaged discrepancy between the 2D (projected) and 3D spindle length

868 quantification ($n = 19$). Circles represent individual spindles, colour is coded according to

869 3D spindle length. Line shows linear regression.



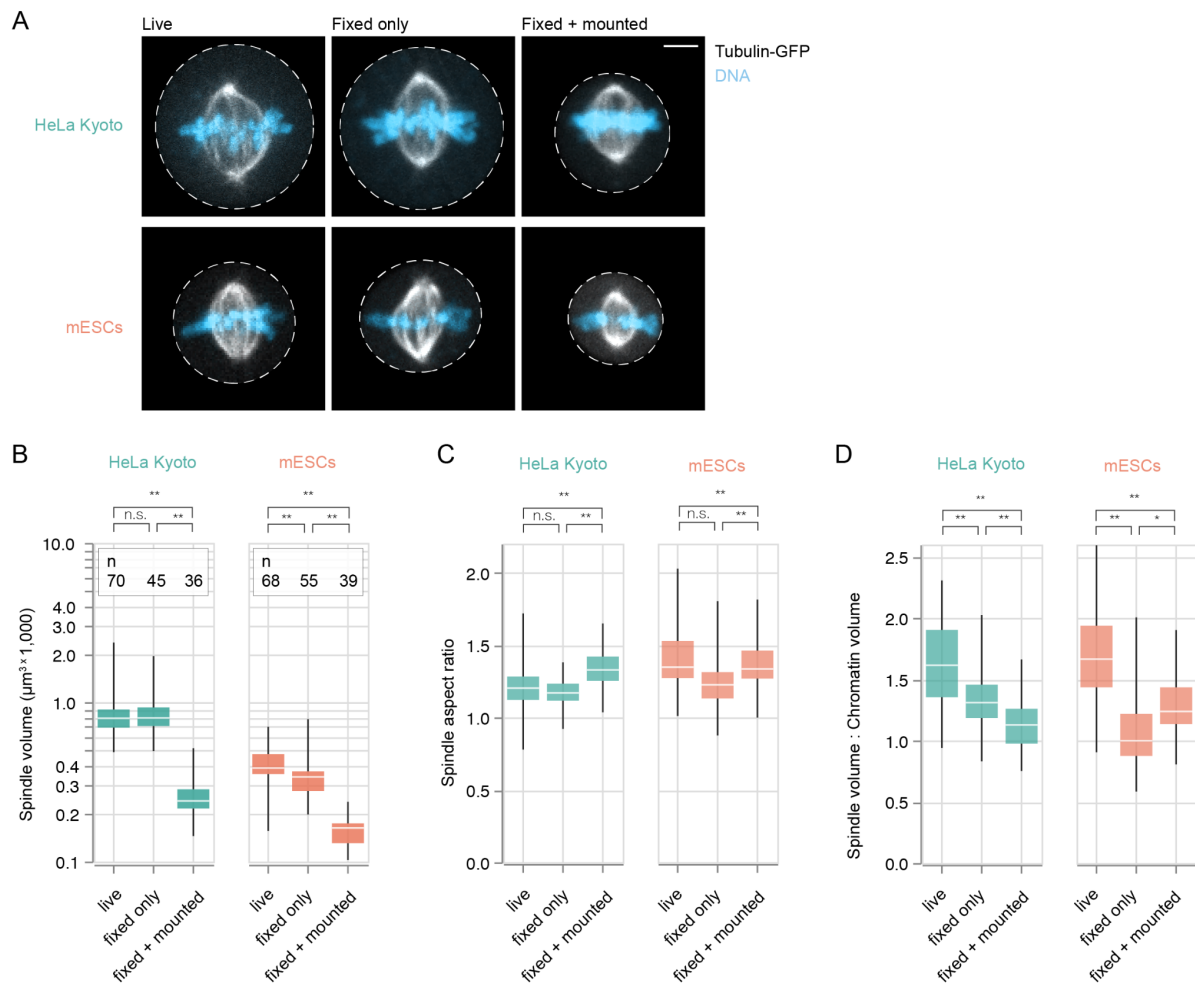
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872 **Figure 2: Spindle3D robustly derives morphometric parameters across a variety**

873 **of cell types and phyla. (A)** Representative live fluorescent 3D spindle data sets from

874 different cells expressing labelled tubulin or microtubule-associated proteins or were
875 treated with SiR-tubulin (white). Chromatin (blue) is visualized with Hoechst, SiR-DNA or
876 H2B-mScarlet. Scale bar: 5 μ m. **(B)** Automated axial registration and segmentation of 2-
877 colour (tubulin gray scale, chromatin blue) input images as shown in (A). Spindle3D
878 exports axially aligned output images containing segmentation masks and spindle pole
879 localization for quality control. Quantification of **(C)** spindle length, **(D)** spindle width, **(E)**
880 spindle aspect ratio, **(F)** spindle volume, **(G)** spindle angle, **(H)** chromatin volume, **(I)**
881 metaphase plate length, and **(J)** chromatin dilation for all cell types. Circles are individual
882 data points and represent a single spindle measurement. Boxes describe the interquartile
883 range. Horizontal line in the box denotes median. Whiskers show minimum and
884 maximum.



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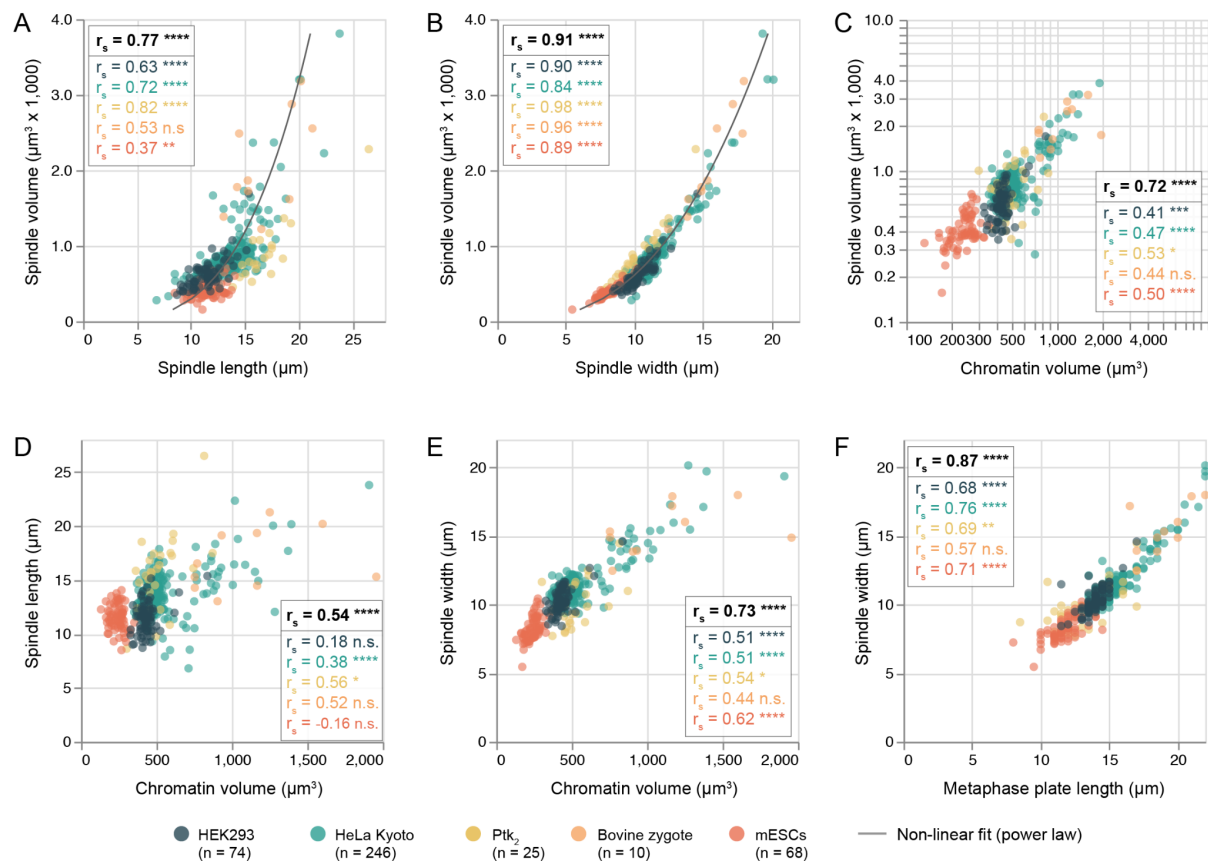
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Figure 3: Fixation and sample preparation alter spindle and chromatin morphology. (A) Fluorescently-tagged tubulin allows for direct comparison of spindle morphology in live and fixed specimens. Left column shows representative mitotic cells (HeLa Kyoto and mESCs lines both stably expressing tubulin-GFP) when imaged live. Cells depicted in the central column were chemically fixed before imaging. Cells in the right column were fixed and embedded in mounting media. Tubulin-GFP signal is in white, DNA in blue. Dotted lines indicate cell boundaries. Scale bar: 5 μm . Fixation and sample preparation introduce artifacts to morphometric parameters such as (B) spindle volume and thus distort geometrical relationships among spindle measures such as (C) spindle aspect ratio and (D) the ratio of spindle volume to chromatin volume. Boxes denote interquartile range, horizontal lines show medians. Whiskers show minimum and maximum. P values from ANOVA with Tukey's test as post-hoc analysis. * $p < 0.05$, ** $p = 0.001$, n.s: not significant ($p > 0.05$).



899

900 **Figure 4: Spindle width, rather than spindle length, reflects spindle volume.**

901 Relationship between **(A)** spindle length and spindle volume and **(B)** spindle width and

902 spindle volume in live spindles of five different cell types. **(C)** Volumetric relationship

903 between chromatin and spindle. Relationship between **(D)** chromatin volume and spindle

904 length and **(E)** chromatin volume and spindle width. **(F)** Relationship between metaphase

905 plate length and spindle width. Circles represent individual cells. Individual data points in

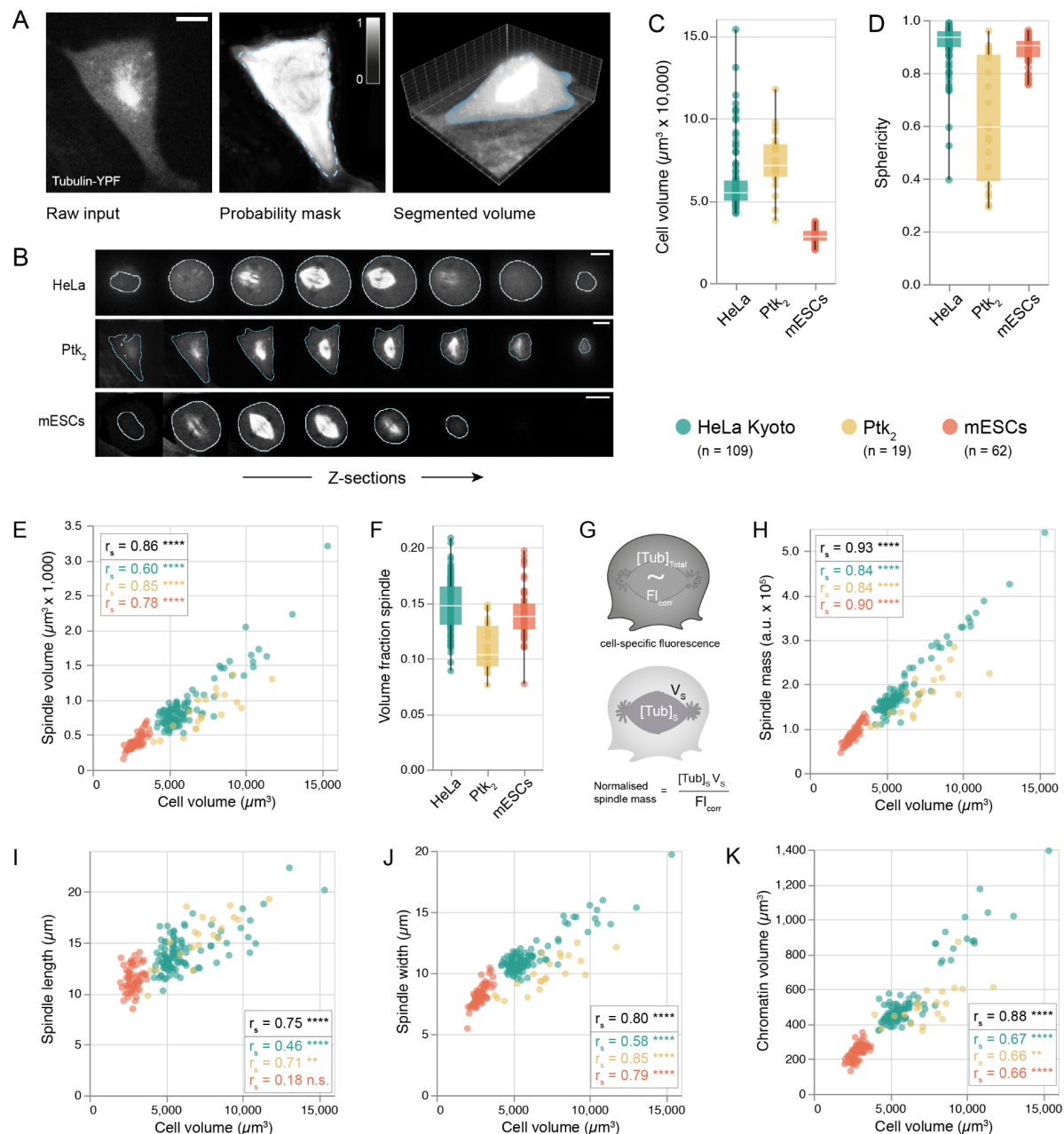
906 (A) and (B) fitted by a power law ($f(x) = a \cdot x^k$, a: spindle length: 2.681 ± 0.535 , exponent:

907 2.191 ± 0.073 ($n = 425$ cells); a: spindle width: 1.739 ± 0.001 , exponent: 2.551 ± 0.000

908 ($n = 425$ cells)). r_s : Spearman's correlation coefficient, black coefficients show correlation

909 for pooled data, coloured coefficients show cell-type resolved correlations. * $p < 0.05$, **

910 $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$, n.s.: not significant ($p > 0.05$).



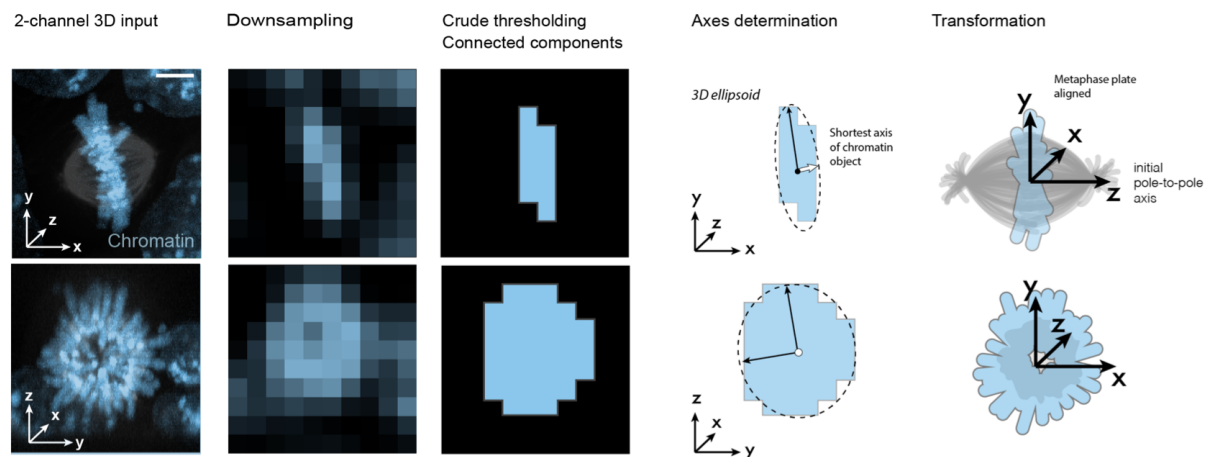
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912 **Figure 5: Spindle volume and chromatin volume scale linearly with cell volume.**

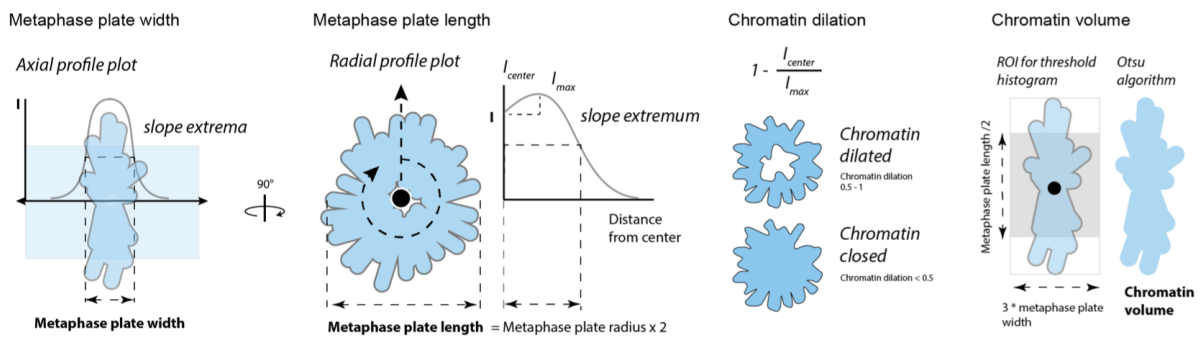
913 **(A)** Rationale for quantifying cell volumes via cytoplasmic tubulin fluorescence by pixel
 914 classification in the segmentation software Ilastik (Berg et al., 2019). Voxels of input
 915 micrographs (left) were converted to probabilities for mitotic cytoplasm (center).
 916 Probability masks were thresholded at 0.5 (dotted line) to produce the final volume mask
 917 (blue, right). Scale bar: 5 μm . **(B)** Z-series showing the cell boundaries (blue) as
 918 determined by pixel classification in three cell lines expressing fluorescent tubulin. Scale
 919 bars: 5 μm . **(C)** Distributions of cell volumes and **(D)** cell sphericity in three cell lines. **(E)**
 920 Bivariate relationships between cell volume and spindle volume and **(F)** distributions

921 showing the fraction of cell volume occupied by the spindle. **(G)** Quantifying spindle mass
922 as the polymer and free tubulin mass within the spindle volume, normalised by a cell-
923 specific fluorescence correction factor proportional to the total fluorescent tubulin
924 concentration. **(H)** Bivariate relationships between cell volume and spindle mass, **(I)** cell
925 volume and spindle length and **(J)** cell volume and spindle width. **(K)** Volumetric
926 relationship between spindle and chromatin. Circles reflect individual cells. r_s :
927 Spearman's correlation coefficient, black coefficients show correlation for pooled data,
928 coloured coefficients show cell-type resolved correlations. * $p < 0.05$, ** $p < 0.005$, *** p
929 < 0.001 , **** $p < 0.0001$, n.s.: not significant ($p > 0.05$). Boxes denote interquartile range,
930 horizontal lines are medians. Whiskers show minimum and maximum.

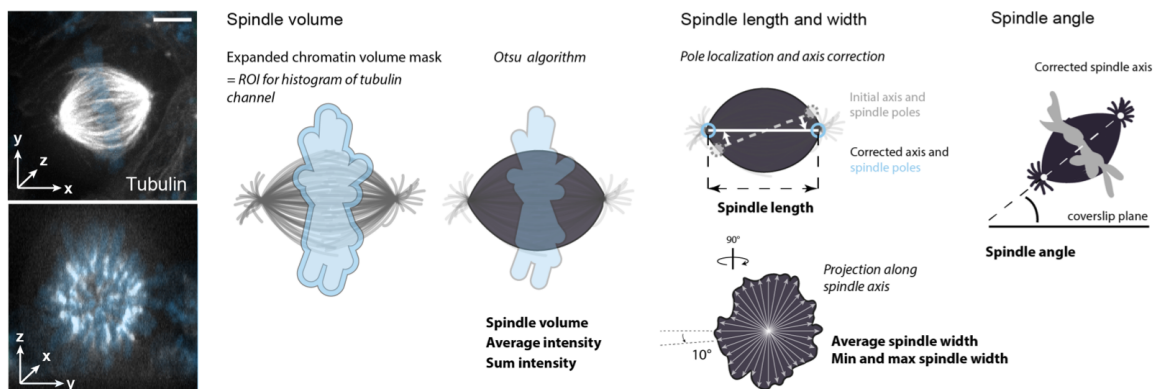
A Metaphase plate detection and orientation



B Chromatin morphometrics



C Spindle morphometrics



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932 **Figure S1: Spindle3D morphometric analysis workflow. (A)** In confocal

933 micrographs, mitotic cells (chromatin shown in blue, tubulin in grayscale) are detected by

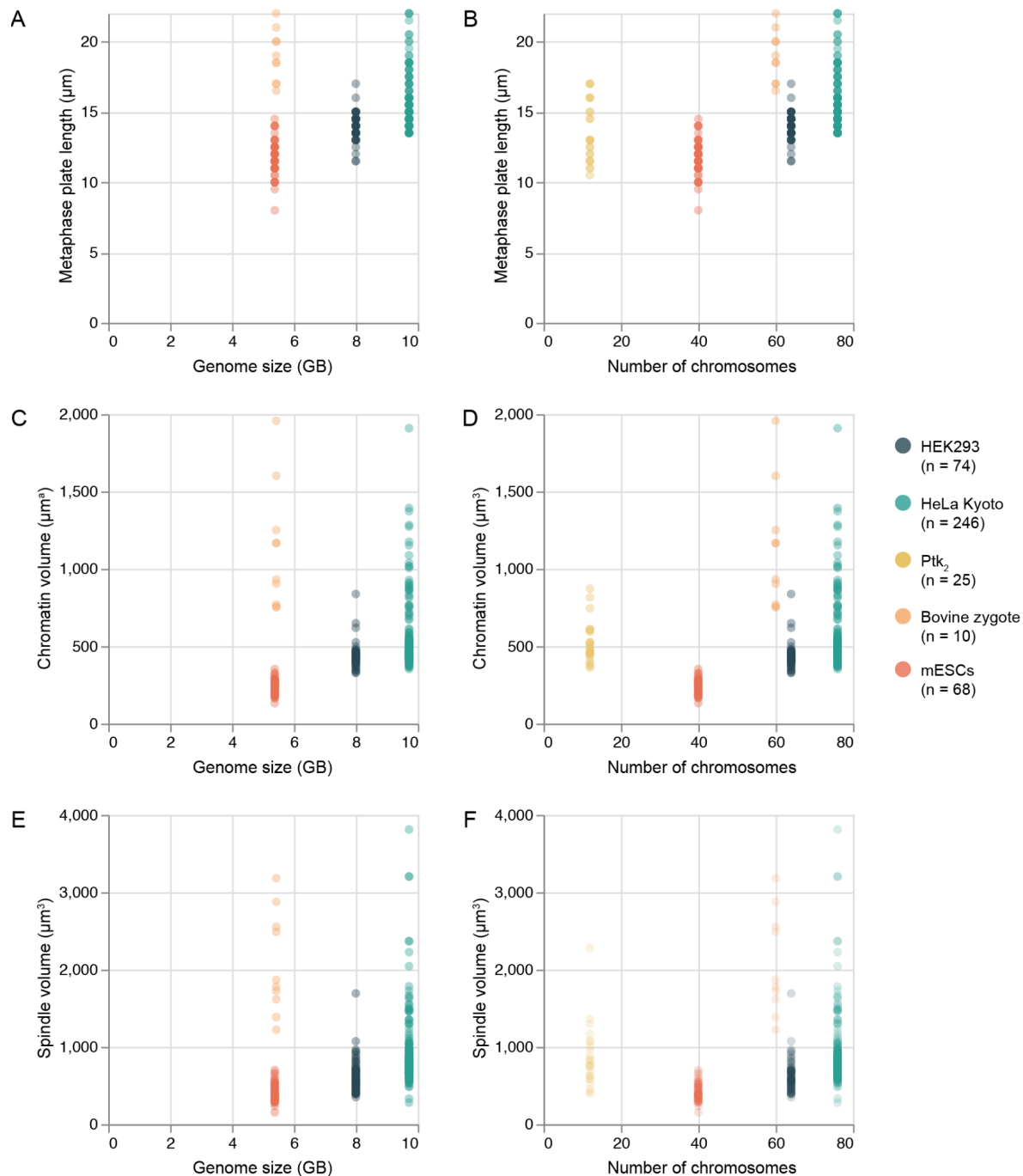
934 crude, histogram-based segmentation and connected-component analysis. The shortest

935 axis of the metaphase plate object is determined and initiates an imaging axis-

936 independent coordinate system. **(B)** In the newly aligned image, radial and axial intensity

937 profiles serve as robust guides to quantify the extents of the usually irregularly shaped

938 chromatin plate. Moreover, radial profiles inform on the magnitude of dilation of the
939 metaphase plate. Based on the extents of the plate, a three-dimensional region of interest
940 is used to limit the pixels considered for histogram-based segmentation of the
941 chromosomes, excluding potentially interfering signals from nuclei in close proximity. **(C)**
942 Analogously, only a fraction of the tubulin channel pixels (the ones immediately bordering
943 the chromatin mask and thus either represent spindle microtubules, or non-spindle
944 tubulin inside the cell) are considered for Otsu thresholding the spindle. Spindle poles are
945 the brightest pixels found within defined radii around the intersections of the initial spindle
946 axis (found in (A)) and the spindle volume mask. This mask is projected along the now
947 corrected spindle axis. The resulting area is radially scanned in 10° steps, to ultimately
948 measure 18 lateral spindle extents, their mean representing the average spindle width.
949 Finally, the tilt of the corrected spindle axis is used to determine the spindle angle.



950

951 **Figure S2: Relationship between genome sizes and spindle and chromatin**

952 **dimensions.** HEK293 cells were described as hypo-triploid (3n-) (Bylund et al., 2004)

953 with an average chromosome number of 64. Considering the median human diploid

954 genome size of 5.72 gigabases (Gb) (NCBI) and the diploid human chromosome

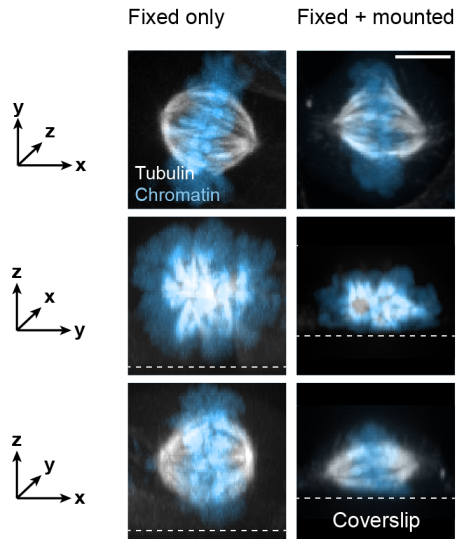
955 number of 46, we estimated the genome size to be approximately 8.00 Gb. HeLa cells were

956 described as hyper-triploid (3n+) (Macville et al., 1999) with an average chromosome

957 number of 76, we estimated the average HeLa genome to amount to approx. 9.72 Gb.

958 The diploid genome of *Mus musculus* corresponds to 40 chromosomes. The median

959 diploid genome size is reported to be 5.38 Gb (NCBI). Analogously, the diploid genome
960 of *Bos taurus* corresponds to 60 chromosomes, the median diploid genome size is 5.44
961 Gb (NCBI). The genome of the marsupial species *Potorous tridactylus* is not yet
962 sequenced, the female diploid chromosome number is 12 (Rens et al., 1999). **(A)** Scatter
963 plot displaying the relationship between genome size and metaphase plate length and
964 **(B)** number of mitotic chromosomes and metaphase plate length. **(C)** Scatter plot
965 displaying the relationship between genome size and chromatin volume and **(B)** number
966 of mitotic chromosomes and chromatin volume. **(E)** Scatter plot displaying the
967 relationship between genome size and spindle volume and **(F)** number of mitotic
968 chromosomes and spindle volume. Circles represent single cells.



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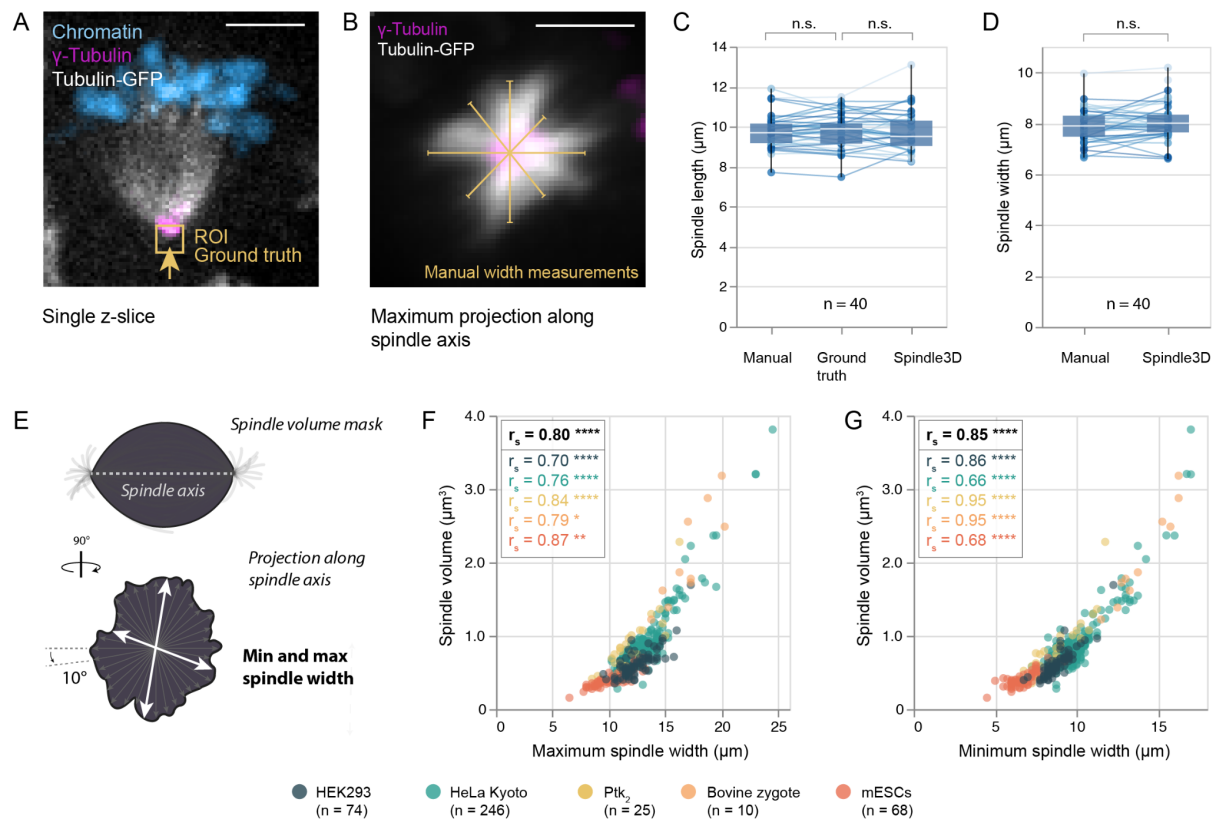
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Figure S3: Sample handling distorts spindle shape. Left column shows projections along the imaging axis (top), along the x axis (middle) and along the y axis (bottom) of a chemically fixed mouse embryonic stem cell (mESC) imaged in 1x PBS. Analogously, the right column shows a mESC that, after fixation, was embedded in mounting solution and mounted on a cover slide. Tubulin-GFP is shown in grayscale, Hoechst-labelled chromatin in blue. The dotted lines indicate the plane of the cover glass. Scale bar: 5 μm .



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Figure S4: Performance accuracy of manual versus automated spindle

measurements. (A) Micrograph (single z-slice) of a tubulin-GFP (gray scale) expressing mouse embryonic stem cell (mESC) fixed at mitosis. Antibody stainings were used to detect γ -tubulin (magenta). DNA is stained with Hoechst (blue). The arrow and region of interest (ROI, yellow) highlight the outer edge of the γ -tubulin signal, the position considered as ground truth spindle pole. Scale bar: 5 μm . **(B)** Maximum projection along the spindle axis of a tubulin GFP-expressing mitotic mESC with labelled centrosomes (γ -tubulin, magenta). Four manually drawn spindle width measurements (yellow) were averaged to yield the reference spindle width. Scale bar: 5 μm . **(C)** Box plots show distributions of spindle length measurements ($n = 40$) derived by manually placing spindle poles within the tubulin-only 3D image (“Manual”), or by manually placing spindle poles within the γ -tubulin-only 3D image (“Ground truth”) or by subjecting the chromatin/tubulin stack to analysis by Spindle3D. **(D)** Box plots show distributions of spindle width measurements ($n = 40$) derived manually or via Spindle3D. Boxes reflect the interquartile range, whiskers show the minimum and maximum. The medians are shown as horizontal white lines inside the boxes. Circles reflect measurements on individual spindles and are linked across the methods by lines. Hypothesis testing was performed using the

994 Wilcoxon signed-rank test. N.s: not significant ($p > 0.05$). **(E)** Rationale for determining
995 the minimum and maximum spindle width after segmentation in Spindle3D. **(F)** Scatter
996 plots showing the relationship between the maximum spindle width and spindle volume,
997 **(G)** between the minimum spindle width and spindle volume. Circles represent individual
998 cells. r_s : Spearman correlation coefficient. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p <$
999 0.0001 , n.s.: not significant ($p > 0.05$).

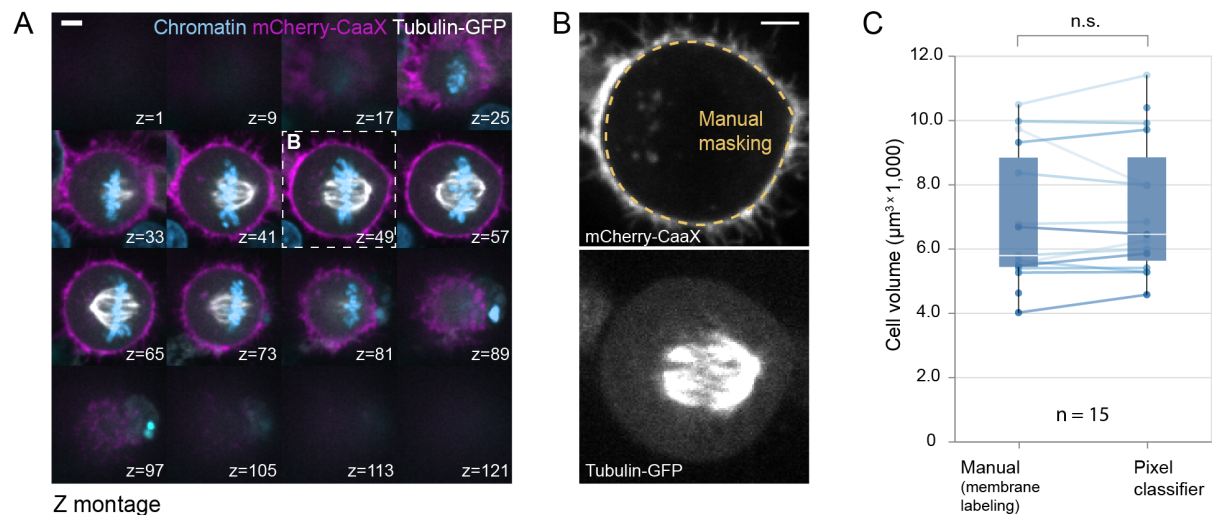
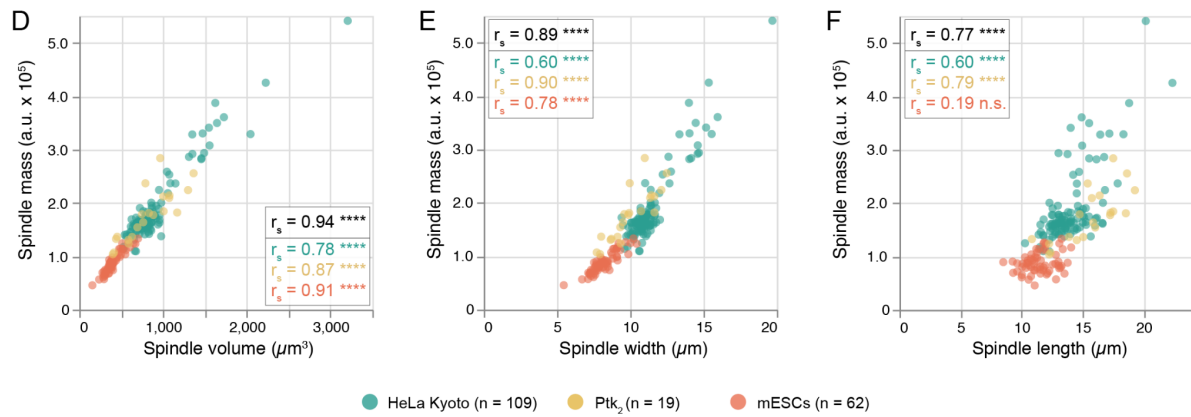


Figure S5: Performance accuracy of manual versus automated cell volume measurements. (A) Z-montage showing a mitotic HeLa Kyoto cell expressing tubulin-GFP and mCherry-CaaX. Scale bar: 5 μm . (B) Isolated slice of (A) highlighting the cell membrane landmark channel (top) with the manually traced cell boundary (yellow) and the tubulin-GFP channel (bottom) used in pixel classification-based segmentation. Scale bar: 5 μm . (C) Distributions showing cell volumes ($n = 15$) as determined in the landmark channels versus through pixel classification in the fluorescent tubulin channel. Boxes reflect the interquartile range, whiskers show the minimum and maximum. The medians are shown as horizontal white lines inside the boxes. Circles reflect measurements on individual spindles and are linked across the methods by lines. Hypothesis testing was performed using the Wilcoxon signed-rank test. N.s.: not significant ($p > 0.05$).



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Figure S6: Spindle mass is directly proportional to spindle volume. (A) Scatter plot displaying the relationship between spindle volume and spindle mass. **(B)** The relationship between spindle width and spindle mass and **(C)** the relationship between spindle length and spindle mass. Circles represent individual cells. r_s : Spearman correlation coefficient. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$, n.s.: not significant ($p > 0.05$).