Effective Cell Membrane Tension is Independent of Substrate Stiffness

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Abstract

Most animal cells are surrounded by a cell membrane and an underlying actomyosin cortex. Both structures are linked with each other, and they are under tension. Membrane tension and cortical tension both influence many cellular processes, including cell migration, division, and endocytosis. However, while actomyosin tension is regulated by substrate stiffness, how membrane tension responds to mechanical substrate properties is currently poorly understood. Here, we probed the effective membrane tension of neurons and fibroblasts cultured on glass and polyacrylamide substrates of varying stiffness using optical tweezers. In contrast to actomyosin-based traction forces, both peak forces and steady state tether forces of cells cultured on hydrogels were independent of substrate stiffness and did not change after blocking myosin II activity using blebbistatin, indicating that tether and traction forces are not directly linked with each other. Peak forces on hydrogels were about twice as high in fibroblasts if compared to neurons, indicating stronger membrane-cortex adhesion in fibroblasts. Finally, tether forces were generally higher in cells cultured on hydrogels compared to cells cultured on glass, which we attribute to substrate-dependent alterations of the actomyosin cortex and an inverse relationship between tension along stress fibres and cortical tension. Our results provide new insights into the complex regulation of membrane tension, and they pave the way for a deeper understanding of biological processes instructed by it.

Introduction

The cell membrane forms the interface between the interior of a cell and its environment. As a lipid bilayer surrounded by aqueous solutions, cell membranes are usually under tension. Different external forces are thought to contribute to membrane tension, including osmotic pressure and interactions with the force generating cytoskeleton. The plasma membrane is supported by the underlying actomyosin cortex, and molecular linkers form tight connections between the two layers.

Many cellular processes, such as the regulation of cell morphology, migration, division, stem cell fate choice, and endo- and exocytosis, are regulated by membrane tension[1–6]. Despite its biological importance[7–9], membrane tension is still difficult to quantify. In plane membrane tension is defined as the force per unit length acting tangentially on the membrane. However, because of the membrane's coupling to cytoskeletal elements, measurements of the 'effective' membrane tension usually contain contributions of the underlying actomyosin cortex and other force-generating elements, as membrane–cytoskeleton adhesion has to be overcome[10,11].

The actomyosin cortex is coupled to the extracellular environment through integral membrane proteins such as integrins and cadherins. It generates tensile forces which are exerted on the environment. These forces, and hence cytoskeletal tension, increase with substrate stiffness [12,13], suggesting that the measured effective membrane tension should increase on stiffer substrates. However, the regulation of membrane tension by substrate mechanics is currently poorly understood.

Results and discussion

We here measured the effective membrane tension of cells cultured on substrates of varying stiffness by pulling thin membrane tethers from the cell using a membrane-adherent bead held in an optical trap (OT)[2,14,15] (Fig. 1a, b; Fig. S1). Throughout a pull, the force on the tether can be continuously monitored, resulting in a characteristic force curve (Fig. 1c). The force rises quickly after initiation of the pull and reaches a peak before decreasing sharply. We attribute this peak force (PF) to the initial local detachment of plasma membrane from the actomyosin cortex (Fig. 1a). Subsequently, the tether is pulled until it reaches a set length. During this elongation process, the plasma membrane slides over the actin cortex while bonds between the membrane and the lipid binding proteins located in or at the cortex are broken and quickly re-established[16]. Following the elongation phase, the bead is held in a stable position and the steady-state force (SSF) is measured (Fig. 1b). The SSF is thought to reflect a combination of the in-plane plasma membrane tension and the membrane-cortex adhesion, which is in turn influenced by the cortical tension[16,17].

Tether forces are independent of hydrogel stiffness but PFs on glass depend on cell type

To test the effect of substrate mechanics and cortical tension on membrane tension, we performed tether pulls in fibroblasts, which possess a fully developed actomyosin cortex, and in neuronal axons, whose sub-membranous cytoskeleton is characterized by actin rings spaced by spectrin tetramers. Cells were cultured on glass as well as on hydrogels within a stiffness range adjusted to match their natural environment. For fibroblasts, hydrogel stiffnesses ranged from

82 100Pa (very soft; similar to subcutaneous adipose tissue), to 10kPa (stiff; already in the stiffness
83 range of many fibrotic tissues)[18]. For neurons, the highest substrate stiffness used was 1kPa,
84 corresponding to the highest stiffness experienced by these cells *in vivo*[19].

85 Peak forces were about twice as high in fibroblasts if compared to neuronal axons (Fig. 1e, h). In
86 both cell types, PFs were similar across all tested hydrogel substrates. In neurons, also PFs
87 measured in axons cultured on glass were similar to those seen on hydrogels (Fig. 1h). In
88 fibroblasts, however, we found significantly lower PFs when cultured on glass compared to those
89 cultured on hydrogels (Fig. 1e).

90 SSFs were significantly higher for fibroblasts and neurons cultured on hydrogels compared to
91 those cultured on glass (Fig. 1f, i). Similar to PFs, there was also no significant change in the SSFs
92 among the cells cultured on differently stiff hydrogels.

93 In contrast, actomyosin-based traction forces scale with substrate stiffnesses for both neurons
94 and fibroblasts[22]. Also in our culture conditions, cellular traction forces significantly increased on
95 stiffer substrates in both cell types within the investigated stiffness range (Fig. 1j, k), while tether
96 forces were independent of hydrogel substrate stiffness (Fig.1d-i). These data indicated that
97 traction forces and tether forces are not tightly coupled.

98

99 ## Tether forces on hydrogels but not SSFs on glass are independent of actomyosin contractility

100 Cellular cortical tension is mediated by actomyosin contractility[20]. In order to further examine
101 the interplay between cortical tension and membrane tension, we measured tether forces in

102 fibroblasts cultured on glass and 10 kPa hydrogels, where we would expect the highest cortical
103 tension[22], following treatment with the myosin inhibitor blebbistatin.

104 Blebbistatin treatment had no effect on tether forces in fibroblasts cultured on hydrogels (Fig. 2).
105 While PFs also did not change significantly after blocking actomyosin contractility in fibroblasts
106 cultured on glass, SSFs increased on glass following blebbistatin treatment[21] (Fig 2b).

107

108 ## Discussion

109 Here we found that the effective membrane tension, which is thought to scale with in-plane
110 membrane tension, does not change as a function of hydrogel substrate stiffness within a
111 physiologically relevant stiffness range in either fibroblasts or neurons (Fig. 1d-i). In contrast,
112 actomyosin-based traction forces increased on stiffer substrates (Fig. 1j-k), suggesting that
113 membrane tension is not linked to cellular contractility[28]. In line with this thought, the
114 application of the myosin II blocker, blebbistatin, did not alter the effective membrane tension in
115 cells cultured on hydrogels (Fig. 2).

116 However, we found differences in effective membrane tension between cells cultured on
117 hydrogels and glass. In fibroblasts, both PFs and SSFs were significantly lower in cells grown on
118 glass in comparison to those grown on hydrogels (Fig. 1d-f). On glass, the application of
119 blebbistatin led to a significant increase in effective membrane tension; both PFs and SSFs rose
120 to levels found in fibroblasts cultured on hydrogels (Fig. 2). In neurons, SSFs were also
121 significantly lower on glass than on hydrogels, whereas PFs on glass were similar to those found
122 on hydrogels of different stiffnesses (Fig. 1g-i).

123 Peak forces as a readout of membrane-cortex adhesion

124 Our results suggest that the largest contribution to the measured PF is the initial force required
125 to detach the membrane from the cortex, which is strongly related to membrane-cortex
126 adhesion (in analogy to attempting to open Velcro from the middle). Fibroblasts have a fully
127 developed actomyosin cortex, and proteins such as ezrin/radixin/moesin (ERM) proteins can
128 connect membrane proteins to the actin cytoskeleton throughout the cell surface. Neuronal
129 axons, on the other hand, have a highly ordered, periodic actin-spectrin network underneath
130 their membrane[23] (Fig. 3b), and much less area for ERM proteins to connect the membrane to
131 the actin rings, suggesting that membrane-cortex adhesion in axons is very low. Hence, this
132 difference in the structure of the cell cortex may explain why PFs are about twice as high in
133 fibroblasts than in neuronal axons cultured on physiologically stiff hydrogels.

134 The cortical structure, and hence likely also membrane-cortex adhesion, of most cell types varies
135 depending on, for example, the mitotic state[24,20] or the cell spread area[25]. The actin
136 cytoskeleton in fibroblasts changes drastically between hydrogel and glass surfaces (Fig. S2).
137 Glass is orders of magnitude stiffer than hydrogels, and it has different surface properties. These
138 mechanical and chemical differences between the substrates may not only lead to changes in
139 the actin cytoskeleton but also cause changes in membrane-cortex adhesion, explaining why PFs
140 in fibroblasts are higher on hydrogels than on glass. In neurons, where the actin-spectrin
141 network is preserved on the different substrates and membrane-cortex adhesion is likely low in
142 either environment, PFs were similar on hydrogel and glass substrates.

143 Interpretation of Steady State Forces

144 The significantly lower SSFs on glass compared to hydrogels in both cell types could result from
145 changes in either the in-plane membrane tension and/or the adhesion between cortex and
146 plasma membrane. As depicted in Figure 3, these differences in SSFs could be explained by a
147 purely mechanical model[26].

148 Particularly on very stiff glass substrates, fibroblasts developed strong stress fibres. Recent
149 experiments suggested that perturbations of stress fibres lead to an increase in tension of the
150 nearby cortex[27]. Thus, the contraction of stress fibres on glass might release tension in the cortex
151 and the membrane between focal adhesion sites (Fig. 3a), explaining why SSFs in fibroblasts
152 were lower on glass than on hydrogels (Fig. 1f), and why blebbistatin treatment, which relaxes
153 stress fibres, led to a significant increase in SSFs on glass but not on hydrogels (Fig. 2b).

154 However, blebbistatin treatment does not only affect stress fibres but also cortical tension. The
155 lack of a measurable change of SSFs after blebbistatin treatment in fibroblasts cultured on
156 hydrogels suggests either that the influence of the cortical tension on the SSFs is negligible, or
157 that the cortical tension changes very little under this treatment.

158 In developing neurons, grow cones at the leading tips of growing axons pull on their axons. The
159 magnitude of forces generated by growth cones increases with substrate stiffness (Fig. 1k),
160 suggesting that tension in the actin-spectrin cortex increases near the growth cone on stiffer
161 substrates and thus affects the plasma membrane tension in a similar way as described above
162 for fibroblasts (Fig. 3).

163

164 ## Conclusion

165 We found that effective membrane tension of cells cultured on hydrogels is independent of
166 substrate stiffness and traction forces. This robustness suggests that membrane tension might
167 either not be influenced by cortical tension, or it is actively maintained within a certain range.
168 Another possibility is that traction forces transmitted to the cortex might dissipate quickly so
169 that overall cortical tension would not scale with local traction forces[22]. If cortical and
170 membrane tension are coupled, changes in membrane tension on the different hydrogels would
171 happen only locally[29] near focal adhesions and cannot be seen when probed further away.
172 Future technological developments enabling highly resolved membrane tension measurements
173 will shed light on these important unresolved questions.

174

175 ## Author Contributions

176 J. Mc H., E. K., and K. A. performed tether pulling experiments. J. Mc H. analysed the results
177 from OT experiments. E. K. cultured 3T3 fibroblasts and prepared hydrogels. S. K. F. dissected
178 and cultured Xenopus laevis RGCs and prepared hydrogels. A.D. performed TFM experiments
179 with 3T3 fibroblasts. R. G. performed the TFM experiments with neurons and all TFM analyses. E.
180 K. and E. P. performed the immunostainings. J. Mc H. and E. K. designed experiments with 3T3
181 fibroblasts. J. Mc H., S. K. F., K. A., and U. F. K. designed pulling experiments with X. laevis RGCs.
182 S. K. F., E. K., U.F.K. and K. F. conceived the project. E. K., J. Mc H., S. K. F., U.F.K. and K.F. wrote
183 the manuscript.

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192 ## References

193 1. Raucher, D. & Sheetz, M. P. Cell Spreading and Lamellipodial Extension Rate Is Regulated by
194 Membrane Tension. *J. Cell Biol.* **148**, 127–136 (2000).
195 2. Nussenzveig, H. M. Cell membrane biophysics with optical tweezers. *Eur. Biophys. J.* **47**, 499–
196 514 (2018).
197 3. Gauthier, N. C., Fardin, M. A., Roca-Cusachs, P. & Sheetz, M. P. Temporary increase in plasma
198 membrane tension coordinates the activation of exocytosis and contraction during cell
199 spreading. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 14467–14472 (2011).
200 4. Keren, K. *et al.* Mechanism of shape determination in motile cells. *Nature* **453**, 475–480
201 (2008).
202 5. De Belly, H. *et al.* Membrane Tension Gates ERK-Mediated Regulation of Pluripotent Cell Fate.
203 *Cell Stem Cell* **28**, 273-284.e6 (2021).
204 6. Dai, J. & Sheetz, M. P. Regulation of endocytosis, exocytosis, and shape by membrane tension.
205 *Cold Spring Harb. Symp. Quant. Biol.* **60**, 567–571 (1995).
206 7. Sheetz, M. P. Cell control by membrane–cytoskeleton adhesion. *Nat. Rev. Mol. Cell Biol.* **2**,
207 392–396 (2001).
208 8. Pontes, B., Monzo, P. & Gauthier, N. C. Membrane tension: A challenging but universal
209 physical parameter in cell biology. *Semin. Cell Dev. Biol.* **71**, 30–41 (2017).
210 9. Pontes, B. *et al.* Membrane tension controls adhesion positioning at the leading edge of cells.
211 *J. Cell Biol.* **216**, 2959–2977 (2017).
212 10. Salbreux, G., Charras, G. & Paluch, E. Actin cortex mechanics and cellular morphogenesis.
213 *Trends Cell Biol.* **22**, 536–545 (2012).

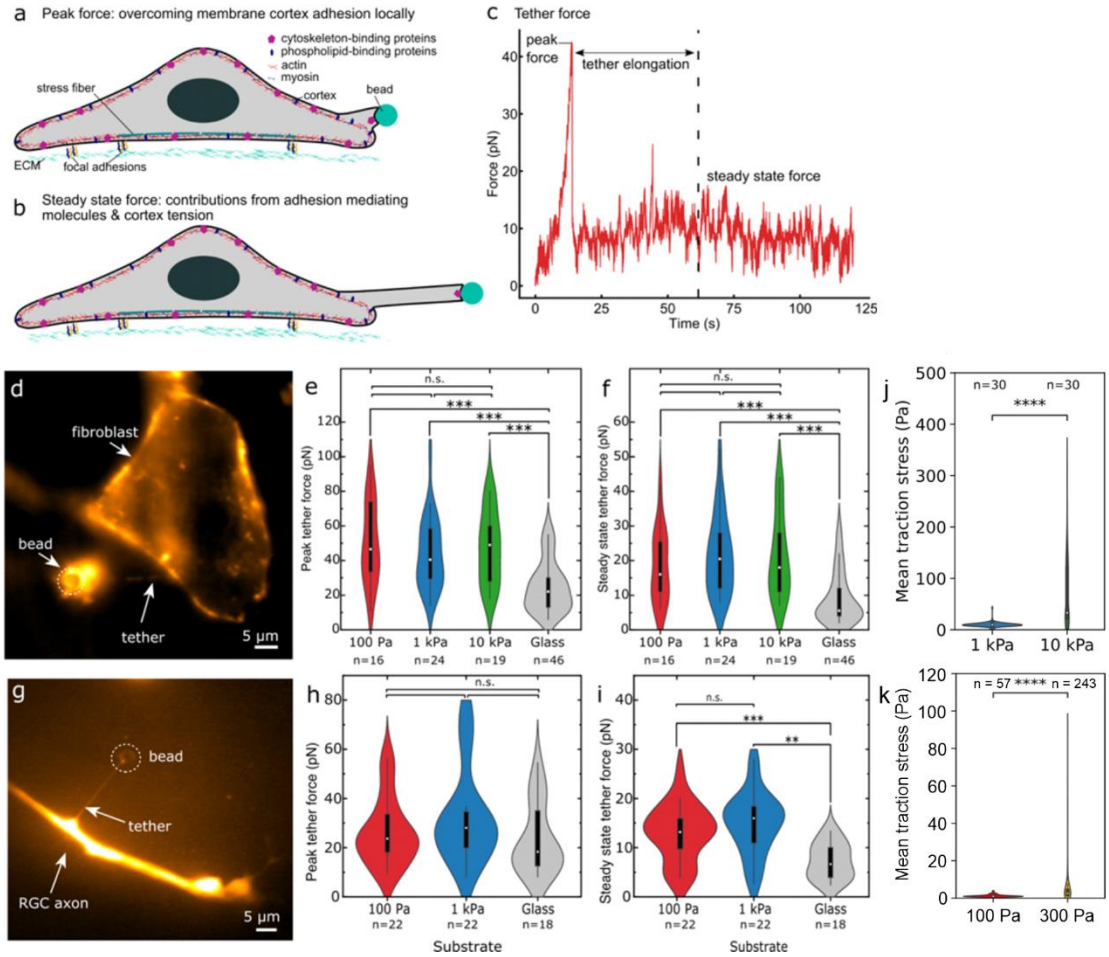
214 11. Clark, A. G., Wartlick, O., Salbreux, G. & Paluch, E. K. Stresses at the Cell Surface during
215 Animal Cell Morphogenesis. *Curr. Biol.* **24**, R484–R494 (2014).
216 12. Sabass, B., Gardel, M. L., Waterman, C. M. & Schwarz, U. S. High Resolution Traction
217 Force Microscopy Based on Experimental and Computational Advances. *Biophys. J.* **94**, 207–
218 220 (2008).
219 13. Califano, J. P. & Reinhart-King, C. A. Substrate Stiffness and Cell Area Predict Cellular
220 Traction Stresses in Single Cells and Cells in Contact. *Cell. Mol. Bioeng.* **3**, 68–75 (2010).
221 14. Sheetz, M. P. Cell control by membrane–cytoskeleton adhesion. *Nat. Rev. Mol. Cell Biol.*
222 **2**, 392–396 (2001).
223 15. Datar, A., Bornschlögl, T., Bassereau, P., Prost, J. & Pullarkat, P. A. Dynamics of membrane
224 tethers reveal novel aspects of cytoskeleton-membrane interactions in axons. *Biophys. J.* **108**,
225 489–497 (2015).
226 16. Gauthier, N. C., Masters, T. A. & Sheetz, M. P. Mechanical feedback between membrane
227 tension and dynamics. *Trends Cell Biol.* **22**, 527–535 (2012).
228 17. Sens, P. & Plastino, J. Membrane tension and cytoskeleton organization in cell motility. *J.*
229 *Phys. Condens. Matter* **27**, (2015).
230 18. Wells, R. G. Tissue Mechanics and Fibrosis. *Biochim. Biophys. Acta* **1832**, 884–890 (2013).
231 19. Franze, K., Janmey, P. A. & Guck, J. Mechanics in Neuronal Development and Repair.
232 *Annu. Rev. Biomed. Eng.* **15**, 227–251 (2013).
233 20. Chugh, P. *et al.* Actin cortex architecture regulates cell surface tension. *Nat. Cell Biol.* **19**,
234 689–697 (2017).

235 21. Lieber, A. D., Yehudai-Resheff, S., Barnhart, E. L., Theriot, J. A. & Keren, K. Membrane
236 Tension in Rapidly Moving Cells Is Determined by Cytoskeletal Forces. *Curr. Biol.* **23**, 1409–
237 1417 (2013).
238 22. Rheinlaender, J. *et al.* Cortical cell stiffness is independent of substrate mechanics. *Nat.*
239 *Mater.* **19**, 1019–1025 (2020).
240 23. Xu, K., Zhong, G. & Zhuang, X. Actin, spectrin, and associated proteins form a periodic
241 cytoskeletal structure in axons. *Science* **339**, 452–456 (2013).
242 24. Chaigne, A. *et al.* A soft cortex is essential for asymmetric spindle positioning in mouse
243 oocytes. *Nat. Cell Biol.* **15**, 958–966 (2013).
244 25. Kumar, R., Saha, S. & Sinha, B. Cell spread area and traction forces determine myosin-II-
245 based cortex thickness regulation. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1866**, 118516
246 (2019).
247 26. Walcott, S. & Sun, S. X. A mechanical model of actin stress fiber formation and substrate
248 elasticity sensing in adherent cells. *Proc. Natl. Acad. Sci.* **107**, 7757–7762 (2010).
249 27. Vignaud, T. *et al.* Stress fibres are embedded in a contractile cortical network. *Nat.*
250 *Mater.* 1–11 (2020) doi:10.1038/s41563-020-00825-z.
251 28. Mandal, K. *et al.* Soft hyaluronic gel promotes cell spreading, stress fibers, focal adhesion,
252 membrane tension by phosphoinositide signaling, not traction force. *ACS Nano* **13**, 203–214
253 (2019).
254 29. Shi, Z., Graber, Z. T., Baumgart, T., Stone, H. A. & Cohen, A. E. Cell membranes resist flow.
255 *Cell* **175**, 1769-1779.e13 (2018).

256 30. Raucher, D. *et al.* Phosphatidylinositol 4,5-bisphosphate functions as a second messenger
257 that regulates cytoskeleton-plasma membrane adhesion. *Cell* **100**, 221–228 (2000).
258 31. Pontes, B. *et al.* Cell cytoskeleton and tether extraction. *Biophys. J.* **101**, 43–52 (2011).
259 32. Pontes, B. *et al.* Membrane elastic properties and cell function. *PLoS One* **8**, e67708
260 (2013).
261 33. Wallingford, J. B., Liu, K. J. & Zheng, Y. Xenopus. *Curr. Biol.* **20**, 263–264 (2010).
262 34. Koser, D. E. *et al.* Mechanosensing is critical for axon growth in the developing brain. *Nat.*
263 *Neurosci.* **19**, 1592–1598 (2016).
264 35. Nieuwkoop, P. D. & Faber, J. *Normal table of xenopus laevis (daudin)*. (Routledge, 1994).
265 36. Swift, J. *et al.* Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed
266 differentiation. *Science* **341**, (2013).
267 37. Moshayedi, P. *et al.* Mechanosensitivity of astrocytes on optimized polyacrylamide gels
268 analyzed by quantitative morphometry. *J. Phys. Condens. Matter* **22**, (2010).
269 38. Mc Hugh, J., Andresen, K. & Keyser, U. F. Cation dependent electroosmotic flow in glass
270 nanopores. *Appl. Phys. Lett.* **115**, 113702 (2019).
271 39. Gittes, F. & Schmidt, C. F. Chapter 8 signals and noise in micromechanical measurements.
272 in *Methods in cell biology.* vol. 55 129–156 (Academic Press, 1997).
273 40. Hochmuth, R. M., Shao, J. Y., Dai, J. & Sheetz, M. P. Deformation and flow of membrane
274 into tethers extracted from neuronal growth cones. *Biophys. J.* **70**, 358–369 (1996).
275 41. Moshayedi, P. *et al.* The relationship between glial cell mechanosensitivity and foreign
276 body reactions in the central nervous system. *Biomaterials* **35**, 3919–3925 (2014).

42. Corder, G. W. & Foreman, D. I. *Nonparametric statistics for non-statisticians: A step-by-step approach*. (Wiley-Blackwell, 2009).

281 ## Figures



282

283 *Figure 1: Peak and steady state tether forces provide different insights into cell properties. a) When an*
284 *OT pull is initiated, the local membrane-cortex adhesion first needs to be overcome, resulting in the*
285 *peak force. b) After tether extension, the bead is held stationary, and the steady state force, which*
286 *scales with membrane tension, is recorded. c) A typical force curve recorded during a tether pull shows*
287 *a peak (see (a)) at the beginning of the pull and a steady state force when the tether is held at its*
288 *maximum extension (see (b). (d-f, j) 3T3 fibroblasts and (g-I, k) Xenopus retinal ganglion cell axons*
289 *on different substrates; shear moduli are provided in Pa. d) Fluorescence image of a lipid tether pulled*
290 *from a 3T3 fibroblast. e) Peak tether forces (PFs) and f) steady state tether forces (SSFs) of fibroblasts*

291 *are similar on all hydrogel substrates irrespective of their stiffness and higher compared to on glass. g)*

292 *Fluorescence image of a lipid tether pulled from an axon. h) PFs in axons are similar on all hydrogel*

293 *and glass substrates. i) SSFs are similar on all hydrogels and higher if compared to glass. j-k) Traction*

294 *forces measured for (j) 3T3 fibroblasts and (k) neuronal axons. Both cell types exerted higher traction*

295 *forces on stiff hydrogels compared to softer ones.*

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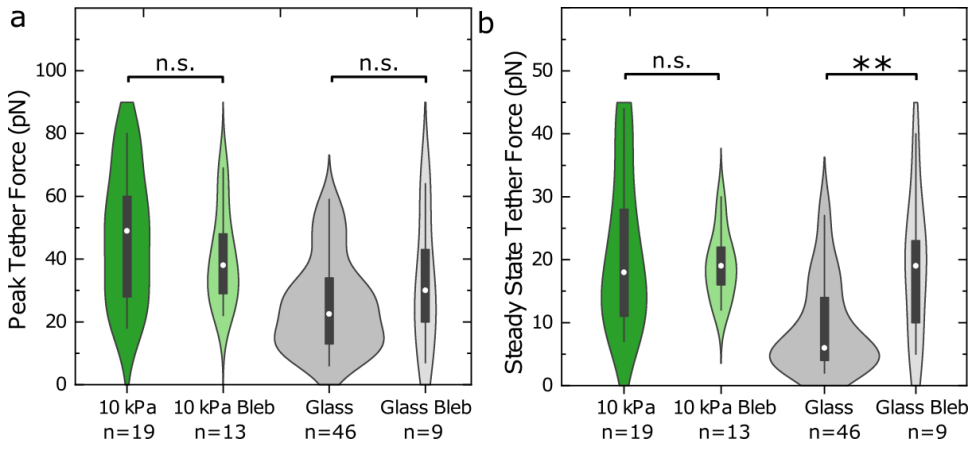

Figure 2: The effect of actomyosin contractility on PFs and SSFs in fibroblasts. a) Blebbistatin application did not significantly alter PFs on glass and hydrogel substrates. b) SSFs are unaffected in cells growing on hydrogels but significantly increase on glass substrates after blebbistatin treatment.

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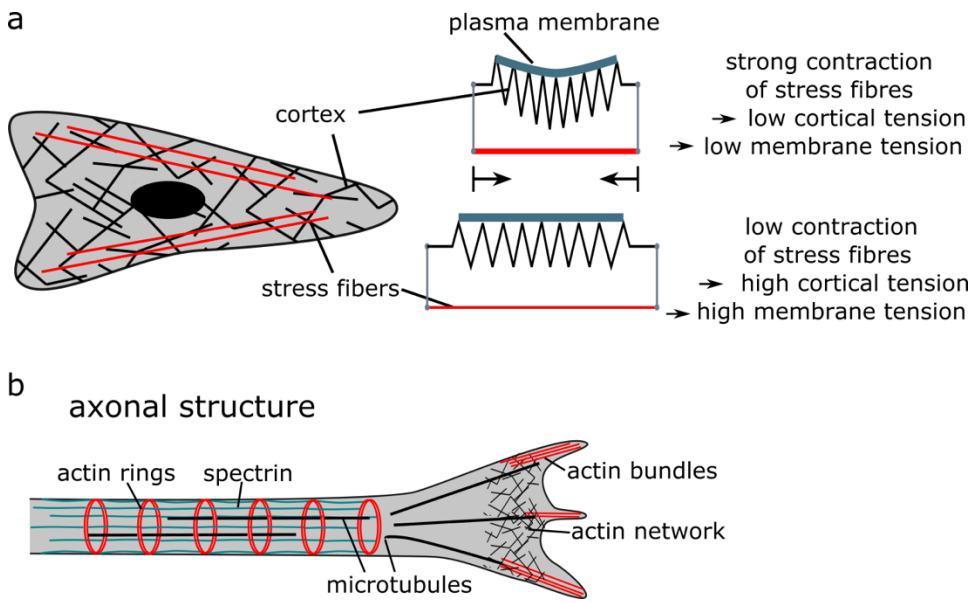

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304

305 *Figure 3: Toy model of substrate type-dependent effective membrane tension. a) Schematic*

306 *representation of the cell cortex and stress fibres in fibroblasts. The strong contraction of stress fibres*

307 *on glass substrates might relax the tension in the cortex and thus reduce membrane tension. The*

308 *relaxation of these stress fibres, on the other hand, might increase the cortical tension. The top model*

309 *illustrates the tension in untreated fibroblasts grown on glass. The bottom shows the tension in the*

310 *relevant components in fibroblasts on glass treated with blebbistatin or in untreated fibroblasts*

311 *grown on hydrogels. b) The axon has a periodic actin ring-spectrin network underneath its*

312 *membrane*[23]*, which is likely rather static and poorly adhered to the membrane.*

313