Production of dynein and kinesin motor ensembles on DNA origami nanostructures for single molecule observation
--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60369R1
Full Title:	Production of dynein and kinesin motor ensembles on DNA origami nanostructures for single molecule observation
Keywords:	Cytoskeleton, dynein, kinesin, molecular motors, DNA origami, single molecule methods
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Northampton, MA, United States

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July 2, 2019

Dear Dr. Steindel,

We are pleased to submit our revised manuscript entitled "**Production of dynein and kinesin motor ensembles on DNA origami for single molecule observation**."

We appreciate the investment of time and energy by the editors at *JoVE* and the reviewers who offered careful commentary and suggestions on our manuscript. We have addressed each point made by both editors and reviewers, and have included every suggestion in our revised manuscript. All major changes to the manuscript are listed in blue text.

We have also included a Response to Reviewers, where we enumerate each change. Our responses are also listed in blue text.

We appreciate the opportunity to submit our revised manuscript to *JoVE*!

Sincerely,

Nathan D. Derr, Ph.D.
Assistant Professor of Biological Sciences
Director, Smith College Center for Microscopy and Imaging

1 **TITLE:**
2 **Production of Dynein And Kinesin Motor Ensembles on DNA Origami Nanostructures for Single**
3 **Molecule Observation**
4
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17 **KEYWORDS:**
18 cytoskeleton, dynein, kinesin, molecular motors, DNA origami, single molecule methods
19
20 **SUMMARY:**
21 The goal of this protocol is to form ensembles of molecular motors on DNA origami
22 nanostructures and observe the ensemble motility using total internal reflection fluorescence
23 microscopy.
24
25 **ABSTRACT:**
26 Cytoskeletal motors are responsible for a wide variety of functions in eukaryotic cells, including
27 mitosis, cargo transport, cellular motility, and others. Many of these functions require motors to
28 operate in ensembles. Despite a wealth of knowledge about the mechanisms of individual
29 cytoskeletal motors, comparatively less is known about the mechanisms and emergent behaviors
30 of motor ensembles, examples of which include changes to ensemble processivity and velocity
31 with changing motor number, location, and configuration. Structural DNA nanotechnology, and
32 the specific technique of DNA origami, enables the molecular construction of well-defined
33 architectures of motor ensembles. The shape of cargo structures as well as the type, number and
34 placement of motors on the structure can all be controlled. Here, we provide detailed protocols
35 for producing these ensembles and observing them using total internal reflection fluorescence
36 microscopy. Although these techniques have been specifically applied for cytoskeletal motors,
37 the methods are generalizable to other proteins that assemble in complexes to accomplish their
38 tasks. Overall, the DNA origami method for creating well-defined ensembles of motor proteins
39 provides a powerful tool for dissecting the mechanisms that lead to emergent motile behavior.
40
41 **INTRODUCTION:**
42 Dynein and kinesin are cytoskeletal motor proteins responsible for myriad functions in eukaryotic
43 cells[1]. By converting the chemical energy of ATP hydrolysis into productive work, these motors
44 translocate on microtubules to haul and distribute various intracellular cargos. They also

45 coordinate in the massive intracellular rearrangements associated with mitosis, where they
46 exhibit orchestrated forces that contribute to the positioning and separation of chromosomes.
47 Structural, biochemical, and biophysical assays, including single molecule observations, have
48 revealed the mechanisms of these motors at the individual level (well-reviewed in previous
49 works[2-4]). However, many of the motors' tasks require them to work in small ensembles of both
50 similar and mixed motor types. Comparatively less is understood about the mechanisms that
51 coordinate the activity and ultimate emergent motility of these ensembles[5,6]. This knowledge
52 gap is due, in part, to the difficulty in creating ensembles with controllable features, such as
53 motor type and copy number. Over the past decade, the molecular construction techniques of
54 DNA origami have been employed to solve this problem. For the microtubule based motors, some
55 examples of these investigations include single molecule observations of ensembles of
56 cytoplasmic dynein-1[7-9], intraflagellar dynein[11], various kinesin motors[12,13], and mixtures of both
57 dyneins and kinesins[7,14,15]. Here, we provide details of the purification and oligonucleotide
58 labeling of motors from yeast[7,16-20], the folding and purification of segmented DNA origami with
59 tunable compliance[8], and the imaging of the yeast motors propelling the chassis structures[7,18].

61 Constructing motor ensembles for in vitro single molecule observation requires three primary
62 efforts. The first is the expression, purification and labeling of motor constructs suitable for
63 attaching to DNA origami. The second is the production and purification of defined DNA origami
64 structures (often termed "chassis"). And the third is the conjugation of the motors to the chassis
65 structure followed by observation using total internal reflection fluorescence (TIRF) microscopy.
66 Here, we provide established protocols for this process for recombinant microtubule-based
67 motors purified from the yeast *Saccharomyces cerevisiae*[7,16-19]. DNA origami-based motor
68 ensembles have been investigated using both recombinant kinesin[15] and dynein[7,8,18] constructs
69 produced in this yeast expression system[16-19]. This protocol is valid for these constructs, given
70 that they are controlled by the galactose induced promoter, and fused to the same protein tags
71 for purification (ZZ and TEV protease linker) and for DNA oligo conjugation (SNAPtag).

73 Specific yeast strains produce specific motor constructs. For example, the dynein used to study
74 the role of cargo compliance was purified from strain RPY1084[7,8]. In general, strains containing
75 motor constructs with the appropriate genetic modifications for expression and purification can
76 be requested from the laboratories having published the use of those motors. Constructs with
77 novel attributes such as mutations or tags can be made using recombinant genetic techniques,
78 such as lithium acetate transformation[21] and commercial kits. Detailed protocols for creating
79 modified motor proteins in yeast for single molecule studies have been published[19]. In addition
80 to the motors being fused to the SNAPtag, the oligos used to label the motors must be conjugated
81 to the SNAP substrate, benzylguanine (BG); previously published protocols describe the
82 formation and purification of BG-oligo conjugates[18]. The overall strategy described here has also
83 been employed for actin-based motors (see previous works for examples[22-24]), and motors
84 purified from other organisms and expression systems (see previous works for examples[7,9-14]).

86 Polymerized microtubules (MTs) are used in these experiments in two different procedures. MT
87 affinity purification of functional motors requires MTs that are not labelled with other functional
88 groups, while the motor-ensemble motility TIRF assay requires MTs labeled with biotin and

89 fluorophores. In all cases, MTs are stabilized with taxol to prevent denaturation. The MT affinity
90 purification step is used to remove any non-motile motors with a high MT affinity, as these
91 motors can alter ensemble motility if conjugated to a chassis. During this process, active motors
92 unbind the MTs and remain in solution, while tight-binding motors spin down in the MT pellet.
93 This helps ensure all motors on the chassis are from an active population.
94
95 A variety of DNA origami structures have been used to study cytoskeletal motor ensembles. As
96 the mechanistic understanding of ensemble transport has increased, the DNA origami structures
97 employed in experiments have grown in complexity. In principle, any structure could be adapted
98 for this purpose provided it is modified to include single-stranded DNA attachment sites for
99 motors and fluorophores. Specific chassis designs and attributes may be useful for probing
100 particular questions about the emergent behavior of motors ensembles. For example, rigid rods
101 have been used to develop foundational knowledge of how copy number affects transport by
102 teams of dyneins and kinesins[7,15,18], and 2D platforms have been used to study myosin ensemble
103 navigation of actin networks[22]. Structures with variable or tunable flexibility have been used to
104 understand the roles of elastic coupling between motors and to probe how stepping
105 synchronization affects motility[8,24]. More recently, spherical structures are being used to gain
106 insight into how geometrical constraints to motor-track binding affect the dynamics of motility[25].
107
108 In this protocol, we offer specific steps for ensemble experiments on segmented chassis with
109 variable rigidity. Binding sites on the chassis are sometimes referred to as "handles", while
110 complementary DNA sequences that bind these handles are termed "antihandles". The number
111 of motors on these chassis is determined by which segments contain extended handle staples
112 with complementarity to the antihandle oligo on the oligo-labeled motors. Using different
113 handle sequences on different segments allows for binding of different types of motors to specific
114 locations on the chassis. The chassis detailed here is composed of 7 sequential rigid segments,
115 each comprised of 12 double-stranded DNA helices arranged in 2 concentric rings[8]. The rigid
116 segments contain the motor handles and are connected through regions that can be either
117 flexible single-stranded DNA or rigid double-stranded DNA, depending on the absence or
118 presence, respectively, of "linker" staples. The compliance of the chassis structure is thus
119 determined by the presence or absence of these "linker" staples. See previous reports for further
120 details and specific DNA sequences[8]. In addition, multiple methods can be used to purify
121 chassis[26]. The rate-zonal glycerol gradient centrifugation method[27] is described here.
122
123 **PROTOCOL:**
124
125 1. **Growth, expression and harvesting of motor proteins controlled by a galactose induced**
126 **promoter**
127
128 1.1. Using a yeast-peptone-dextrose (YPD) culture plate and a sterile inoculating stick, streak
129 desired frozen yeast strain and incubate for 3–4 days at 30 °C.
130

131 1.2. Day 1 of culture growth: In the afternoon, add 10 mL of YP culture media with 2% dextrose
132 to a 1" diameter glass culture tube and inoculate it with a single colony from the plate. Grow in
133 a rapidly rotating roller drum at 30 °C overnight.

134

135 1.3. Day 2: In the afternoon, transfer the 10 mL of culture to a 250 mL flask containing 50 mL of
136 YP culture media with 2% raffinose. Incubate overnight at 30 °C while shaking at 250 rpm on an
137 orbital shaker.

138

139 1.4. Day 3: In the afternoon, transfer the 60 mL of culture to a 3 L flask containing 1 L of YP culture
140 media with 2% galactose. Incubate overnight at 30 °C while shaking at 250 rpm on an orbital
141 shaker.

142

143 1.5. Day 4: Starting in the mid-morning, monitor the optical density (OD) of the culture at 600 nm
144 every 2 h. When the culture is between an OD 1.5 and 2, continue with the following steps to
145 harvest the cells. Harvesting outside of this OD range may reduce yield due to low cell counts
146 before OD 1.5, or cellular quiescence and protein degradation above OD 2.

147

148 1.6. Decant the cell culture into centrifuge bottles and spin at ~6200 x g at 4 °C for 8 min. Pour
149 off the supernatant and discard.

150

151 1.7. Resuspend the cell pellet in the bottle with double-distilled H_2O (ddH_2O).

152

153 1.8. Spin the cells at ~6200 x g at 4 °C for 8 min. Pour off the supernatant and discard.

154

155 1.9. Depending on the viscosity of the cell pellet, add up to 2 mL of ddH_2O to create a solution
156 fluid enough for pipetting.

157

158 1.10. Using a motorized pipette controller fitted with a 10 mL pipette, slowly dispense the cell
159 slurry into liquid nitrogen one drop at a time. This will produce frozen pellets of yeast cells.

160

161 1.11. Store the frozen cells at -80 °C until ready to purify.

162

163 **2. Purification of motor proteins from yeast cells**

164

165 2.1. **Cell lysis and soluble protein extraction**

166

167 2.1.1. Prepare 1 mL of a fresh solution of 0.1 M PMSF in pure ethanol. Wait to add the PMSF to
168 aqueous buffers as it is unstable in water; also, avoid exposure to water until within 20 min (the
169 approximate half-life of PMSF in water) of use.

170

171 2.1.2. Prepare 50 mL of 4x lysis buffer on ice with supplements from the 5x stock of lysis buffer,
172 but do not add PMSF until just before the buffer is applied to the yeast pellet.

173

174   2.1.3. Grind the liquid nitrogen-frozen yeast pellets into a fine powder with a blade-type coffee
175   grinder that has been pre-chilled with liquid nitrogen.

176

177   Note: This protein extraction typically starts with the cell pellet harvested from 2 L of yeast
178   culture. The starting amount of yeast culture can be adjusted up or down with commensurate
179   adjustments to the volumes of IgG beads and DNA oligos in steps 2.2.2 and 2.3.1 below.

180

181   2.1.4. Aliquot 15 mL of the 4x lysis buffer with DTT and Mg-ATP on ice and add PMSF to the buffer
182   to achieve a final concentration of 2 mM, completing the preparation of the 4x lysis buffer with
183   supplements.

184

185   2.1.5. Collect the yeast powder into a pre-chilled ~100 mL glass beaker on ice. Add a small volume
186   of the 4x lysis buffer with supplements into the powder so that the final concentration of the
187   buffer does not exceed 1x. Typically, add ~1.5 mL of the buffer for every 10 mL of yeast powder.

188

189   2.1.6. Quickly thaw the powder to the liquid phase by placing the beaker in a 37 °C water bath
190   with constant stirring using a spatula.

191

192   2.1.7. Place the beaker of lysate back on ice immediately after thawing, estimate the lysate
193   volume using a 50 mL conical tube, and add more 4x lysis buffer with supplements so that the
194   final concentration of the buffer is ~1x. Typically, 2 L of yeast culture yields a total of 25–35 mL
195   of lysate containing 1x lysis buffer.

196

197   2.1.8. Evenly distribute the lysate to centrifuge bottles on ice. Carefully balance the bottles to a
198   mass difference no more than 0.01 g between each pair, ensuring that each bottle is above the
199   minimum volume required to prevent bottle collapse. Centrifuge at ~290,000 x g for 25 min at
200   4 °C.

201

202   2.1.9. Collect the supernatant containing soluble proteins in a 50 mL conical tube on ice, and
203   discard the pellet containing cell debris and large organelles. Avoid collecting any cloudy portions
204   of the supernatant, as they can clog the gravity-flow column used in subsequent steps. Save 10
205   μL of the supernatant for SDS-PAGE analysis.

206

207   **2.2. IgG affinity purification**

208

209   2.2.1. During the spin above, set up a gravity-flow chromatography column on ice or in a cold
210   room.

211

212   2.2.2. Transfer 200 μL of 50% IgG affinity bead slurry to the column using a P-1000 pipette tip cut
213   with a razor blade to enlarge the diameter of its entry port. If purifying more or less than 2 L of
214   cell culture pellet, adjust the volume of bead slurry proportionately, with a minimum volume of
215   100 μL.

216

217 2.2.3. Aliquot another 15 mL of the 4x lysis buffer with DTT and Mg-ATP and add PMSF to the
218 buffer to achieve a final concentration of 2mM. Dilute the 4x buffer on ice to 1x by adding ddH$_2$O.
219
220 2.2.4. Wash the beads 2x with 5 mL of 1x lysis buffer with supplements.
221
222 2.2.5. Resuspend the beads in 200 µL of 1x lysis buffer with supplements.
223
224 2.2.6. Add the bead suspension to the protein extract obtained from centrifugation and incubate
225 the mixture at 4 °C for 1 h with gentle rotation.
226
227 2.2.7. During the incubation, prepare 25 mL of the wash buffer (recipe detailed in **Table 1**) on ice
228 and 50 mL of 1x TEV buffer (recipe detailed in **Table 1**) on ice.
229
230 2.2.8. After the 1 h incubation, filter the lysate-bead mixture on ice or in the cold room through
231 the same chromatography column used in step 2.2.2 above. Save 10 µL of the flow-through for
232 SDS-PAGE analysis.
233
234 2.2.9. Wash the remaining motor-bound beads on ice 2x with 5 mL of wash buffer. Save 10 µL of
235 the first wash for SDS-PAGE analysis.
236
237 2.2.10. Wash the beads on ice once with 5 mL of 1x TEV buffer. Allow the buffer to fully drain
238 from the column.
239
240 2.3. **Labeling with DNA oligonucleotides and TEV cleavage**
241
242 2.3.1. Remove the chromatography column from the setup and cap the bottom of the column.
243 Within the same column, incubate the motor-beads with 100 µL of 1x TEV buffer containing 10–
244 20 µM of the purified BG-oligo at room temperature (RT) for 10–15 min. If purifying more or less
245 than 2 L of cell culture pellet, adjust the volume of 1x TEV buffer and purified BG-oligos
246 proportionately. Increase the incubation time according to manufacturer's instructions if needed
247 to increase the yield and rate of motor labeling, but be aware that longer incubation times may
248 also increase the proportion of dysfunctional motors due to protein denaturation at RT.
249
250 2.3.2. Gently resuspend the beads every minute of the incubation.
251
252 2.3.3. Wash the labeled motor-beads 4x with 4 mL of 1x TEV buffer using the same
253 chromatography column and setup as before. Allow the final wash to fully drain from the column.
254
255 2.3.4. Cap the bottom of the column, resuspend the motor-beads in no more than 200 µL of 1x
256 TEV Buffer, and transfer to a 2 mL round-bottom microcentrifuge tube.
257
258 2.3.5. Incubate the suspension with ~0.3 units of TEV protease per µL of motor-bead mixture at
259 16 °C for 1 h with gentle rotations. The tube should be mounted so as to minimize the total
260 surface area of the tube with which the beads come into contact.

261

2.3.6. Centrifuge the tube in a microcentrifuge at 21,130 x *g* for 30 s in a cold room to concentrate the mixture at the bottom of the tube.

2.3.7. Still in the cold room, use a cut P-1000 pipette tip to transfer the mixture to a spin column and centrifuge at 21,130 x *g* for 30 s. Collect the filtrate containing the TEV-cleaved, purified motors. The TEV protease will be in the filtrate as well as the motors.

2.3.8. Save 10 µL of the filtrate for SDS-PAGE analysis. Aliquot the remaining filtrate in volumes of 2 µL for TIRF experiments or 50 µL for microtubule affinity purification. Flash freeze the aliquots in liquid nitrogen and store at -80 °C.

2.3.9. Resuspend the beads remaining in the filter in 1x TEV buffer for SDS-PAGE analysis. Use the same volume of 1x TEV buffer as in step 2.3.4 above.

3. **Microtubule (MT) polymerization**

3.1. Preparation of tubulin mixes for TIRF assays

3.1.1. In a cold room, separately dissolve the lyophilized tubulin of each type (unlabeled bovine tubulin, biotinylated tubulin, and fluorescent tubulin) in reconstitution buffer (recipe detailed in **Table 2**) to make a final concentration of 10 mg/mL. Let sit on ice for 10 min.

3.1.1.1. Mix the following components and let sit on ice for 10 min in the cold room (final concentrations are indicated parenthetically): 18 µL of bovine tubulin (~8.2 mg/mL), 2 µL of biotinylated tubulin (~0.9 mg/mL), and 2 µL of fluorescent tubulin (~0.9 mg/mL) .

3.1.2. Prepare 3 µL aliquots of the tubulin mixture and flash freeze in liquid nitrogen. Store the mixtures at -80 °C.

3.2. Preparation of tubulin for MT affinity purification of motors

3.2.1. In a cold room, dissolve lyophilized bovine tubulin in the reconstitution buffer to make a final concentration of 10 mg/mL. Let sit on ice for 10 min.

3.2.2. Prepare 3 µL aliquots of the tubulin mixture and flash freeze in liquid nitrogen. Store the mixtures at -80 °C.

3.3. Polymerization of tubulin into MTs

3.3.1. Remove a 3 µL aliquot of tubulin from the -80 °C freezer and very quickly and briefly thaw to the liquid phase by holding the bottom of the tube. Immediately place on ice and incubate for at least 3 min.

305   3.3.2. Gently layer 3 µL of 2x polymerization mix (recipe detailed in **Table 2**) on top of the tubulin
306   solution. Mix by gently flicking the tube, but do not mix by pipetting, as shear forces may disrupt
307   MT nucleation and elongation.
308
309   3.3.3. Incubate the mixture in a water bath at 37 °C for 30 min.
310
311   3.3.4. Gently layer 6 µL of RT 1x BRB80 with supplements (recipe detailed in **Table 2**) on top and
312   mix by flicking. Do not mix by pipetting.
313
314   3.3.5. Incubate the mixture at 37 °C for at least 10 min.
315
316   3.3.6. Proceed to MT affinity purification, or if doing TIRF assays, incubate MTs in the dark at RT
317   overnight to form longer MTs.
318
319   3.3.7. Store polymerized MTs for weeks in the dark at RT.
320
321   4. **Microtubule (MT) affinity purification**
322
323   4.1. **Removal of unpolymerized tubulins by centrifugation**
324
325   4.1.1. Mix the following components to make a 60% glycerol cushion: 1x BRB80 (without
326   supplements; recipe detailed in **Table 2**), 20 µM Taxol (dissolved in DMSO), 1 mM DTT, and 60%
327   glycerol.
328
329   4.1.2. Transfer 60 µL of the glycerol cushion to an ultracentrifuge tube. Gently layer 12 µL of the
330   unlabeled MTs polymerized previously on top of the cushion. To prevent shearing of MTs,
331   transfer them using a cut pipette tip.
332
333   4.1.3. Centrifuge the mixture at ~97,300 x g at 22 °C for 15 min. After the spin, the excess tubulins
334   remain in the top liquid layer, while the MTs form a pellet at the bottom. Mark the outer edge
335   of the tube before the spin to help locate the pellet, as the pellet may not be visible with the
336   naked eye.
337
338   4.1.4. Carefully remove the liquid layer (~12 µL) above the glycerol cushion and save 10 µL for
339   SDS-PAGE analysis.
340
341   4.1.5. Gently rinse the interface between the liquid layer and cushion with 20 µL of 1x BRB80
342   with supplements. Remove and discard the 20 µL rinse.
343
344   4.1.6. Carefully remove the glycerol cushion (~60 µL) and save 10 µL for SDS-PAGE analysis. Be
345   careful not to disturb the MT pellet.
346
347   4.1.7. Gently rinse the pellet with 60 µL of 1x BRB80 with supplements (recipe detailed in **Table
348   2**). Be careful not to disturb the MT pellet. Discard the rinse solution.

349

350 4.1.8. Resuspend the MT pellet in 24 µL of Taxol-supplemented lysis buffer (recipe detailed in
351 **Table 3**) using a cut pipette tip.

352

353 **4.2. Purification of functional motors by MT-based affinity chromatography**

354

355 4.3. Remove two 50 µL aliquots of motors purified from yeast from the -80C freezer and quickly
356 thaw to the liquid phase. Immediately place on ice.

357

358 4.4. Add the following components to a new ultracentrifuge tube in the indicated order and
359 incubate the mixture at RT for 10 min: (1) 100 µL of the motors purified from yeast, (2) 29 µL of
360 5x ATP/Taxol mix (recipe detailed in **Table 3**), then (3) 12 µL of purified MTs transferred with a
361 cut pipette tip.

362

363 4.5. Centrifuge the mixture at ~97,300 x g and 22 °C for 15 min. After the spin, functional motors
364 remain in the supernatant, and MTs form a pellet, along with the non-functional motors bound
365 to them.

366

367 4.6. Collect the supernatant, flash freeze in 2 µL aliquots, and store at -80 °C.

368

369 4.7. Save 10 µL of the supernatant for SDS-PAGE analysis. Resuspend the pellet in 141 µL of 1x
370 BRB80 for SDS-PAGE analysis, too. Use protein standards, such as actin, to create a standard
371 curve for quantifying the concentration of the purified motors as previously detailed[17].

372

373 4.8. Use this concentration information when conjugating motors to the chassis in step 6.1.3.

374

375 5. **Production of segmented DNA origami chassis**

376

377 5.1. **Formation of chassis**

378

379 5.1.1. Order the staple oligonucleotides listed in previously published tables[8] in 96 well plates
380 wet at 250 µM in Tris buffer, or dry and then resuspend them to 250 µM with Tris buffer.

381

382 5.1.2. Create a pool of the core staples by mixing 5 µL of each core staple (see table S1 in Driller-
383 Colangelo 2016[8]).

384

385 5.1.3. Create a pool of the linker staples by mixing 5 µL of each linker staple (see linker staple
386 table in Driller-Colangelo 2016[8]).

387

388 5.1.4. Create a pool of the fluorophore binding staples by mixing 5 µL of each fluorophore
389 handle staple (see fluorophore handle table in Driller-Colangelo 2016[8]).

390

391 5.1.5. Mix 50 µL folding reactions with the following components: 1x Folding buffer; 100 nM 8064
392 scaffold; 600 nM core staple pool; 600 nM fluorophore staple pool; 9 µM fluorophore strand (see

393 fluorophore antihandle table in Driller-Colangelo 2016[8]); for each motor binding site, either 4.2
394 µM extended handle strands or 600 nM strands without handles as desired (see motor
395 handle/antihandle staples table in Driller-Colangelo 2016[8]); 600 nM linkers as desired[8]; additional
396 6 mM $MgCl_2$; and water.
397
398 5.1.6. Fold in a thermal cycler using the following program: Rapid heating to 80 °C, cooling in
399 single degree increments to 65 °C over 75 min, then additional cooling in single degree
400 increments to 30 °C over 17.5 h.
401
402 5.1.7. Assay folding quality on a 2% agarose gel in 0.5x TBE buffer (see **Table 4** for recipe)
403 supplemented with 11 mM $MgCl_2$ and DNA gel stain (see **Table of Materials**). Run gel in 0.5x TBE
404 buffer supplemented with 11 mM $MgCl_2$ at 70 V for 60-90 min. Run gels in an ice water bath or
405 in a cold room to prevent excessive heating and subsequent denaturation of chassis structures.
406
407 5.1.8. Image the gel using conditions suitable for the DNA gel stain used in step 5.1.7.
408
409 **5.2. Purification of chassis**
410
411 5.2.1. In the late afternoon the day before purification, create glycerol gradients by gently
412 layering 80 µL each of 45%, 40%, 35%, 30%, 25%, 20%, and 15% glycerol in 1x origami folding
413 buffer in a centrifuge tube (see **Table 4** for recipe). Boundaries between layers should be slightly
414 visible.
415
416 5.2.2. Incubate gradients at 4 °C overnight.
417
418 5.2.3. The next morning, add 45% glycerol in 1x origami folding buffer to the folded chassis
419 solution to a final concentration of 10% glycerol. Mix gently and layer on the top of the gradient
420 in the centrifuge tube.
421
422 5.2.4. Spin the gradient with the chassis at 243,000 *x* g for 130 min at 4 °C.
423
424 5.2.5. Collect 50 µL fractions from the tube in a top to bottom direction.
425
426 5.2.6. Cast a 2% agarose gel in 0.5x TBE buffer supplemented with 11 mM $MgCl_2$ and DNA gel
427 stain.
428
429 5.2.7. Load 5 µL of each fraction on the gel and run gel in 0.5x TBE buffer supplemented with 11
430 mM $MgCl_2$ for 90–120 min at 70 V. Run gels in an ice water bath or in a cold room to prevent
431 excessive heating and subsequent denaturation of chassis structures.
432
433 5.2.8. Image the gel using conditions suitable for the DNA gel stain used in step 5.2.6.
434
435 5.2.9. Select fractions for future experiments that exhibit well-folded monomeric structures free
436 of unincorporated staples.

437

438 5.2.10. Quantified concentrations of selected fractions using appropriate spectroscopic
439 methods, such as UV absorption at 260 nm. Use this concentration information when conjugating
440 motors to the chassis in step 6.1.3.

441

442 6. **Making slide assay chambers**

443

444 6.1. Make a slide assay chamber by sticking two strips of double-sided tape to a glass slide and
445 placing a coverslip on top. The chamber is the narrow space sandwiched between the coverslip
446 and glass slide, flanked by the two strips of tape. **Figure 1** illustrates the assay chamber.

447

448 6.2. When preparing the slide with solutions, use a pipette to flow any fluid into the chamber
449 from one side, and use a strip of filter paper to collect the flow of the fluid on the other side.

450

451 7. **Motor-ensemble motility TIRF assay**

452

453 **7.1. Conjugation of motors to DNA chassis**

454

455 7.1.1. On ice, prepare fresh 1 mL each of DTT-supplemented BRB80, Taxol-supplemented lysis
456 buffer, and casein-Taxol-supplemented lysis buffer (recipes detailed in **Table 5**). Transfer 200 μL
457 of each buffer to a RT tube.

458

459 7.1.2. Still on ice, use the cold casein-Taxol-supplemented lysis buffer to prepare the 4x energy
460 and 4x scavenger mixes (recipes detailed in **Table 5**).

461

462 7.1.3. Incubate 10 μL of ~300 nM purified motors with 5 μL of ~10 nM DNA chassis on ice for 15-
463 30 min. These concentrations of motors have been shown to saturate the chassis' motor binding
464 sites for this incubation time.

465

466 NOTE: motor occupancy is not 100%, likely due to stochastically missing handle staples in
467 individual chassis structures[8,28].

468

469 7.1.4. During the incubation, dilute the biotin- and fluorophore-labeled MTs 100x with the RT
470 Taxol-supplemented lysis buffer, and prepare a gel filtration resin appropriate for size exclusion
471 column chromatography by following the procedure outlined below.

472

473 **7.2. Removal of excess motors from motor-chassis conjugates by size exclusion column**
474 **chromatography**

475

476 7.2.1. In a 50 mL conical tube, wash 5 mL of the gel filtration resin 2x with 45 mL of ddH$_2$O. For
477 each wash, mix the resin with water in the conical tube, and spin the mixture at 460 x g for 1 min.
478 Discard the supernatant and save the resin.

479

480 7.2.2. Wash the resin 2x with 45 mL of 1x lysis buffer (recipe detailed in **Table 5**) using the same
481 method described above.

482

483 7.2.3. Resuspend the washed resin in 1x lysis buffer in a 1:1 ratio, so that the resin becomes a
484 ~50% slurry in buffer. The washed resin can be stored at 4 °C for at least 1 month.

485

486 7.2.4. Transfer 800 μL of the resin suspension to a spin column. Drain the excess buffer by gravity
487 flow for 5 min. Remove any remaining buffer with a 2s spin at 1,000 x g. The final resin volume
488 should be around 350–400 μL.

489

490 7.2.5. Dilute the motor-chassis mix with the casein-Taxol-supplemented lysis buffer to a final
491 volume of 50 μL. Transfer the diluted mix to the spin column packed with resin.

492

493 7.2.6. Centrifuge the spin column at 1,000 x g for 6 s (this time is inclusive of the acceleration and
494 deceleration time). Collect the pure motor-chassis conjugates by saving the filtrate and discard
495 the column which retains the excess motors.

496

497 7.3. **Preparation of slides for imaging**

498

499 7.3.1. Flow 13 μL of 1 mg/mL biotinylated bovine serum albumin (biotin-BSA) into a slide assay
500 chamber. Incubate for 2 min to allow binding of BSA to glass.

501

502 7.3.2. Wash the chamber 2x with 20 μL of the RT DTT-supplemented BRB80 by flowing in the
503 buffer on one side of the chamber and wicking the excess fluid away from the other side using a
504 strip of filter paper.

505

506 7.3.3. Flow in 20 μL of 0.5 mg/mL streptavidin. Incubate for 2 min to allow binding of streptavidin
507 to the biotin on the BSA.

508

509 7.3.4. Wash the chamber 2x with 20 μL of the RT Taxol-supplemented lysis buffer.

510

511 7.3.5. Gently flow in 20 μL of the diluted MTs with a cut pipette tip. Incubate for 2 min to allow
512 binding between the biotin on MTs and streptavidin.

513

514 7.3.6. Wash 2x with 20 μL of the RT casein-Taxol-supplemented lysis buffer. Incubate for 2 min
515 to allow casein to permeate the entire chamber.

516

517 7.3.7. Dilute the purified motor-chassis conjugates 5x to 10x to single-molecule conditions (~10-
518 100 pM) with the cold casein-Taxol-supplemented lysis buffer. Mix together the following
519 components to produce a final motor-chassis mixture: 10 μL of the motor-chassis dilution, 5 μL
520 of 4x energy mix, and 5 μL of 4x scavenger mix.

521

522 7.3.8. Flow in 20 μL of the final motor-chassis mixture to the assay slide chamber and proceed to
523 TIRF microscopy.

524
525   7.4. **Imaging and data acquisition**

526
527   7.4.1. Image the slide immediately with a TIRF microscope. Typically, each slide remains useable
528   for between 30 and 60 min.

529
530   7.4.2. Acquire a still image in the MT channel, and a movie in the chassis channel. For the motors
531   in this protocol, 10 min movies with a frame rate of 0.5 fps and exposure time of 200 ms are
532   appropriate.

533
534   7.4.3. From the chassis movie, generate one kymograph for each MT in ImageJ or a similar image
535   processing software[29]. Analyze the velocities and run lengths of motor-chassis ensembles by
536   measuring the slopes and horizontal distances of the runs on the kymograph[30].

537
538   **REPRESENTATIVE RESULTS:**
539   Successful purifications of motors and chassis structures were assayed by gel electrophoresis.
540   SDS-PAGE analysis confirmed the successful extraction of dynein from yeast (**Figure 2**), as the
541   final filtrate collected in step 2.3.7 showed a clear, sharp band at the position of ~350 kDa. As
542   expected, this dynein band was absent from the flowthrough and wash that removed unwanted
543   proteins, and the beads from which dynein was cleaved. The observation suggests that the IgG
544   affinity purification and TEV protease cleavage were both highly efficient. Additionally, TEV
545   protease was also present in the final filtrate and formed a clear band at ~50 kDa.

546
547   The successful MT affinity purification of dynein and kinesin proteins was also confirmed with
548   SDS-PAGE analysis (**Figure 3**). While dynein showed up as a clear single band at ~350 kDa, kinesin
549   showed up as slightly smearing multiple bands at ~120 kDa, possibly due to the presence of both
550   phosphorylated and dephosphorylated forms of the protein[14] and variable yields in oligo-
551   labeling. A comparison between the dynein and kinesin bands before and after this MT affinity
552   purification revealed a reduction in the motor concentration, as indicated by the decrease in the
553   band intensity, which was likely due to the removal of non-functional motors. Despite the
554   reduction, the concentrations of motors retained were sufficient for effective TIRF assays. In
555   addition to the TEV protease, a noticeable amount of tubulin (~51 kDa) was present in the final
556   supernatant, most likely due to the gradual decomposition of MTs during the experiment, or
557   incomplete removal of the excess tubulin through centrifugation. However, the consistent
558   motility of motor-chassis ensembles shown in TIRF assays suggests that tubulin and TEV protease
559   did not interfere with motor functions (see **Figure 6**).

560
561   Folding of DNA origami structures was assayed by agarose gel electrophoresis. **Figure 4** depicts
562   the results of a gel analyzing the folding of a flexible segmented chassis with 2 motor binding
563   sites. The shift in mobility between the pure unfolded scaffold strand (Lane 1) and the folding
564   reaction (Lane 2) indicates origami folding. Additionally, the folding reaction in Lane 2 indicates
565   the presence of some multimerization of chassis structures. Multimerization typically occurs, and
566   requires subsequent purification of the well-folded monomeric structures. The unincorporated
567   excess staples were also visible, displaying a high degree of mobility through the gel.

568
569   Folded origami reactions were purified to remove excess unincorporated staples and multimers
570   of the chassis structure. **Figure 5** shows the results of a glycerol gradient purification of a flexible
571   chassis with 7 motor binding sites. The early fractions correspond to the low glycerol density at
572   the top of the tube. They contain the excess staples. The late fractions correspond to the high
573   glycerol densities at the bottom of the tube and contain multimers and aggregates of folded
574   structures. In this gel, fraction 7 indicates a suitable fraction containing well-folded monomeric
575   chassis. Note that the well-folded structure is isolated from both excess staples and multimers.
576   While this gel is representative, and fraction 7 often contains useable chassis, each purification
577   experiment yields slightly different results and fractions should always be assayed to determine
578   which fraction is best for motility assays.
579
580   The motility of motor-chassis ensembles is easily detectable and measurable on the kymographs
581   generated from TIRF movies. For instance, the kymographs (**Figure 6**) of flexible chassis
582   conjugated to seven dynein proteins ("7D" ensembles) show highly processive runs at relatively
583   consistent velocities, demonstrating that the ensembles were active and motile in the
584   reconstituted system, and that MT affinity purification successfully removed most of the non-
585   functional, immobile dyneins that could slow or stall the ensembles. The same TIRF experiment
586   has been successfully performed on other chassis types with different compliance and motor
587   numbers to reveal the effects of these factors on dynein teamwork[8,9].
588
589   **FIGURE AND TABLE LEGENDS:**
590
591   **Figure 1. Schematic of the slide assay chamber.** The coverslip sits atop two strips of double-
592   sided tape. Solutions are pipetted in one side, and extracted with filter paper on the other.
593
594   **Figure 2. SDS-PAGE analysis of the purification of dynein from yeast.** Yeast cells were lysed and
595   centrifuged to collect the lysate (lane 1) containing total soluble proteins. The affinity between
596   IgG on column beads and the ZZ tag on the recombinant dynein construct was exploited for this
597   purification. The flow-through (lane 2) was collected from the mixture of the lysate and beads
598   after it passed through a chromatography column. The dynein-bound beads were washed with
599   buffers, and the first wash with the wash buffer (lane 3) was collected. Dynein was then
600   conjugated to a DNA oligo. After TEV protease cleavage, centrifugation in a spin column
601   separated the filtrate containing dynein (lane 4) and the residual beads (lane 5). All samples
602   collected from the purification were denatured with 1x LDS Sample Buffer and 1x Reducing
603   Agent, and loaded onto a 4–12% Bis-Tris gel. The gel was run in 1x MOPS buffer at 200 V for ~1
604   h, and stained with SYPRO Red Protein Gel Stain for imaging under UV light. Notably, the clear,
605   sharp band at ~350 kDa in lane 4 indicates the presence of concentrated purified dynein in the
606   final filtrate, while the band at ~50 kDa in the same lane indicates the co-presence of TEV
607   protease.
608
609   **Figure 3. SDS-PAGE analysis of the MT affinity purification of functional dynein and kinesin**
610   **proteins.** Kinesin and dynein purified from yeast (lanes 1 and 3) were mixed with polymerized
611   MTs and ATP, and ultracentrifugation was performed to separate the functional motors in the

612 supernatants (lanes 2 and 4) from the non-functional motors that co-pellet with MTs. The motor
613 samples before and after purification were denatured with 1x LDS Sample Buffer and 1x Reducing
614 Agent, and loaded onto a 4–12% Bis-Tris gel. The gel was run in 1x MOPS buffer at 200 V for ~1
615 h, and stained with SYPRO Red Protein Gel Stain for imaging under UV light. The intensity of the
616 motor bands (~120 kDa for kinesin, and ~350 kDa for dynein) appeared to decrease after the MT
617 affinity purification, indicating a reduction in the motor concentrations. Noticeable
618 concentrations of tubulin and TEV protease were present in the post-purification motor samples.
619
620 **Figure 4. Agarose gel analysis of folded DNA origami structure.** Folded DNA origami structures
621 are assessed by gel electrophoresis. Lane 1 is the pure unfolded scaffold strand while lane 2 is
622 the product of the folding reaction. Gel was run at 70 V in an ice water bath for 90 min in 0.5x
623 TBE buffer supplemented with 11 mM $MgCl_2$. A shift in mobility indicates origami folding.
624 Unincorporated staples and chassis multimers were also visible in Lane 2.
625
626 **Figure 5. Agarose gel analysis of glycerol gradient purification of DNA origami chassis.** The
627 quality of the glycerol gradient purification can be determined by gel electrophoresis of the
628 fractions from the centrifuge gradient. Low number fractions are from the top of the tube and
629 correspond to low density of glycerol. High numbered fractions are from the bottom of the tube
630 and correspond to high densities of glycerol. The excess staples, monomeric chassis, and
631 multimeric chassis are all visible. Fraction 7 contains chassis suitable for TIRF microscopy as they
632 are monomeric and excess staples are absent. Gel was run at 70 V in an ice water bath for 120
633 min in 0.5x TBE buffer supplemented with 11 mM $MgCl_2$.
634
635 **Figure 6. Kymographs showing the motility of dynein-chassis ensembles on single MTs.** Dynein
636 proteins extracted from yeast and purified with MTs were conjugated to flexible chassis
637 structures. Each chassis had seven binding sites for dynein and formed a "7D" ensemble. The
638 movement of these ensembles on MTs was recorded in a 10 min movie (200 ms exposure, 0.5
639 fps) during a TIRF assay. Kymographs from two MTs were generated from this movie in ImageJ
640 by tracing along a single MT. The vertical and horizontal red bars in the top left corner of each
641 image indicate 2 min and 20 µm, respectively. Each bright line records the movement of one
642 ensemble, with the inverse of the slope indicating the velocity and horizontal displacement
643 indicating the run length. With TIRF assays and kymography, the motility of motor-chassis
644 ensembles becomes easily detectable and directly measurable.
645
646 **Table 1. Buffers for the IgG affinity purification of motor proteins from yeast cells (Protocol**
647 **section 2).**
648
649 **Table 2. Buffers for the polymerization of microtubules (Protocol section 3).**
650
651 **Table 3. Buffers for the microtubule affinity purification of functional motor proteins (Protocol**
652 **section 4).**
653
654 **Table 4. Buffers for the production of segmented DNA origami chassis (Protocol section 5).**
655

656 **Table 5. Buffers (a) and mixes (b) for the motor-ensemble motility TIRF assay (Protocol section
657 7).** Details on the Oxygen Scavenger System used to make the Scavenger Mix can be found
658 elsewhere[17].

659

660 **DISCUSSION:**

661

662 The molecular construction techniques of DNA origami provide a unique way to construct motor
663 ensembles with defined architectures, motor numbers, and types, enabling studies of how
664 emergent behavior arises from specific motor configurations[31]. As structural and cellular studies
665 continue to elucidate examples of cytoskeletal motors working in teams, techniques for isolating
666 and investigating the biophysical and biochemical mechanisms of motors in ensembles are
667 growing in utility. For example, cryo-EM has shown that dynactin can bind 2 individual dynein
668 motors, and that such pairings have different motility than the individual motors[10]. In addition,
669 DNA-based construction was used to determine if mammalian dynein, when activated by
670 dynactin and bicD2, could match the force of kinesin-1 in a tug of war scenario[14]. In another
671 mixed-motor study, DNA origami was used to decouple the effects of opposite polarity motors
672 and their regulatory binding proteins by spatially separating them on an origami structure[15]. As
673 more structural and regulatory determinants of motility are found, DNA-origami-based
674 techniques should prove useful in determining the specific biochemical and biophysical
675 contributors to the emergent motility of motor ensembles. The molecular construction
676 techniques enabled by DNA origami are particularly useful because the emergent
677 phenomenological outcomes of ensembles are difficult to predict. This is due in part to the
678 myriad factors that contribute to the motility of the individual motors within the ensemble[5,6,31].

679

680 Examples of our previous structures include monolithic rods and segmented rods with variable
681 rigidity. Current efforts explore spherical structures as well[25]. Others have employed
682 morphologies such as planar structures and rods[22,24]. Likewise, by using motor handles with
683 orthogonal DNA sequences, different types of motors can be bound to the same chassis
684 structure[7,14,15,18]. This approach enables studies of the opposing actions of dyneins and kinesins,
685 minus- and plus-end directed kinesins, and minus- and plus-end directed myosins. It also enables
686 the introduction of a mutant among otherwise wild-type ensembles, allowing the specific
687 biochemical contributors to the emergent motility to be deciphered[7]. Because of the ability to
688 bind multiple fluorophores to each individual structure, imaging in TIRF and subsequent analysis
689 by kymography or particle tracking is possible. Previous reports show analysis of kymography
690 data and statistical evaluation of chassis structures with variable compliance[8]. While cytoskeletal
691 motors have proven to be an exciting early application of using DNA origami as a molecular
692 breadboard[32], other proteins and protein systems will also benefit from these methods.

693

704

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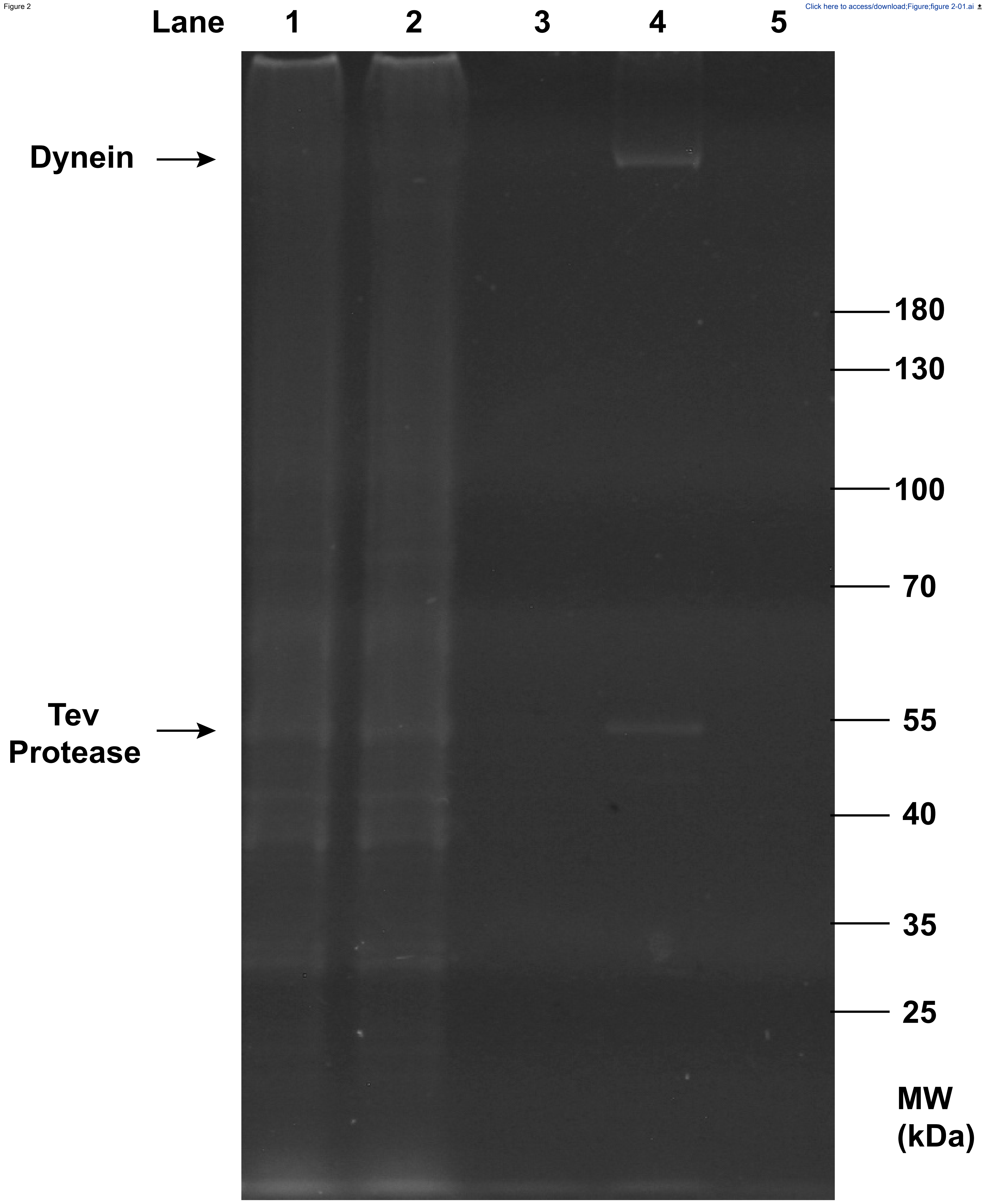786   doi:10.1093/nar/gks1070 (2013).

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799
800
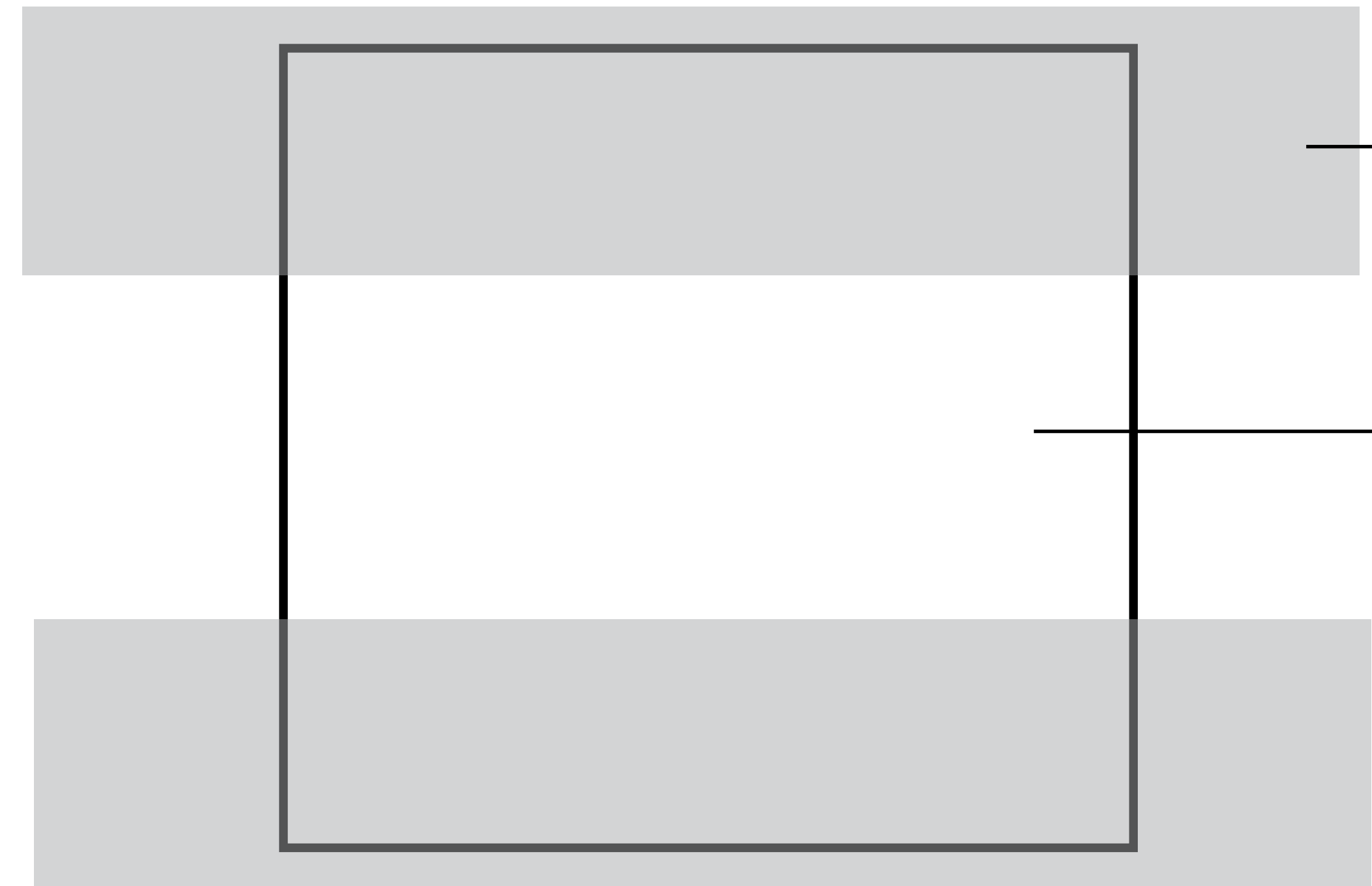
Figure 1

Double-sided Tape

Coverslip

Glass Slide

Figure 2

Figure 3

Lane   **1**   **2**   **3**   **4**

Dynein ⟶

— 180

— 130

Kinesin ⟶

— 100

— 70

Tubulin ⟶

Tev protease ⟶

— 55

— 40

— 35

— 25

MW

(kDa)

Figure 4

Click here to access/download;Figure;Fig 4.ai ⬆

Lane 1 2

Multi-meric chassis

Monomeric chassis

Unfolded scaffold

Unincorporated staples

Figure 5

Fraction 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Multi-meric chassis

Monomeric chassis

Unincorporated staples
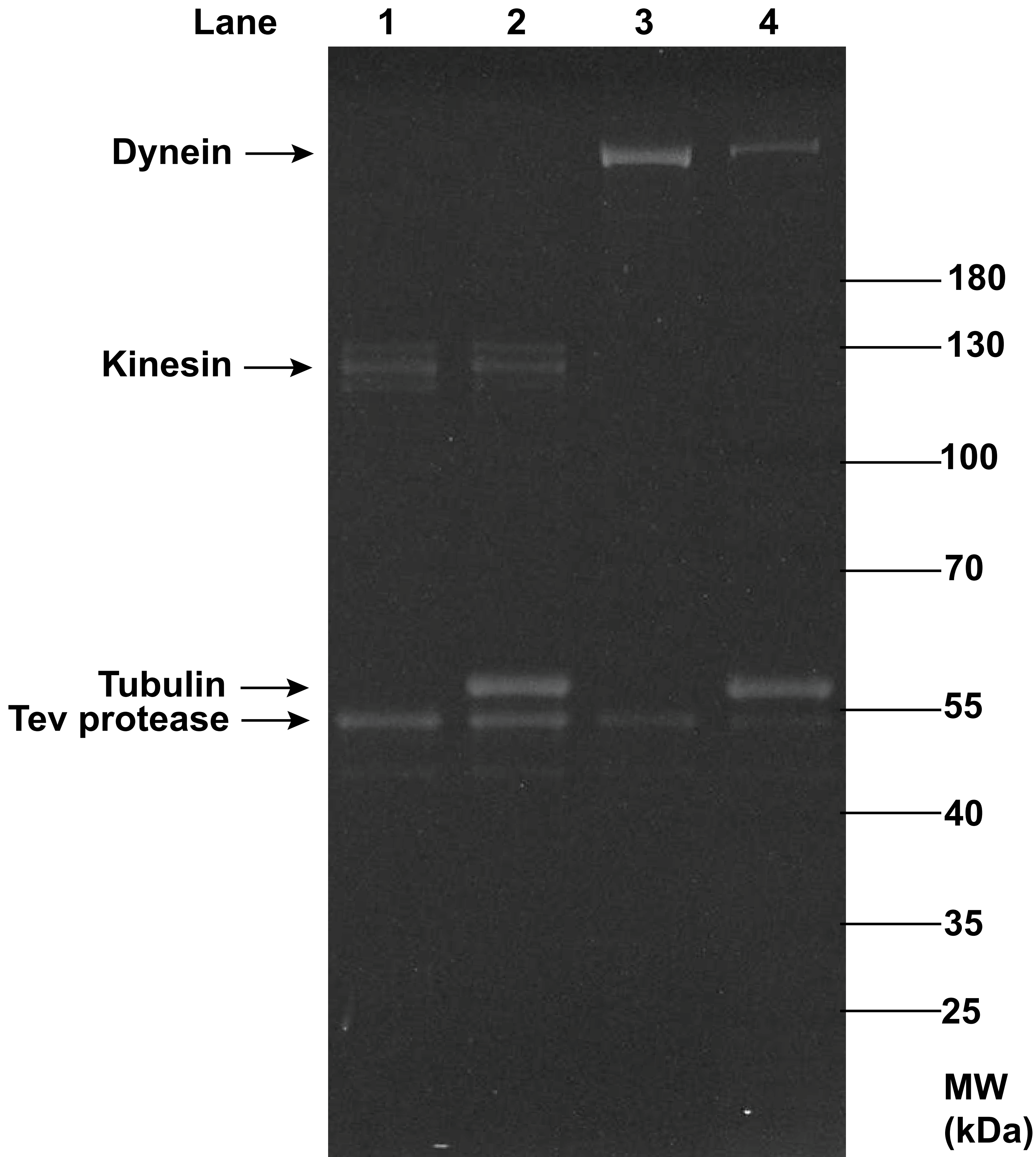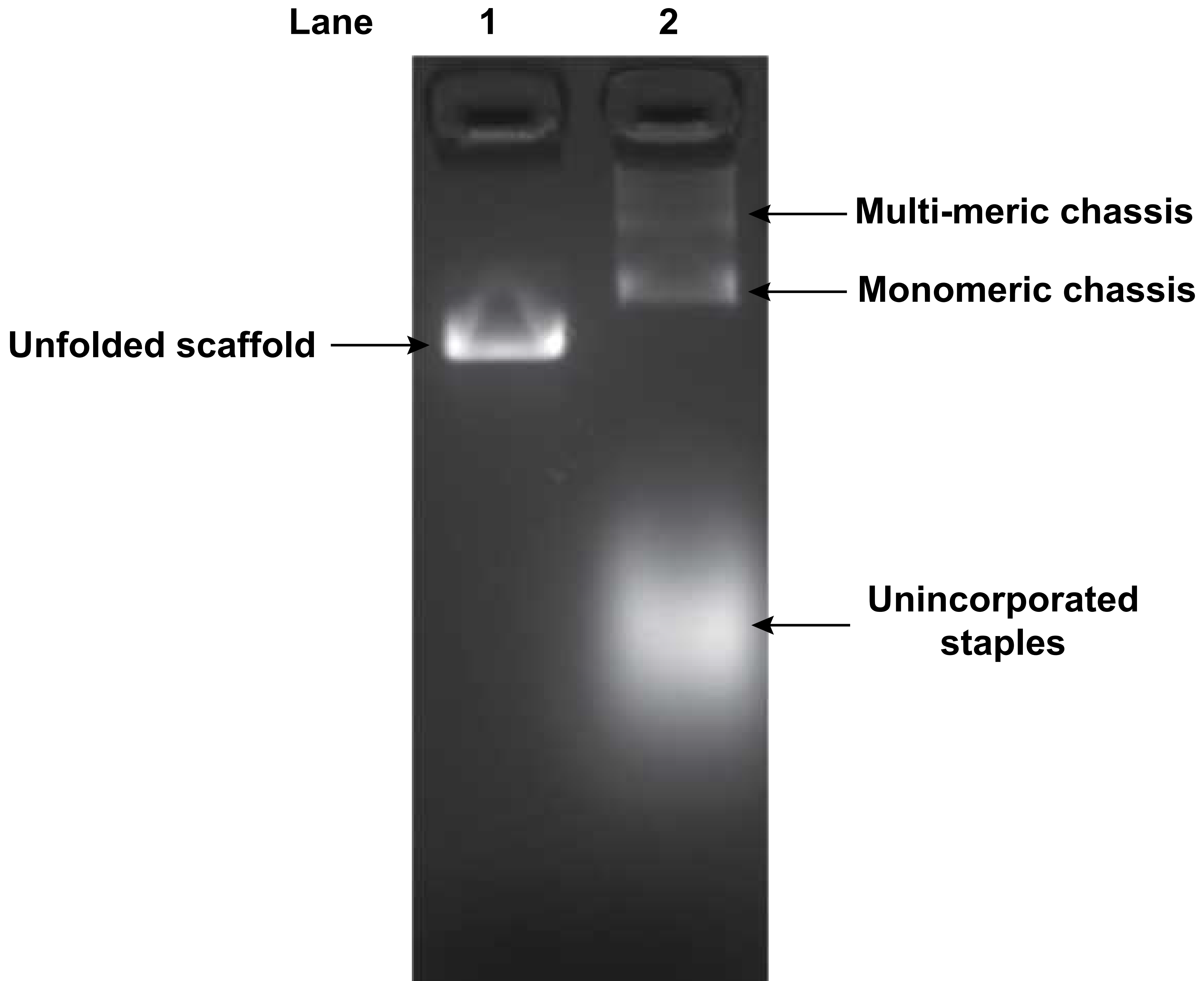
Figure 6

Time

Position on the MT

Table 1
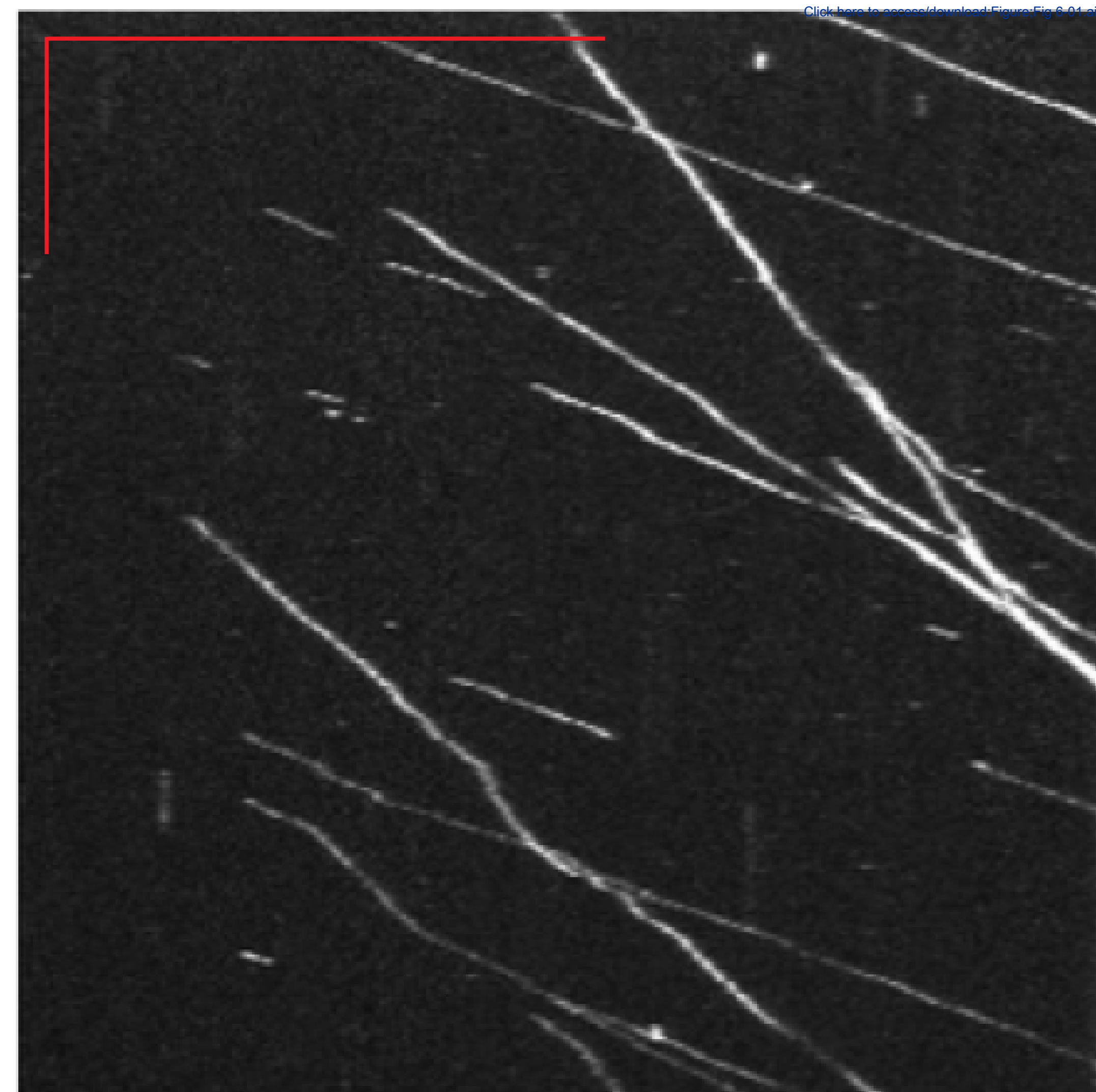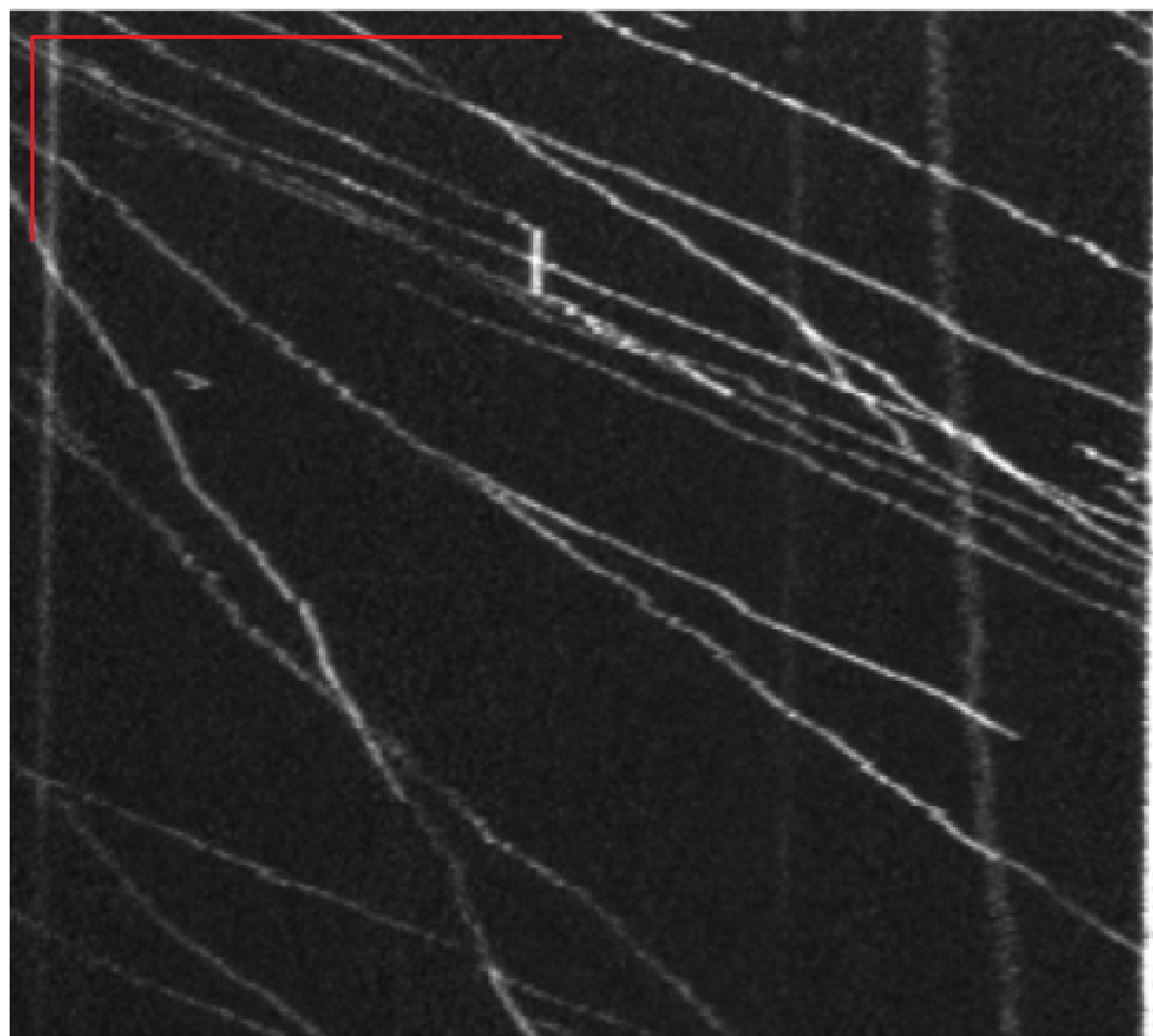
Buffer Name	Composition	Step(s) Used
5x Lysis Buffer	150 mM HEPES (pH 7.4)	-
	250 mM KAcetate	
	10 mM MgAcetate	
	5 mM EGTA (pH 7.5)	
	50% glycerol	
4x Lysis Buffer With Supplements	4x Lysis Buffer	2.1-2.2
	4 mM DTT	
	0.4 mM Mg-ATP	
	2 mM PMSF	
Wash Buffer	1x Lysis Buffer With Supplements	2.2
	250 mM KCl	
	0.1% Triton X-100	
5x TEV Buffer	50 mM Tris-HCl (pH 8.0)	-
	150 mM KCl	
	10% Glycerol	
1x TEV Buffer	1x TEV Buffer	2.2-2.3
	1 mM DTT	
	0.1 mM Mg-ATP	
	0.1% Triton X-100	
	0.5 mM PMSF	

Comment

Filter sterilize the buffer. It can be stored at RT in a properly sealed container for a year.

Make this 4x buffer from the 5x lysis buffer above. Prepare a buffer without PMSF first, and add the compound (dissolved in pure ethanol) to a small aliquot of the buffer right before each step that requires it.

To make the buffer, add KCl, Triton X-100, and ddH$_2$O to the 4x lysis buffer with DTT and Mg-ATP, but do not add PMSF until right before use.

Filter sterilize the buffer. It can be stored at RT in a properly sealed container for a year.

To make the buffer, add DTT, Mg-ATP, Triton X-100, and ddH$_2$O to the 5x stock TEV buffer, but do not add PMSF until right before use.

Table 2

Buffer Name	Composition	Step(s) Used
5x BRB80	400 mM PIPES	-
	10 mM MgCl$_2$	
	5 mM EGTA	
	Adjust pH to 6.8 with KOH	
1x BRB80 With Supplements	1x BRB80	3.3 & 4.1-4.2
	20 μM Taxol (dissolved in DMSO)	
	1 mM DTT	
2x Polymerization Mix	2x BRB80 (without supplements)	3.3
	2 mM DTT	
	2 mM Mg-GTP	
	20% DMSO	
Reconstitution Buffer	1x BRB80 (without supplements)	3.1
	1 mM DTT	
	1 mM Mg-GTP	

Comment

Filter sterilize the buffer. It can be stored at RT in a properly sealed container for a year.

Must be freshly made from the 5x BRB80 stock for every experiment.

Flash freeze the mix in small aliquots and store at -80 ^{o}C.

Must be freshly made for every experiment.

Table 3

Buffer Name	Composition	Step(s) Used
Taxol-Supplemented Lysis Buffer	1x Lysis Buffer 20 μM Taxol (dissolved in DMSO) 1 mM DTT	4.1
5x ATP/Taxol Mix	1x Lysis Buffer 25 mM Mg-ATP 50 μM Taxol (dissolved in DMSO) 5 mM DTT	4.2

Comment

Must be freshly made for every purification.

Must be freshly made for every purification.

Table 4

Buffer Name	Composition	Step(s) Used
20x Origami Folding Buffer	100 mM Tris pH 8.0 20 mM EDTA 200 mM MgCl$_2$	-
1x Origami Folding Buffer	5 mM Tris pH 8.0 1 mM EDTA 10 mM MgCl$_2$	5.1-5.2
0.5x TBE	45 mM Tris pH 8.0 45 mM Boric Acid 1 mM EDTA	5.1-5.2

Comment

Can be stored at RT in a properly sealed container for up to a year.

Make the buffer fresh by diluting the 20x stock with ddH$_2$O before every experiment.

Can be stored at RT in a properly sealed container for up to a year.

Buffer Name	Composition	Step(s) Used
DTT-Supplemented BRB80	1x BRB80 1 mM DTT	7.3
Taxol-Supplemented Lysis Buffer	1x Lysis Buffer 20 µM Taxol (dissolved in DMSO) 1 mM DTT	7.1 & 7.3
Casein-Taxol-Supplemented Lysis Buffer	1x Lysis Buffer 20 µM Taxol (dissolved in DMSO) 1 mM DTT ~2.5 mg/ml Casein (dissolved in Tris-HCl at pH 8.0)	7.1-7.3
1x Lysis Buffer	30 mM HEPES (pH 7.4) 50 mM KAcetate 2 mM MgAcetate 1 mM EGTA (pH 7.5) 10% glycerol	7.2
4x Energy Mix	22.5 µL 1x Casein-Taxol-Supplemenfted Lysis Buffer 1 µL 0.1 M Mg-ATP 1 µL 40% Glucose 0.5 µL β-Mercaptoethanol	7.3
4x Scavenger Mix	24 µL 1x Casein-Taxol-Supplemented Lysis Buffer 1 µL 1x Oxygen Scavenger System	7.3

Comment
Must be freshly made before every TIRF experiment.

Must be freshly made before every TIRF experiment.

Must be freshly made before every TIRF experiment.

Make this buffer fresh by diluting the 5x stock (recipe detailed in Table 1) with ddH$_2$O.

Must be freshly made before every TIRF experiment; volumes indicated are for a final volume of 25 µL.

Must be freshly made before every TIRF experiment; volumes indicated are for a final volume of 25 µL.

Table of Materials

Name of Material/ Equipment	Company
2 mL Round Bottom Tube	USA Scientific
Biotin labeled tubulin protein: porcine brain, >99% pure	Cytoskeleton.com
Biotin-BSA	Sigma
Bottle Assembly, Polycarbonate, 250 mL, 62 x 120 mm	Beckman Coulter
Bottle, with Cap Assembly, Polycarbonate, 10.4 mL, 16 x 76 mm	Beckman Coulter
Centrifugal Filter Unit	Millipore Sigma
IgG Sepharose 6 Fast Flow, 10 mL	GE Healthcare
Micro Bio-Spin Chromatography Columns, empty	Bio-Rad
P8064 Scaffold	Tilibit
Poly-Prep Chromatography Columns	Bio-Rad
ProTev Protease	Promega
Scotch Double Sided Tape with Dispenser	amazon.com
Sephacryl S-500 HR	GE Healthcare
Streptavidin	Thermo Fisher
SYBR Safe DNA stain	Invitrogen
Tubulin protein (>99% pure): porcine brain	Cytoskeleton.com
Tubulin, HiLyte 647	Cytoskeleton.com
Ultra-Clear Centrifuge Tubes	Beckman Coulter

Catalog	Comments/Description
1620-2700	
T333P-A	
A8549-10MG	
356013	
355603	
UFC30VV00	
17096901	
7326204EDU	
2 mL at 400nM	
731-1550	
V6101	
N/A	
17061310	
434302	
T240-B	
TL670M-A	
344090	

This piece of the submission is being sent via mail.

Response to review

We thank the editors and reviewers for their careful and considerate review of our manuscript.
With the suggested revisions, we believe the manuscript will better serve the community and aid
in the adoption of these techniques. We particularly appreciate the investment of time and
energy to review, and we are grateful that the methods we have developed will be made more
accessible through the production of these detailed protocols. We have addressed each point
made by the editors and reviewers, and enumerate these changes on a point-by-point basis
below. Both our responses to the reviewers and major changes in the manuscript are in blue
text. By addressing all of these points, we believe we have made this protocol both stronger
and more clear. (Please note that some changes we made based on the reviewers' comments
have been moved out of the protocol itself and into the *Introduction* as per the style
requirements of *JoVE*.)

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are
no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x
11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin,
single spacing within paragraphs, and spaces between all paragraphs and protocol steps/
substeps.
3. Please ensure that all references appear as numbered superscripts after the appropriate
statement(s).
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark
symbols (™), registered symbols (®), and company names before an instrument or reagent.
Please limit the use of commercial language from your manuscript and use generic terms
instead. All commercial products should be sufficiently referenced in the Table of Materials and
Reagents.
For example: Beckman Coulter, GE Healthcare, ProTEV, Millipore, 3M, Sephacryl,

We have made these needed edits.

Protocol:
1. Everything in the protocol should be in a numbered step in the imperative tense and of no
more than 4 sentences, header, or 'Note'. Please move the introductory paragraphs of the
protocol sections to the Introduction, Results, or Discussion (as appropriate) or break into steps.
Please make recipes (2.4, 3.4, 4.3, 5.3, 7.5) into imperative steps or, alternatively, make into
tables.

We have made these needed edits.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step
performed? Alternatively, add references to published material specifying how to perform the

protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We believe we have made these needed edits. Please let us know if we have missed anything.

Figures, Tables, and Figure Legends:
1. Please remove the titles and Figure Legends from the uploaded figures. Please instead include all the Figure Legends together at the end of the Representative Results in the manuscript.

We have made these needed edits.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
In this methods article, Hu and Deer provide detailed protocols for the purification of the microtubule (MT)-associated motor proteins, kinesin and cytoplasmic dynein, and the generation of polymerized MTs and DNA origami. I believe the provided protocols are sufficient to reproduce the discussed experiments. The presented procedures are straightforward and relatively easy to implement in a lab. The protocol is written with care and I feel that it will be a timely and well-cited contribution. In conclusion, I believe the methods article is appropriate for JoVE and would recommend publication, subject to the following minor changes:

Minor Concerns:

1. The authors write in line 47 that "By converting the chemical energy of ATP into productive work and force generation, […]". As work is the product of force times the distance over which the force is applied, I recommend to write either "By converting the chemical energy of ATP hydrolysis into productive work, […]" or "By converting the chemical energy of ATP hydrolysis into force generation, […]".

Thank you for catching this imprecise language. We have made the suggested edit.

2. In lines 623 to 626, the authors write that "The kymographs (Figure 6) of flexible chassis conjugated to seven dynein proteins ("7D" ensembles) show highly processive runs at relatively consistent and high velocities, which demonstrate the coordination and cooperation of multiple dynein motors in transporting one cargo." This statement is not correct. As the authors have shown in their Science paper (Fig. 2B in Derr et al. Science 2012), the velocity of the chassis with the 7 dynein motors is significantly lower than the velocity of a single dynein. This result suggests that yeast dynein motors, which are highly processive on their own, are not coordinating with each other and instead interfere. That the processivity of the multi-motor chassis is increased compared to the processivity of a single dynein, can be easily explained by the reduced likelihood of the chassis to detach from the MT as a result of the multiple MT-chassis dynein linkages. I recommend that the authors modify this sentence.

Reviewer #2:

Manuscript Summary:

This manuscript describes the procedure for assembling a complex hybrid nanostructure, consisting of a DNA-origami 'chassis' and purified molecular motors kinesin and dynein, and undertaking microscopy studies of the resulting nanostructures to elucidate motor mechanisms. It covers all the necessary steps, from yeast motor protein expression and purification, DNA origami assembly, loading of proteins onto the origami, and TIRF imaging.

This is an extremely comprehensive range of skills, in a very interdisciplinary project area, and so a JOVE video would be highly useful in broadening the applicability of this method. The efficacy of the protocol is well demonstrated, with reference to a number of recent high-impact publications. This protocol will be useful to scientists working in both motor protein and DNA origami areas. It is unlikely that many groups have sufficient background in both areas to develop such a protocol independently, so this video would act to bridge the expertise gap between the two areas. As many applications of DNA origami involve protein functionalization, this video would also serve as a useful example of such a protocol that could be adapted for other proteins.

Generally the discussion of background context and merits of the technique are unbiased and suitable. However, the protocol contains a large range of skills, and there is a high likelihood of scientists from diverse expertise backgrounds undertaking the protocol. Thus, some additional background discussion is required, as specified below. There are also a number of specific points that should be clarified.

Major Concerns:
None

Minor Concerns:

General discussion:

Abstract, Line 21: 'mechanisms the lead to emergent motile behavior'
Add a more specific example of the types of property that could be measured, eg 'how run length and velocity can vary with motor number'.

Thank you for this suggestion. We have added a phrase about motile properties to be measured.

Introduction, Line 78: 'segmented DNA origami with tunable compliance'
This sections needs more discussion on different DNA origami chassis/cargo designs and properties. For example, some examples of the different shapes, and a brief discussion of why

one would be chosen over another. As written it would not be clear to someone who is not already familiar with the referenced DNA origami papers.

We have added several new sentences to illustrate these ideas more fully.

5.1, Line 405: 'The compliance of the chassis structure is determined by the presence or absence of "linker" staples'
Similar to above comment, term 'Linkers' isn't very obvious in this conext without looking up reference 7. Could describe it more specifically here, eg 'alternating regions of rigid DNA-origami helix bundles and regions of flexible ss or dsDNA, which act as linkers'

Thank you for noticing this. We have more fully explained these ideas as well.

1.1, Line 92: 'streak desired frozen yeast strain'
Additional detail needs to be added either here or before this on the yeast strain. This section would not be clear to those without detailed protein production experience, and could use some more references. For example, how is the strain selected? Does the yeast strain already contain the construct of interest? If so, where was this obtained from?

We have more thoroughly explained about the yeast strain and referenced a Methods paper that details how to create modified yeast proteins for single molecule assays.

4, Line 336: 'Microtubule (MT) affinity purification'
Would be good to give some background rationale for this step. Why do only non-functional motors bind to MTs under these conditions? 1-2 sentences would be suitable. Are there other potential affinity purifications that could be helpful?

Thank you for the advice. We have elaborated on the background rationale for this purification method. (We are unaware of other affinity purification methods that could be helpful at this step, so have not elaborated on that point.)

6.1.3, Line 504: 'A high absolute and relative concentration of motors enables saturation of the chassis' motor binding sites.'
Accurate control of motor stoichiometry is one of the key benefits of using DNA origami as a chassis for these experiments. Given this, it is necessary to give further discussion on how accurate binding site occupancy is at this point in the method, and in the background section. How are motor and chassis concentration estimated? What are appropriate ranges equivalent to 'high' and 'low' for good preparations? What is expected occupancy of motor binding sites, and how robust are these values?

We have added more details to address these concerns. We have added more details in sections 4.2.5 on PAGE analysis of motors and in section 5.2.10 on quantification of chassis concentration. We have also discussed the occupancy in section 6.1.3 as suggested.

6.4.3, line 557: ' generating kymographs from the chassis movies.'

Should provide suggestion of software or methods for this. Could provide references for methods papers covering this. This is mentioned in Figure caption for Figure 6, but should be mentioned earlier.

We have added more details on kymograph generation and analysis in Protocol section 6.4.3, suggested ImageJ as the software, and cited a method paper.

Specific points to clarify:

1.4, Line 103: 'When the culture is between an OD 1.5 and 2'
Can you suggest a time frequency for checking OD, eg. Every hour? For those not familiar with yeast culture, is shape of growth curve known? Is it better to under or overshoot the suggested OD range? Do you know how sensitive yield is to OD at this point?

We do not have data on all of these points ,but we have some more information here as suggested so as to facilitate adoption of this protocol by others.

2.1.4, Line 141: 'add PMSF to the buffer'
What would be an appropriate time between addition of PMSF to lysis buffer and addition of lysis buffer to yeast powder? 1 min? <5 min? Should clarify what time frame is meant by 'immediately' here.

We have clarified the time frame here.

2.1.5, Line 142: 'pre-chilled glass beaker on ice'
What is appropriate size for beaker?

We have included the beaker size that we typically use in our experiment.

2.1.7 Line 148: 'estimate the lysate volume, and add more 4x lysis buffer'
Add an example of typical/expected volumes for this point

We have added the expected volumes for this step.

2.1.8 Lines 153-156: 'The bottles must be carefully balanced in the rotor'
Balancing of centrifuge is key step, can you reference another JOVE video that shows this step if it is not shown directly in this video?

This will be in our video.

2.2.3, Line 171: '4x buffer prepared previously'
Does this require fresh PMSF?

We have clarified this point as suggested.

2.3.1, Line 193: '100 l of 1x TEV buffer containing ~15 uM of the purified BG-oligo'

You gives some indication of reaction yield dependence on reaction time, can you give some information on dependence on oligo concentration? How sensitive is it to oligo concentration, would be better to specify, eg. 10-20 uM instead of ~15 uM.

We agree 10-20 uM is a better instruction for this step and have changed it accordingly. We have added a comment on concentration as well.

2.3.2, Line 299: 'do not mix by pipetting'
Add a brief dicussion of why this is important, does pipetting/vortexing disrupt MT nucleation?

We have added this discussion.

5.1.1, Line 408: 'at 250 M concentration in Tris buffer'
Plate oligos can also be ordered dry and normalised, then resuspended, this is a more commonly available shipping option for many locations.

We have amended this step as suggested.

5.1.5, Line 418: '600 nM sore staple pool'
Typo, should be 'core' not 'sore'

This typo has been fixed.

5.1.7, Line 430 & 5.2.7, Line 456: 2% agarose gel in 0.5x TBE buffer supplemented with 11 mM MgCl2'
Should specificy that gel running buffer also requires 11 mM MgCl2.

Thank you for noticing these omissions. We have made the suggested edits.

5.2.10, Line 463: 'Concentrations of selected fractions can be quantified using appropriate spectroscopic methods.'
Give some examples here, eg. UV absorption at 260 nm.

We have added this suggestion.