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1 **TITLE:**
2 Magnetic Tweezers in a Microplate Format
3

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16 **KEYWORDS:**
17 mechanobiology, tweezers, 3D-printed, magnets, FRET, ensemble
18

19 **SUMMARY:**
20 Here, we describe the use of a novel microplate assay to enable mechanical manipulation of
21 biomolecules while performing ensemble biochemical assays. This is achieved using a microplate
22 lid modified with magnets to create multiple static magnetic tweezers across the microplate.
23

24 **ABSTRACT:**
25 Mechanobiology describes how the physical forces and mechanical properties of biological
26 material contribute to physiology and disease. Typically, these approaches are limited single-
27 molecule methods, which restricts their availability. To address this need, a microplate assay was
28 developed that enables mechanical manipulation while performing standard biochemical assays.
29 This is achieved using magnets incorporated into a microplate lid to create multiple magnetic
30 tweezers. In this format, force is exerted across biomolecules connected to paramagnetic beads,
31 equivalent to a typical magnetic tweezer. The study demonstrates the application of this tool
32 with FRET-based assays to monitor protein conformations. However, this approach is widely
33 applicable to different biological systems ranging from measuring enzymatic activity through to
34 the activation of signaling pathways in live cells.
35

36 **INTRODUCTION:**
37 Mechanobiology focuses on understanding how the propagation of physical forces within and
38 between cells regulates cellular activity^{1,2} and how this correlates with the organization and
39 dynamics of both proteins and cells.
40

41 Single-molecule force measurements have revealed how force is used in biological systems, from
42 single proteins to whole cells and tissues³⁻⁷. These challenging experiments require specialized
43 equipment and technical expertise. Conversely, standard biochemical assays can be performed
44 at higher throughput in readily available commercial equipment.

45
46 Here, the study describes a mechanobiology assay that enables magnetic tweezer-based
47 manipulation and biochemical assays to be performed together⁸. Magnets are placed on a 3D
48 printed microplate lid (**Figure 1A–D**), enabling the use of commercial plate readers for the assays.
49 Force is applied across the biomolecule of interest by coupling the molecule to paramagnetic
50 particles. The magnets then exert tension across the molecule. Altering the distance between the
51 particles and magnets adjusts the exert force across the biomolecule (**Figure 1E**).

52
53 We represent the use of this assay using the actin-based molecular motor, Myosin VI. Myosin VI
54 is regulated by intramolecular backfolding⁹. Myosin VI has been shown to exist in an auto-
55 inhibited state, whereby the binding of partner proteins, such as NDP52, triggers the unfolding
56 of myosin VI^{10,11}. To perform these assays, we will use a dual-labeled construct of the myosin VI
57 tail domain with an N-terminal GFP and a C-Terminal RFP whereby backfolding of the protein
58 generates Fluorescence Resonance Energy Transfer (FRET) between GFP and RFP. The N-
59 terminus also carries a biotinylation tag to immobilize the protein on the surface. We use this
60 assay in combination with FRET measurements to show how force can impact myosin VI back-
61 folding.

62 63 **PROTOCOL:**

64
65 Sample proteins required for this experiment and a list of reagents are found in the **Table of**
66 **Materials**. Equivalent proteins should be produced for the user's system of study to measure
67 conformation changes.

68 69 **1. 3D printed magnetic lid**

70
71 1.1. Design a microplate lid to house the magnets within a 24-well microplate. An example
72 CAD file can be downloaded from GitHub¹². The measurements are shown in **Figure 1**.

73
74 NOTE: The height of the pedestal can be altered to change the distance between magnets and
75 surface to alter the applied force. In addition, the final force exerted upon the particle depends
76 on the particle size and gap between the pair of magnets. Equations to calculate the applied force
77 for 1 μm and 2.8 μm particles at 0.5 mm, 1 mm, and 2 mm gaps are given in Dos Santos et al.⁸.
78 For the experiments described here with 2.8 μm particles and a 2 mm gap, use Equation 1.

79
80 Equation 1: $FORCE = -0.0078 + 43.5\left(\frac{-(Z-0.4)}{1.58}\right) - 30.4\left(\frac{-(Z-0.4)}{0.319}\right)$

81
82 1.2. 3D print a microplate lid using Polyethylene Terephthalate Glycol (PETG) on a 3D printer
83 system.

84
85 NOTE: Use a printer with a layer resolution of 0.02 mm to enable accuracy and reproducibility.
86 The impact of printer accuracy was discussed in Dos Santos et al.⁸.

87

88 1.3. Attach pairs of 5 mm cube Neodymium N42 magnets using adhesive to the pedestals
89 with the magnetic poles orientated to create the field directed towards the bottom of the
90 microplate.

91

92 1.4. Leave at least 1 position empty to act as a no-magnet control.

93

94 1.5. Store in a sealed container of sufficient size to readily place and remove the lid.

95

96 **2. Microplate surface modification**

97

98 2.1. Take a clean non-treated glass-bottomed 24-well microplate.

99

100 2.2. Wash the wells 3x with 500 μ L of wash buffer (50 mM Tris-HCl (pH 7.5) and 50 mM NaCl)
101 at room temperature.

102

103 2.3. Add 200 μ L of biotin-BSA (0.2 mg/mL) in the wash buffer and leave it undisturbed for 15
104 min at room temperature.

105

106 NOTE: Biotin-BSA is used for both passivation and attachment. Other passivation and surface
107 attachment strategies can be used. For first-time use and optimization, the concentration of
108 biotin-BSA can be varied up to 1 mg/mL.

109

110 2.4. Wash the wells 3x with 500 μ L of wash buffer.

111

112 2.5. Add 200 μ L of streptavidin (20 μ g/mL) in the wash buffer and leave it undisturbed for 15
113 min at room temperature.

114

115 NOTE: For first-time use, the concentration of streptavidin can be varied up to 1 mg/mL.

116

117 2.6. Remove the solution and then wash the wells 3x with wash buffer.

118

119 2.7. Cover the wells with 500 μ L of wash buffer.

120

121 2.8. Store at 4 °C until ready to use. Plates should be used on the same day.

122

123 **3. Sample preparation: Protein immobilization**

124

125 3.1. Allow the surface-modified microplate to reach room temperature.

126

127 3.2. Wash the wells with 500 μ L of wash buffer.

128

129 3.3. Add 100 nM biotin-eGFP-Myosin VI Tail-mRFP (stock sample protein) and incubate for 15
130 min at room temperature in wash buffer.

131

132 NOTE: This example protein is produced as described by Dos Santos et al.⁸. Concentrations and
133 incubations times can vary depending upon the protein.

134

135 3.4. Wash the wells 3x with 500 μ L of wash buffer.

136

137 3.5. Set up the plate reader to record fluorescence spectra for GFP (excitation 490 nm and
138 emission 510–600 nm) and RFP (excitation 560 nm and emission 580–650nm) to confirm the
139 presence of the fusion proteins.

140

141 3.6. If there is a lack of protein, increase the concentration at step 3.3.

142

143 NOTE: Poor binding could relate to inadequate biotin-BSA and/or streptavidin binding, and
144 therefore, the amounts could be increased 10-fold.

145

146 3.7. If possible, perform a well-scan measurement to determine if the fusion protein is bound
147 across the microplate (**Figure 2**).

148

149 NOTE: This measurement will record the fluorescence intensity at specific points across the
150 microplate well. It will reveal if the protein is bound across the well.

151

152 3.8. Ensure that the signal is uniform (within 5%) in the center of the well where the assay
153 measurement occurs.

154

155 4. Magnetic bead preparation

156

157 4.1. Vortex the vial of 2.8 μ m paramagnetic beads with recombinant Protein A (**Table of**
158 **Materials**) for 30 s to resuspend the beads.

159

160 4.2. Transfer the volume corresponding to 1 mg of paramagnetic beads to a 1.5 mL tube.

161

162 4.3. Place the tube in a magnetic isolator (**Table of Materials**) and then remove the
163 supernatant.

164

165 4.4. Resuspend the beads in 200 μ L of PBS by gently pipetting the solution up and down.

166

167 4.5. Place the tube in the magnetic isolator and then remove the supernatant.

168

169 4.6. Repeat the wash step 3x.

170

171 4.7. Dilute 10 μ g of Anti-RFP antibody in 200 μ L of PBS.

172

173 4.8. Isolate the beads in the magnetic isolator and then resuspend in the antibody solution.

174

175 4.9. Rotate at 20 rpm for 10 min at room temperature on a benchtop rotator.

176

177 4.10. Place the tube in the magnetic isolator and remove the supernatant.

178

179 4.11. Wash 3x in PBS.

180

181 4.12. Resuspend the beads in 200 μ L of wash buffer.

182

183 4.13. To confirm antibody immobilization on the beads, incubate the antibody-loaded beads
184 with a solution of $>1 \mu$ M RFP fusion protein.

185

186 NOTE: Confirm visually whether the protein is isolated by the beads.

187

188 4.14. Alternatively, measure the fluorescence intensity of the supernatant (excitation 560 nm
189 and emission 580–650nm) for RFP in a fluorescence spectrometer.

190

191 **5. Sample preparation: Bead attachment**

192

193 5.1. Add 10 μ g of the paramagnetic bead-antibody fusion diluted in wash buffer (200 μ L) to
194 the protein-bound microplate wells.

195

196 5.2. Do not place beads in one control well to check for the effect of beads on the fluorescent
197 signal. Prepare a control sample without antibodies to determine any non-specific interactions
198 with the beads.

199

200 5.3. Incubate the microplate at room temperature for 30 min.

201

202 5.4. Wash the wells 3x with 500 μ L of wash buffer.

203

204 5.5. If available locally, check that the beads are bound to the surface on a brightfield
205 microscope. If there is a lack of beads, increase the concentration in step 5.1 to 500 μ g/mL.

206

207 **6. Data acquisition**

208

209 6.1. Place the microplate, without lid, into a fluorescent plate reader at 25 $^{\circ}$ C.

210

211 6.2. Set up the plate reader to record fluorescence spectra for GFP (excitation 490 nm and
212 emission 510–600 nm) and RFP (excitation 560 nm and emission 580–650 nm) to confirm the
213 presence of the fusion proteins.

214

215 NOTE: Measure/record the fluorescence spectra through the bottom of the microplate to place
216 the lid on top. Therefore, a suitable microplate reader should be selected.

217

218 6.3. Record a FRET fluorescence spectrum (excitation 490 nm and emission 510–650nm), or
219 monitor fluorescence at 510 nm and 610 nm with excitation at 490 nm. This is set up according

220 to the manufacturer's guidance.

221

222 6.4. Remove the microplate from the reader and add the magnetic lid.

223

224 6.5. Return to the plate reader and leave the sample for 2 min.

225

226 6.6. Repeat the FRET measurement, as in step 6.3.

227

228 7. Data analysis

229

230 7.1. Export the data for import into a spreadsheet (e.g., Excel).

231

232 7.2. Manually extract the data for Donor GFP at 510 nm and acceptor RFP at 610 nm.

233

234 7.3. Calculate relative FRET using Equation 2 for each condition.

235

236 Equation 2:
$$FRET = \frac{A}{(D+A)}$$

237

238 A: acceptor intensity (RFP 610 nm), D: donor intensity (GFP 510 nm).

239

240 NOTE: Use the recombinant RFP under the same excitation and emission conditions to control
241 for the background excitation of the acceptor.

242

243 REPRESENTATIVE RESULTS:

244 **Figure 2** shows an example of a well-scan measurement where the fluorescence intensity of GFP
245 has been recorded at 1 mm intervals across the microplate well. Typical fluorescence
246 measurements are performed at the center position of the microplate well (position 8,8 in **Figure**
247 **2**); it is, therefore, important that there is bound protein at this location. As shown in **Figure 2**,
248 the intensity is highest in the center of the well within a radius of a few millimeters. Typically,
249 immobilization is better away from the edges of the well, potentially due to protein binding to
250 the side walls. The poor signal due to lack of protein binding or defects in biotin-BSA and/or
251 streptavidin would be identified here. A fluorescent streptavidin conjugate could be used to
252 confirm biotin-BSA on the surface. Likewise, a control protein consisting of biotin-GFP would
253 determine if streptavidin is bound to the biotin-BSA surface. As mentioned in the protocol, the
254 amount of biotin-BSA, streptavidin, and protein can be increased.

255

256 **Figure 3** provides example data for the forced-induced unfolding of myosin VI. The biotin-eGFP-
257 Myosin VI Tail-mRFP construct¹¹ was immobilized on the microplate surface and coupled to the
258 paramagnetic beads using an anti-RFP antibody (**Figure 3A**). In this scenario, a high FRET state is
259 found when myosin VI is backfolded where the fluorescent proteins are in close proximity.

260

261 A FRET spectrum was recorded in the absence of the magnetic lid (**Figure 3B**), where a signal at
262 610 nm can be observed. If no signal is seen at either 510 nm or 610 nm, individual fluorescence

263 spectra should be recorded for GFP and RFP to determine if the fusion is intact. The experiment
264 was then repeated in the presence of the lid with magnets generating an assumed force of 1.8
265 pN, arising from a 0.5 mm pedestal with a 2 mm gap between magnets. The intensity at 610 nm
266 was lost, indicating a loss of FRET due to a conformation change. The relative FRET was calculated
267 using Equation 1, where a decrease is observed following the addition of force. A change in
268 conformation triggering the FRET change will lead to an intensity decrease in 610 nm (acceptor)
269 and an intensity increase in 510 nm (donor). If only one changes, then the effect is likely to be
270 the disruption of the fluorescent proteins rather than the conformation change.

271
272 The experiment was repeated over a range of forces where magnets were positioned at different
273 heights relative to the bottom of the microplate. Based on the height, the gap between magnets,
274 and particle size, the force applied to the sample (assumed force) can be calculated based on
275 equations⁸. The Relative FRET decreased upon the addition of force up to 1 pN, but it did not
276 change for greater forces (**Figure 3C**). The absence of anti-RFP antibodies or paramagnetic beads
277 stops the force application upon myosin VI, and therefore there is no change in Relative FRET.
278 The presence of the antibody or beads could cause a change in the fluorescence signal due to
279 environmental changes¹³. This may impact the FRET calculation, so it is critical to perform the
280 control experiments. Lastly, the application of high force could remove the construct from the
281 surface or cause a break in the protein. Therefore, it is important to ensure there is still a signal
282 from both GFP and RFP following a decrease in Relative FRET.

283
284 **FIGURE AND TABLE LEGENDS:**
285 **Figure 1: Example design for 3D printed magnetic lid.** (A) Side and (B) top-down projections
286 showing the position of the pedestals which hold the magnets. The pedestals in rows 1 and 2 are
287 at 0.5 cm, 0.4 cm, 0.3 cm, 0.2 cm and 0.1 cm, respectively. The measurements stated here will
288 differ for each brand and type of microplate. (C) The pedestals determine the force applied to
289 the sample. The width of the divider (black) can be altered to change the space between the
290 magnets. This will alter the strength of the magnetic field, as detailed in Dos Santos et al.⁸. The
291 height of the spacer (blue) can be varied to adjust the distance between the magnets and the
292 surface to alter the force applied on the sample, as described in Dos Santos et al.⁸. (D) Image of
293 an example 3D printed lid. (E) Principle for exerting forces on biomolecules. A molecule of interest
294 is attached to the surface and bound to a paramagnetic particle. A pair of 5 mm cube neodymium
295 magnets attached to the lid (grey) exert force on the beads through their magnetic field.
296 Increasing the proximity of the magnets to the beads leads to higher forces exerted across the
297 molecule. In this assay, the proximity is altered using different size pedestals.

298
299 **Figure 2: Well scan depicting bound protein on the surface of the microplate.** The eGFP-Myosin
300 VI Tail-mRFP construct was immobilized on the surface through an N-terminal biotinylation tag,
301 as shown in **Figure 3A**. A well-scan measurement was performed across the microplate well at 1
302 mm intervals monitoring the fluorescence of GFP (excitation at 490 nm and emission at 515 nm).
303 The GFP fluorescence intensity numbers are presented as a heat plot.

304
305 **Figure 3: Representative results for the force-induced unfolding of myosin VI.** (A) Diagram of
306 the assay format. The eGFP-Myosin VI Tail-mRFP construct was immobilized through an N-

307 terminal biotinylation tag (BRS). The C-terminus was coupled to Protein-A paramagnetic beads
308 through an anti-RFP. When no force is applied, the protein is folded, which generates a high FRET
309 signal. The application of force (F) triggers unfolding, leading to a low FRET state. **(B)** Example
310 fluorescence spectra following GFP excitation in the absence (red) and presence of 1.8 pN of
311 force (dark blue). INSET: Relative FRET calculated using Equation 1 for 3 replicates. The color
312 legend matches the spectra. **(C)** Plot of Relative FRET obtained under different forces by varying
313 the pedestal height from 0.1–1 cm. Data are also shown for control experiments in the absence
314 of the antibody and beads. Error bars represent SEM from 3 independent experiments.

315

316 **DISCUSSION:**

317 This approach enables force-based measurements to be readily applied in a microplate using
318 fluorescent plate readers. Importantly, this assay format assumes there is functional protein
319 when it is bound to a surface. Therefore, prior knowledge is required before embarking on these
320 measurements to ensure there is protein activity. It is also beneficial to make sure that the
321 binding of molecules to the paramagnetic beads and surface is optimized for each system.

322

323 This concept can be modified to function across all types of microplates; however, the magnet
324 selection will need to be adapted for microplates above 24 wells, as magnets cannot fit in the
325 wells. Magnets can still be placed on top of the wells; however, in a 24 well plate, this does not
326 lead to stray fields across the other wells⁸; in smaller wells, such as 96 well plates, there may be
327 field variations between the wells. Lastly, it is possible to use the same approach while
328 performing fluorescence microscopy on an inverted microscope.

329

330 This approach does not have the force accuracy found within single-molecule measurements and
331 therefore aims to complement those methodologies. Furthermore, this approach applies a fixed
332 amount of force to the sample and does not allow forces to be varied within a well or monitor
333 forces exerted by a protein. Moreover, assumptions are made which does not apply to single-
334 molecule measurements. For example, we cannot determine the number of molecules attached
335 to each bead. However, the molecule attachment is assumed to be the same for each bead-tether
336 pairing; therefore, relative force changes can be measured. In addition, we cannot determine if
337 a molecule is bound by a paramagnetic particle, and therefore we do not know what fraction of
338 the biomolecule population is generating the signal.

339

340 In summary, using this approach, it is now possible to quantitatively study biological processes
341 using established assays under mechanical load. Moreover, multiple conditions can be tested
342 simultaneously, thereby increasing the throughput.

343

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347

348 **DISCLOSURES:**

349 The authors declare no competing interests.

350

351 **REFERENCES:**

- 352 1. Jansen, K. A. et al. A guide to mechanobiology: Where biology and physics meet.
353 *Biochimica et Biophysica Acta*. **1853**, 3043–3052 (2015).
- 354 2. Dos Santos, A., Toseland, C. P. Regulation of nuclear mechanics and the impact on dna
355 damage. *International Journal of Molecular Sciences*. **22** (6), 3178 (2021).
- 356 3. Dos Santos, A. et al. DNA damage alters nuclear mechanics through chromatin
357 reorganization. *Nucleic Acids Research*. **49** (1), 340–353 (2020).
- 358 4. Elosegui-Artola, A. et al. Force triggers YAP nuclear entry by regulating transport across
359 nuclear pores. *Cell*. **171**, 1397–1410 e1314, (2017).
- 360 5. Lherbette, M. et al. Atomic force microscopy micro-rheology reveals large structural
361 inhomogeneities in single cell-nuclei. *Scientific Reports*. **7**, 8116 (2017).
- 362 6. Seo, D. et al. A mechanogenetic toolkit for interrogating cell signaling in space and time.
363 *Cell*. **165**, 1507–1518 (2016).
- 364 7. Yao, M. et al. The mechanical response of talin. *Nature Communications* **7**, 11966 (2016).
- 365 8. Dos Santos, A., Fili, N., Pearson, D. S., Hari-Gupta, Y., Toseland, C. P. High-throughput
366 mechanobiology: Force modulation of ensemble biochemical and cell-based assays. *Biophysical*
367 *Journal*. **120**, 631–641 (2021).
- 368 9. Fili, N., Toseland, C. P. Unconventional myosins: How regulation meets function.
369 *International Journal of Molecular Sciences*. **21** (1), 67 (2019).
- 370 10. Fili, N. et al. Competition between two high- and low-affinity protein-binding sites in
371 myosin VI controls its cellular function. *Journal of Biological Chemistry*. **295**, 337–347 (2020).
- 372 11. Fili, N. et al. NDP52 activates nuclear myosin VI to enhance RNA polymerase II
373 transcription. *Nature Communications*. **8**, 1871 (2017).
- 374 12. MagPlate at GitHub <<https://github.com/cptoseland/MagPlate>> (2021)
- 375 13. Toseland, C. P. Fluorescent labeling and modification of proteins. *Journal of Chemical*
376 *Biology*. **6**, 85–95 (2013).

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378

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