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Using Microfluidics and Fluorescence Microscopy to Study the Assembly Dynamics of Single Actin Filaments and Bundles

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1

1 **TITLE:**

2 Using Microfluidics and Fluorescence Microscopy to Study the Assembly Dynamics of Single
3 Actin Filaments and Bundles

4

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19

20 **SUMMARY:**

21 We present protocols for simple actin filament microfluidic assays, in combination with
22 fluorescence microscopy, that allow one to accurately monitor individual actin filaments in real-
23 time while sequentially exposing them to different protein solutions.

24

25 **ABSTRACT:**

26 In order to decipher the complex molecular mechanisms that regulate the assembly and
27 disassembly of actin filaments, it is a great asset to monitor individual reactions live in well-
28 controlled conditions. To do so, live single-filament experiments have emerged over the past 20
29 years, mostly using total internal reflection fluorescence (TIRF) microscopy, and have provided
30 a trove of key results. In 2011, in order to further expand the possibilities of these experiments
31 and to avoid recurring problematic artifacts, we introduced simple microfluidics in these assays.
32 This study details our basic protocol, where individual actin filaments are anchored by one end
33 to the passivated coverslip surface, align with the flow, and can be successively exposed to
34 different protein solutions. We also present the protocols for specific applications and explain
35 how controlled mechanical forces can be applied, thanks to the viscous drag of the flowing
36 solution. We highlight the technical caveats of these experiments and briefly present possible
37 developments based on this technique. These protocols and explanations, along with today's
38 availability of easy-to-use microfluidics equipment, should allow non-specialists to implement
39 this assay in their labs.

40

41 **INTRODUCTION:**

42 The assembly and disassembly of actin filaments and actin filament networks are controlled by
43 several biochemical reactions and depend on the mechanical context. In order to gain insight
44 into these complex mechanisms, it is invaluable to be able to observe individual reactions on

2

45 individual filaments (in sufficiently large numbers). Over the past decades, the observation of
46 dynamic actin filaments in real-time, mostly using total internal reflection fluorescence (TIRF)
47 microscopy, has emerged as a key technique and has provided an impressive list of results that
48 could not have been obtained with bulk solution biochemical assays¹.

49

50 To achieve this, one needs to maintain fluorescently labeled actin filaments close to the surface
51 of the microscope coverslip while exposing them to solutions of actin-binding proteins (ABPs),
52 which can also be fluorescently labeled. Doing so provides a means to monitor events taking
53 place on individual filaments in well-controlled biochemical conditions, and thus quantify
54 reaction rates. However, a number of specific limitations should be considered. Artificially
55 maintaining filaments close to the surface, often thanks to multiple anchoring points or by using
56 a crowding agent such as methylcellulose, can alter their behavior (e.g., causing pauses in their
57 polymerization and depolymerization²). Tracking the contour of each filament can be
58 challenging, particularly if new filaments or filament fragments accumulate in the field of view
59 over time. The reactions take place in a finite volume where the concentration of actin
60 monomers and ABPs can vary over time, potentially making it difficult to derive accurate rate
61 constants. Finally, renewing or changing the solution of ABPs is difficult to achieve in less than
62 30 s and will often lead to inhomogeneous protein content in the sample.

63

64 A bit over 10 years ago, inspired by what was already done to study individual Deoxyribonucleic
65 Acid (DNA) strands³, we introduced a new technique based on microfluidics to observe and
66 manipulate individual actin filaments⁴. It allows one to circumvent the aforementioned
67 limitations of classical single-filament techniques. In these microfluidics assays, actin filaments
68 are grown from spectrin-actin seeds adsorbed on the coverslip. Filaments are thus anchored by
69 one end only to the bottom of the microfluidic chamber and fluctuate above the surface
70 without sticking. Filaments align with the flow of incoming solutions, thereby easing the
71 monitoring of their contour length and maintaining them in a shallow region above the
72 coverslip where TIRF can be used. Different solutions are simultaneously flowed into the
73 chamber without mixing, and the filaments can be exposed to them sequentially and rapidly.

74

75 Here, we propose a series of basic protocols to set up single-actin-filament microfluidics assays
76 in the lab. Coverslips and microfluidics chambers can be prepared in advance (in half a day), and
77 the experiment itself, where several biochemical conditions can be tested, is done in less than a
78 day.

79

80 **PROTOCOL:**

81

82 **1. Microfluidic chamber preparation**

83

84 1.1. Select a SU-8 master mold with several chamber patterns. Typical chambers are cross-
85 shaped with three inlets and one outlet, 20 μm high and 800 μm wide (**Figure 1**). Such master
86 molds can be purchased from external companies or made in academic laboratories (e.g.,
87 Gicquel, Y. et al.⁵).

88

- 89 1.2. Place tape around the edge of the mold.
90
- 91 1.2.1. Put ~50 cm long, 19 mm wide, standard transparent office tape (see **Table of Materials**)
92 on a bench, sticky side up. Place the mold vertically at one end and along the midline of the
93 tape.
94
- 95 1.2.2. Roll the mold to the other end of the tape to create a 1 cm border around the mold.
96 Fold down the tape over the bottom of the mold.
97
- 98 1.3. Prepare Polydimethylsiloxane (PDMS) solution.
99
- 100 1.3.1. In a disposable weighing dish, directly pour 25–30 g of PDMS base (**Table of Materials**).
101 Add 10% weight/weight PDMS curing agent (**Table of Materials**) with a disposable plastic
102 Pasteur pipette.
103
- 104 1.3.2. Mix manually and thoroughly with a plastic stick. Ensure that the curing agent is well
105 incorporated into the PDMS base, even if stirring creates lots of bubbles.
106
- 107 1.4. Degas the PDMS solution in a vacuum desiccator (**Table of Materials**) for at least 5 min
108 at room temperature (RT). Bubbles will expand, rise to the surface, and burst when the vacuum
109 is broken.
110
- 111 1.5. Pour the PDMS solution over the SU-8 mold. Use a plastic stick to scrape and transfer as
112 much of the mixture as possible.
113
- 114 1.6. Degas PDMS for a second time (5 min in the vacuum desiccator). Make sure to get rid of
115 most bubbles (a few small bubbles at the top surface are fine).
116
- 117 1.7. Place the mold in an oven at 70 °C for at least 5 h for the PDMS to reticulate and solidify.
118
- 119 1.8. Remove solid PDMS chambers from the mold.
120
- 121 CAUTION: Silicon wafers for SU-8 molds are extremely fragile, so extensive care must be taken
122 when separating the PDMS from wafers. Work on a hard, flat surface and keep the wafer flat on
123 the surface.
124
- 125 1.8.1. With a razor blade, make a circular cut in the PDMS, about 1 cm away from the edge of
126 the mold. All patterns must be at least 0.5 cm inside the cut. Gently peel off the central PDMS
127 block with gentle tugs.
128
- 129 CAUTION: When peeling off, maintain the SU-8 mold flat on the benchtop to prevent breaking
130 it.
131

132 1.8.2. Place PDMS on clean aluminum foil, the molded surface facing the aluminum foil, to
133 protect its surface from dust and to make patterns more visible.

134

135 1.9. Choose and cut a chamber with a razor blade at least 0.5 cm away from the pattern. The
136 resulting PDMS block is around 0.5 cm high, 1.5 cm wide, and 3 cm long. Pierce three inlets and
137 one outlet with a biopsy punch 0.75 mm I.D. (**Table of Materials**).

138

139 1.10. Clean the PDMS chamber with ultrapure ethanol (**Table of Materials**) and air-dry using a
140 safety blowgun (**Table of Materials**). Place the PDMS with the pattern facing up in a clean Petri
141 dish, and close the dish with its lid.

142

143 2. **Glass coverslip cleaning**

144

145 NOTE: Here, a standard coverslip cleaning procedure, based on a series of sonication steps, is
146 detailed. Other glass coverslip-cleaning procedures have been described in many other
147 publications that can achieve similar satisfying results⁶⁻⁹.

148

149 2.1. Place 10–20 coverslips (40 mm long) on a Polytetrafluoroethylene (PTFE) holder (**Table**
150 **of Materials**). Sonicate the coverslips in 0.5 L of 2% glass cleaning solution (**Table of Materials**)
151 in a 1 L glass beaker (35 °C, 30 min).

152

153 2.2. Dispose the glass cleaning solution and rinse the coverslips extensively with dH₂O in at
154 least three successive 0.5 L baths.

155

156 2.3. Prepare 0.5 L of 2 M KOH in a 1 L glass beaker. Sonicate the coverslips in KOH (RT, 30
157 min). Dispose KOH and rinse the coverslips with dH₂O in at least three 0.5 L baths.

158

159 CAUTION: Use appropriate lab safety protection equipment (gloves, glasses, and lab coat).

160

161 2.4. Transfer and sonicate the coverslips in 0.5 L of ultrapure ethanol (RT, 30 min). Coverslips
162 can be kept in ethanol for up to 2 weeks. Close the beaker with thermoplastic film (**Table of**
163 **Materials**) to prevent evaporation. Before use, dry the coverslip with air flow.

164

165 3. **PDMS chamber assembly**

166

167 3.1. Preheat the hot plate to 100 °C. Place up to three cleaned PDMS chambers and glass
168 coverslips in a clean Petri dish. Place the open Petri dish in a deep Ultraviolet (UV) cleaner ($\lambda =$
169 185 nm, see **Table of Materials**) and expose it to UV light for 3–5 min.

170

171 NOTE: Alternatively, PDMS chambers and coverslips can be exposed to air or oxygen plasma for
172 30 s.

173

174 3.2. Gently position the PDMS chamber over the coverslip. Make sure the two surfaces put
175 in contact were directly exposed to UV. The PDMS automatically sticks to the glass and the
176 chamber becomes clearly visible.

177 3.3. To remove any air trapped at the PDMS-coverslip interface, very gently press the
178 surface with a finger. For a tighter bonding, press more strongly over corners and sides. Make
179 sure that the ceiling of the chamber does not come in contact with the glass surface.

180
181 3.4. Place the chamber with the glass bottom facing the hot plate at 100 °C for 5 min. After
182 this step, the glass-PDMS bonds become permanent, and chambers can only be used once. Use
183 the chamber immediately or store it in a clean Petri dish for up to a week.

184

185 4. [OPTIONAL] Direct passivation and functionalization

186

187 NOTE: Depending on the application, chambers can be passivated and functionalized either
188 once connected to the microfluidic controlling device (see **Table of Materials**) or by manually
189 injecting solutions directly into the chamber with a pipette prior to its connection to the
190 microfluidic device. The latter offers the advantage of consuming less reagent and avoiding
191 potential contamination by flowing the solution through the polyether ether ketone (PEEK)
192 tubing of the microfluidic device. In all the following steps, solutions are injected by directly
193 sticking the pipette tip into the outlet. In order to avoid creating bubbles inside the chamber,
194 make sure to have a tiny droplet sticking out of the pipette tip when plugging the tip into the
195 outlet of the PDMS chamber. Likewise, remove the pipette tip before the entire volume has
196 been injected.

197

198 4.1. Inject 20 µL of PLL-PEG (1 mg/mL in Phosphate-buffered saline (PBS)). Incubate for a
199 minimum of 1 h (or overnight) at RT. To prevent evaporation, place the PDMS chamber in a
200 humid box (e.g., an empty tip box with water in the lower compartment and the PDMS
201 chamber on the tip-holding platform).

202

203 4.2. Inject 20 µL of 100 pM spectrin-actin seeds (in F-buffer, see **Table 1** and **Table 2**). Wait
204 for no more than 1 min. Adjust the seed concentration and timing to tune seed surface density,
205 high enough for large statistics and low enough for filaments not to overlap.

206

207 NOTE: Alternatively, if spectrin-actin seeds are not available, use biotin-functionalized short
208 filament segments that will be immobilized on a streptavidin-coated coverslip^{9,10}.

209

210 4.3. [OPTIONAL] Inject 20 µL of 5% bovine serum albumin (BSA) in F-buffer. Leave at RT for
211 10 min.

212

213 4.4. [OPTIONAL] Inject 20 µL of 1 mg/mL β-casein in F-buffer. Leave at RT for 10 min.

214

215 NOTE: Follow steps 4.3 and/or 4.4 to further passivate the chamber. The choice of passivation
216 depends on the proteins used and does not work equally well on all ABPs. When using actin
217 alone, PLL-PEG or BSA is sufficient.

218

219 5. **Connect microfluidic device**

220

221 NOTE: Use a pressure-based microfluidic system with up to four channels to control flows in the
222 microfluidic chamber (**Figure 1A**, see **Table of Materials**). To avoid bubbles forming in the
223 microfluidic tubing and perturbing flow stability, degas all solutions. Place 5 mL of dH₂O and 10
224 mL of F-buffer stock in a vacuum desiccator connected to a vacuum pump (ultimate vacuum
225 <250 mbar) and degas for at least 1 h at RT.

226

227 5.1. Rinse inlets + outlet tubings with dH₂O (500 µL, 300 mbar).

228

229 5.2. Fill all 2 mL reservoir tubes (see **Table of Materials**) with 300 µL of F-buffer. Set pressure
230 to 300 mbar and let five to eight drops go to waste. Repeat for each channel and set pressure
231 to 0.

232

233 5.3. Connect the outlet and rinse the chamber extensively.

234

235 5.3.1. Set the pressure for the reservoir tube 4 (outlet) to 50 mbar. Once a droplet comes out
236 from the tubing end, connect the tubing to the outlet of the PDMS chamber. The liquid fills in
237 the chamber and comes out of all inlets.

238

239 5.3.2. [OPTIONAL] If the chamber has been directly passivated (section 4), set pressure to 100
240 mbar to rinse the chamber with 50–100 µL of F-buffer (3–5 min). Remove the excess liquid at
241 inlets with a cleaning tissue.

242

243 5.3.3. Set pressure to 20 mbar.

244

245 5.4. Connect inlets.

246

247 5.4.1. Set the pressure for the reservoir tube 1 to 50 mbar. To avoid introducing air bubbles,
248 ensure that a droplet comes out of the tubing and the PDMS inlet.

249

250 5.4.2. Connect the tubing to inlet 1 (the two droplets merging when connecting). Set the
251 pressure to 30 mbar.

252

253 5.4.3. Repeat steps 5.4.1–5.4.2 to connect inlets 2 and 3.

254

255 5.5. Set the pressure of all inlets to 20 mbar and the outlet pressure to 0 mbar. Ensure that
256 the flow rates in the inlets are roughly equal (see Troubleshooting section).

257

258 [Place **Figure 1** here]

259

260 6. **Configuring the setup with standard flow rates**

261

262 NOTE: The computer-controlled pressure system allows easy and precise adjustment of the
263 pressures of all inlets/outlet connected to the PDMS chamber, therefore the control of
264 incoming and outgoing flow rates. Preset configurations can be saved and turned on/off with
265 a single mouse click. Below are the recommended configurations (unless otherwise stated,
266 outlet pressure is set to 0 mbar). See **Table 3** for expected flow rates for these preset
267 configurations. The pressures indicated here must be adjusted depending on the chamber
268 geometry and system configuration.

269

270 6.1. **Change:** Use this preset when changing one or more reservoir(s). It creates a mild
271 backward flow in the tubing of interest to prevent introducing bubbles.

272

273 6.2.1. Set all inlet pressures to 12 mbar and outlet pressure to 5 mbar (**Figure 2B**).

274

275 6.2. **High Flow 'All':** Use this preset to rapidly inject three solutions in parallel. They will
276 reach the chamber within 4 min.

277

278 6.2.1. Set all inlet pressures to 150 mbar.

279

280 6.3. **High Flow 'x':** Use this preset to rapidly inject a solution. It will reach the chamber within
281 3 min (**Figure 3A–C**).

282

283 6.3.1. Set inlet 'x' pressure to 150 mbar (~15 $\mu\text{L}/\text{min}$). The pressure in the other inlets is
284 adjusted to around 100 mbar, such that the resulting flow rate in these inlets is ~500 nL/min.

285

286 6.4. **Mid Flow 'All':** Use this preset to pause the system.

287

288 6.4.1. Set all inlets to 20 mbar (**Figure 2A**).

289

290 6.5. **Mid Flow 'x':** Use this preset to allow the solution 'x' to fill in most of the main channel
291 width (see **Figure 2C,D**), while restricting the other inlet solutions to the channel sides. Actin
292 filaments in the chamber will thus be exposed to the biochemical condition imposed by solution
293 'x' only.

294

295 6.5.1. Set inlet 'x' pressure to 12 mbar. Set the pressure in the other inlets and adjust to ~9
296 mbar, such that their respective flow rates are ~ 150 nL/min.

297

298 [Place **Figure 2** here]

299

300 7. **Changing solution 'x'**

301

302 NOTE: As shown in **Figure 3A–C**, it is important to keep in mind that solutions take minutes to
303 flow from a reservoir tube to the main channel of the chamber. This minimal 'dead' time is
304 imposed by the liquid volume contained in the tubing and the flow profile within the tubing
305 (**Figure 3A–C**).

306

307 7.1. Prepare 200–300 μL of solution in a new reservoir tube. Set pressure to **Change** setting
308 (see section 6).

309

310 7.2. Unscrew the reservoir tube of inlet 'x'. The solution in the tubing will slowly flow
311 backwards, from the chamber to the free tubing tip. The measured flow rate becomes negative
312 (**Figure 2B**).

313

314 7.3. Once a tiny droplet has formed at the tubing tip, screw in the new tube with the fresh
315 solution. Once the tube is correctly tightened to the pressure system, the flow rate of the inlet
316 returns to positive.

317

318 7.4. Set the pressure setting to **High Flow 'x'**.

319

320 7.5. Depending on the microfluidic configuration and chamber geometry, wait for 3–5 min
321 for the solution to fully fill in the tubing and reach the chamber.

322

323 7.6. [OPTIONAL] Follow this process by measuring the increase in fluorescence over time
324 (**Figure 3C**).

325

326 [Place **Figure 3** here]

327

328 8. **Basic single filament experiment: Adenosine diphosphate (ADP)-actin barbed end**
329 **depolymerization**

330

331 NOTE: This section assumes a non-functionalized chamber (section 5 only). If the chamber has
332 been directly functionalized (section 4), start at step 8.4.

333

334 8.1. Surface functionalization with actin filament seeds:

335

336 8.1.1. Change solution 3 to 200 μL of 50 pM spectrin-actin seeds¹¹ in F-buffer (see section 7).

337

338 NOTE: Alternatively, if spectrin-actin seeds are not available, one can use biotin-functionalized
339 short filament segments that will be immobilized on a streptavidin-coated coverslip (refer to^{9,10}
340 for details).

341

342 8.1.2. Inject for 2 min with **High Flow 3**.

343

344 NOTE: Adjust the concentration and time depending on the final seed density.

345

346 8.2. Surface passivation:

347

348 8.2.1. Change tube 3 with 300 μL of 5% BSA in F-buffer.

349

350 8.2.2. Inject for 5 min at **High Flow 3**, followed by 5 min at **Mid Flow 3**. During this second step,
351 reduce pressure in channels 1 and 2 to 7–8 mbar to get a counter flow ~100 nL/min, so that
352 the entire chamber surfaces are BSA passivated.

353

354 NOTE: Since the BSA solution is more viscous, the pressures need to be adjusted accordingly.

355

356 8.3. Change tube 3 to F-buffer and rinse the channel (5 min, **High Flow 3**).

357

358 8.4. Prepare the following 200–300 μL solutions, all proteins being diluted in F-buffer:

359 Inlet 1, polymerization solution: 1 μM 10% Alexa-488 labeled G-actin, 1 μM profilin (**Table 1**).

360 Inlet 2, aging solution: 0.15 μM 10% Alexa-488 labeled G-actin.

361 Inlet 3, depolymerization solution: F-buffer only.

362

363 NOTE: Profilin is used here to prevent spontaneous nucleation and maintain a constant
364 concentration of G-actin.

365

366 8.5. Change tubes 1 to 3 (section 7). Inject using the **High Flow All** preset for 3–4 min. The
367 three solutions have now filled the PEEK tubing and reached the chamber (**Figure 3A**). The glass
368 surface can be exposed to any inlet solution without deadtime (<1 s, **Figure 3D,E**).

369

370 8.6. Switch on the microscope. Set the settings: 150 mW 488 nm excitation laser at 10%–
371 20% power, 100–200 ms camera exposure time, 200–300 nm TIRF penetration depth, 60x
372 objective. These settings are used throughout the manuscript.

373

374 8.7. Filament polymerization (**Figure 4A**)

375

376 8.7.1. Set the pressure setting to **Mid Flow 1** for ~10 min.

377

378 8.7.2. [OPTIONAL] Record polymerization (1 frame/20 s, TIRF). Filaments should polymerize at
379 around 10 subunits/second (sub/s)^{1,12}.

380

381 8.8. Filament aging: Set the pressure setting to **Mid Flow 2** for 15 min. At the critical
382 concentration, 0.15 μM G-actin, filament length will remain constant, and filaments will turn to
383 >99% ADP-F-actin⁴.

384

385 8.9. Depolymerization (**Figure 4A**):

386

387 8.9.1. Start acquisition at 1 frame/5 s, in epifluorescence mode. As there is a very low
388 fluorescence background in channels 2 and 3, it is not necessary to use TIRF.

389

390 8.9.2. After one to two frames, switch to **Mid Flow 3**. Filaments should depolymerize at around
391 10 sub/s (reference¹²).

392

393 8.10. To reset the experiment, break all fluorescently labeled filaments by continuously
394 exposing them to the laser at maximum power for ~2 min. To test different conditions, change
395 solutions 1, 2, or 3 and inject them (**High Flow**, 3–4 min). Repeat steps 8.7–8.9.

396

397 9. Other single-filament experiments

398

399 9.1. Testing the interactions of ABPs with F-actin

400

401 NOTE: Microfluidics has been successfully used to quantify the activity of several side-binding
402 ABPs, such as cofilin, tropomyosin, and Arp2/3. Following the protocol in section 8:

403

404 9.1.1. Change channel 3 to the fluorescent ABP of interest in the F-buffer. Inject (**High Flow 3**,
405 3 min).

406

407 9.1.2. Filament polymerization: Set the pressure setting to **Mid Flow 1** for 10 min.

408

409 9.1.3. ABP binding: Start acquisition with TIRF. Adjust frame rate depending on the ABP
410 concentration. After 1–2 frames, switch to **Mid Flow 3**.

411

412 NOTE: Depending on the ABP, it can also be possible to rapidly (e.g., for less than 5 s) switch to
413 **Mid Flow 2** to further reduce the background fluorescence when taking an image.

414

415 9.1.4. ABP unbinding: While continuing the acquisition, switch to **Mid Flow 2**.

416

417 9.2. Polymerization with formin at the free barbed end

418

419 NOTE: Formins have been shown to affect filament barbed end polymerization. Microfluidics is
420 particularly adapted to measure formin binding and unbinding rates and their impact on
421 filament elongation.

422

423 9.2.1. Prepare the following solutions:

424 Channel 1: 10 nM formin in F-buffer (**Table 1**).

425 Channel 2: 1 μ M 10% Alexa-488 labeled G-actin, 4 μ M profilin.

426 Channel 3: F-buffer.

427

428 9.2.2. Change tubes 1, 2, and 3 (section 7). Inject using the **High Flow All** preset for 3–4 min.

429

430 9.2.3. Initiate filament polymerization: Set the pressure setting to **Mid Flow 2** for 2 min.

431

432 9.2.4. Formin binding to filament barbed end: Set the pressure settings to **Mid Flow 1** for 30 s.

433

434 9.2.5. Formin-mediated polymerization: Set the pressure setting to **Mid Flow 2**. With formin
435 mDia1 at their barbed end, filaments should polymerize at around 50 sub/s^{13–15}.

436

437 9.3. Polymerization/depolymerization from surface-anchored formin

438

439 NOTE: The polymerization and depolymerization rates of formin-decorated barbed ends have
440 been shown to depend on the tension applied to the filament. In microfluidics, the friction of
441 the fluid flow along the filament side generates a tension proportional to the filament length
442 and to the flow rate^{14,16}.

443

444 9.3.1. Use the method in section 8 described above, replacing steps 8.1, 8.2, and 8.3 for
445 surface passivation with:

446

447 9.3.1.1. Change tube 3 to 1 µg/mL anti-His antibody in F-buffer. Inject for 2 min with **High**
448 **Flow 3**.

449

450 9.3.1.2. Change tube 3 with 5% BSA in F-buffer. Inject for 5 min at **High Flow 3**, followed
451 by 5 min at **Mid Flow 3**. During this second step, reduce pressure in channels 1 and 2 to 7–8
452 mbar to get a counter flow ~100 nL/min so that the entire chamber surfaces are BSA
453 passivated.

454

455 9.3.1.3. Change tube 3 to 100 nM His-tagged formin in F-buffer. Inject for 5 min with
456 **High Flow 3**. Change tube 3 with F-buffer. Inject for 5 min with **High Flow 3** to ensure that no
457 formins remain in the tubing.

458

459 9.3.2. Prepare and inject the following solutions (all 200–300 µL, in F-buffer):

460 Channel 1: 1 µM 10% Alexa-488 labeled G-actin

461 Channel 2: 1 µM unlabeled G-actin, 4 µM profilin.

462 Channel 3: F-buffer only.

463

464 9.3.3. Filament nucleation: Expose the surface-anchored formins to G-actin (setting **Mid Flow**
465 **1**).

466

467 9.3.4. Filament polymerization: Expose the chamber to profilin-actin using **Mid Flow 2**.

468

469 9.3.5. Start acquisition: 1 frame/2 s, epifluorescence. With formin mDia1, filaments should
470 polymerize at 50–80 sub/s, depending on the filament length and the flow rate¹⁴.

471

472 9.3.6. Filament depolymerization: Start acquisition (1 frame/4 s, epifluorescence). After 1–2
473 frames, expose the filaments to F-buffer, **Mid Flow 3**. With formin mDia1, filaments should
474 depolymerize at 5–15 sub/s, depending on the filament length and flow rate¹⁴.

475

476 9.4. Actin filaments with unlabeled segments

477

478 NOTE: Actin fluorescent labeling creates several artifacts, such as pauses during
479 depolymerization¹⁷ and altered tropomyosin binding¹⁸. A workaround for these artifacts is to
480 use microfluidics to assemble filaments displaying unlabeled segments.

481

482 9.4.1. Prepare and inject the following solutions (200–300 μ L in F-buffer):

483 Channel 1: 1 μ M unlabeled G-actin, 1 μ M profilin.

484 Channel 2: 0.3 μ M 10% Alexa-488 labeled G-actin.

485

486 9.4.2. Sequentially expose the surface to channel 2 (5 min), channel 1 (10 min), and channel 2
487 (15 min) to generate ADP-actin unlabeled segments with fluorescently labeled segments at
488 each end.

489

490 9.5. Barbed end-anchored filaments with gelsolin

491

492 NOTE: With spectrin-actin seeds, filaments polymerize at their free barbed end while the
493 pointed end is stabilized by the spectrin-actin seed. An alternative is to anchor filaments with a
494 barbed end capper such as gelsolin.

495

496 9.5.1. Prepare a F-actin solution of 4 μ M 10% Alexa-488 labeled G-actin in 20 μ L of F-buffer.
497 Let the actin spontaneously nucleate and polymerize at RT for at least 30 min on the bench.
498 Wrap the tube in aluminum foil to protect it from light.

499

500 9.5.2. Meanwhile, prepare the microfluidic chamber and passivate the surface with a mixture
501 of 5% BSA and 1% biotin-BSA (see step 8.2).

502

503 9.5.3. Rinse channel 3 with F-buffer (2 min at **High Flow 3**). Inject 10 μ g/mL neutravidin in F-
504 buffer (4 min at **High Flow 3**).

505

506 9.5.4. Change tubes to:

507 Channel 1: 10 nM biotin-gelsolin (**Table 1**).

508 Channel 2: F-buffer.

509 Channel 3: 0.4 μ M prepolymerized F-actin.

510

511 9.5.5. Inject all solutions together using the **High Flow All** setting for 3 min.

512

513 9.5.6. Expose the entire chamber to gelsolin (**Mid Flow 1**, 30 s).

514

515 9.5.7. Attach filaments to the surface (**Low Flow 3**: Channel 3 at 3 mbar, Channels 1 and 2 at ~2
516 mbar, for about 2 min).

517

518 9.5.8. [OPTIONAL] If the filament density is too low, repeat step 9.5.6.

519

520 9.5.9. Pointed end depolymerization: Start acquisition (1 frame/30 s, epifluorescence). After
521 1–2 frames, expose filaments to buffer only, **Mid Flow 2**. Filaments should depolymerize at
522 around 0.2 sub/s.

523

524 10. Fascin-induced filament bundle formation and disassembly by ADF/cofilin

525

526 NOTE: To form actin filament bundles, take care to have a sufficiently high filament seed
527 density at the surface of the chamber. When exposed to fascin protein, neighboring filaments
528 that fluctuate laterally will be dynamically cross-linked by fascin proteins. As fascin quickly
529 unbinds from the filament side¹⁹, fascin has to be constantly present in the main flowing
530 solution in order to maintain filament bundling.

531

532 10.1. Follow steps 8.1–8.3.

533

534 10.2. Prepare the following 200–300 μL solutions, all proteins being diluted in F-buffer:

535 Channel 1: Polymerization solution (1 μM 10% Alexa-488 labeled G-actin, 1 μM profilin).

536 Channel 2: Bundling solution (200 nM fascin (**Table 1**), 0.15 μM 10% Alexa-488 labeled G-actin).

537 Channel 3: Disassembly solution (200 nM ADF/cofilin (**Table 1**), 100 nM fascin, 0.15 μM 10%
538 Alexa-488 labeled G-actin).

539

540 10.3. Change tubes 1 to 3 (section 7). Inject using the **High Flow All** preset, for 3–4 min.

541

542 10.4. Filament polymerization: Set the pressure setting to **Mid Flow 1** for ~ 10 min.
543 Polymerization can be imaged with TIRF.

544

545 10.5. Filament bundling (**Figure 4C**): Start image acquisition (1 frame/5 s, epifluorescence).
546 After 1–2 frames, set the pressure setting to **Mid Flow 2** and observe filament bundling.

547

548 10.6. Bundle fragmentation: Start image acquisition (1 frame/5 s, epifluorescence). After 1–2
549 frames, set the pressure setting to **Mid Flow 3** and observe the cofilin-induced disassembly of
550 both single filaments and bundles.

551

552 11. Microfluidic device cleaning procedure

553

554 NOTE: To avoid any contamination from one experiment to another, it is critical to extensively
555 clean and completely dry all the tubings and flow meters after each experiment.

556

557 11.1. Disconnect all tubings from the PDMS chamber and discard the chamber.

558

559 11.2. To clean PEEK tubing and flow meters, tape the tubing ends in an empty 15 mL plastic
560 tube and inject the following solutions at maximum pressure until the reservoir is almost
561 empty:

562 400 μL of F-buffer.

563 400 μL of 0.5 M NaOH.

564 400 μL of pure water.

565 200 μL of isopropanol.

566

567 11.3. Replace with an empty reservoir and blow air until the tubings are fully dry (~ 2 –4 min,
568 maximum pressure).

569

570 12. **Image analysis**

571

572 NOTE: While this manuscript focuses on the method to assemble, manipulate, and visualize
573 single actin filaments in microfluidics, a brief method to analyze acquired movies is provided
574 here. The analysis is performed on 16-bit images, using ImageJ, following section 8.

575

576 12.1. Image treatment is minimal:

577

578 12.1.1. Import the polymerization or depolymerization image stack.

579

580 12.1.2. [OPTIONAL] Homogenize image intensity with function **Subtract Background** (default
581 settings (i.e. 'Rolling ball radius' = 50 pixels)). This is particularly useful if the background
582 fluorescence changes during the course of a movie or if fluorescence illumination is not
583 homogeneous over the field of view.

584

585 12.1.3. Adjust brightness and contrast (background near zero, filaments near maximum).

586

587 12.2. Create filament kymograph:

588

589 12.2.1. Select a filament that does not pause, break or detach. Do not select based on behavior
590 otherwise. Draw a line 1–2 pixels above (**Straight Line** tool). Save the filament number (**Add** in
591 **ROI Manager**).

592

593 12.2.2. Apply the function **Reslice** (Slice count: 5 pixels). Calculate the maximum intensity
594 (function **Zprojection**).

595

596 12.3. Measure the polymerization/depolymerization rate:

597

598 12.3.1. On the kymograph, draw a line along the filament barbed end (**Straight Line** tool, **Figure**
599 **4A**). Measure the line width and height (function **Measure**).

600

601 12.4. Repeat steps 12.2–12.3 over multiple filaments. Calculate the
602 polymerization/depolymerization rates (**Figure 4A**):

603 $v = \frac{w * pix}{2.7 * h * dt}$, where v is the rate (in sub/s), w the line width (pixels), pix the pixel size (nm), h

604 the line height (frames), and dt the time between frames (in second). Here, 2.7 nm corresponds
605 to the effective contribution of an actin subunit to the filament length.

606

607 **REPRESENTATIVE RESULTS:**

608 For all the experiments described above, fluorescently labeled actin filaments should be clearly
609 visible, with good contrast, indicative of low background fluorescence from the surface (**Figure**
610 **4**, see **Supplementary File 1** for troubleshooting of common issues). Actin filaments should also
611 not stick to the surface: when the dominant flow rate is low, the actin filaments' lateral

612 fluctuations should be perceptible when observing them live and allow one to clearly determine
 613 that they are anchored by one of their ends only. Similarly, when using TIRF imaging, their
 614 vertical fluctuations should be visible by changes in intensity along their length and time.
 615 Depending on the applied flow rates, one may need to adjust the TIRF penetration depth to
 616 optimize the image quality of the actin filaments acquired by TIRF.

617
 618 When exposing filaments to polymerization conditions (see section 8), filament elongation
 619 should be regular (i.e., the elongation at the end of the filament is not impeded by surface
 620 interaction or permanent sticking). In addition, the measured filament barbed end elongation
 621 rate should match the expected value according to the actin concentration in the tube^{1,20},
 622 indicating that the tube solution has been correctly flowed up to the microfluidic chamber
 623 (**Figure 4A**). Similarly, when exposed to a buffer solution, filaments should depolymerize
 624 steadily at a rate that reflects their ADP-content⁴ (**Figure 4A**). When exposing already grown
 625 actin filaments to a solution of fluorescently labeled cofilin, cofilin clusters will be nucleated and
 626 grow toward both the pointed and barbed ends (**Figure 4B**) at a rate that is dependent on the
 627 cofilin concentration. When assessing a potential cross-linking activity of an ABP, such as fascin
 628 (**Figure 4C**), close-by actin filaments forming bundles will be easily detected by their higher
 629 fluorescence intensity and a change in their lateral fluctuations.

630
 631 The flow of liquid applies a viscous friction force on actin filaments that are anchored to the
 632 surface of the microfluidic chamber. The friction force coefficient on F-actin is $\eta = 6.10^{-4}$
 633 $\text{pN}\cdot\text{s}/\mu\text{m}^2$, expressed per filament micron length¹⁴. At intermediate flow rates, as filament
 634 height fluctuates around a constant average of 250 nm above the surface, there exists a force
 635 gradient from the free-floating end up to the filament anchoring point. One can therefore
 636 compute the applied tension at any point along the filament, using $F = 6\eta\pi Lv$, where v is the
 637 local flow velocity 250 nm above the surface (**Figure 1B**) and L is the downstream filament
 638 segment length (i.e., from the considered point up to the free end). For higher flow rates, the
 639 filament average height is not constant but increases linearly from the anchoring point to the
 640 free end, remains below 250 nm on average, and will vary depending on the flow rates, thus
 641 leading to a more complex tension force profile along the filament²¹.

642
 643 [Place **Figure 4** here]

644
 645 **FIGURE AND TABLE LEGENDS:**

646 **Figure 1: Injecting solutions through a microfluidic chamber.** (A) Standard microfluidic setup for
 647 single actin filaments experiments. Protein solutions, placed in reservoirs 1–3, are pushed into
 648 the chamber by adjusting the pressure in the gas phase. The generated flow rate is measured
 649 by flow meters. Inside the microfluidic chambers, solutions do not mix and occupy space
 650 depending on the relative pressures applied (here, equal pressure on all inlets). Typical
 651 dimensions: reservoir tubes contain up to 2 mL of solution. PEEK tubing (0.25 mm inner
 652 diameter) connects the reservoirs to the flow meters (after 10 cm of tubing) and then to the
 653 PDMS chamber (after another 70 cm). Silicon tubing and stainless steel tubing couplers are
 654 used to connect the PEEK tubing to the PDMS inlets. The main microfluidic channel is 20–60 μm
 655 high, around 1 mm wide and 1 cm long. (B,C) Flow profiles inside the microfluidic chamber. The

656 fluid generates a parabolic profile across the chamber height: $v(z) = 6z(h-z)R/h^3w$, where h and
 657 w are the chamber height and width, and R is the total flow rate (**B**). When the chamber width
 658 is considerably larger than its height, the flow is nearly uniform across the chamber, except at
 659 the PDMS surfaces, where it goes to zero (**C**). Bottom: Single actin filaments polymerized from
 660 surface-anchored spectrin-actin seeds.

661

662 **Figure 2: The pressure applied to each reservoir controls the partition/spatial distribution of**
 663 **solutions inside the microfluidic chamber. (A)** With equal pressure applied to the reservoirs,
 664 each solution occupies one-third of the chamber. (**B**) When changing a reservoir tube (here
 665 reservoir 3), the effective pressure drops down to zero, creating a backward flow. (**C,D**)
 666 Increasing the relative pressure on one of the reservoirs allows exposure of the glass surface to
 667 a single solution. The field of view in the middle of the chamber can be sequentially exposed to
 668 solutions 1 and 2 by alternating between configuration **Mid Flow 1 (C)** and **Mid Flow 2 (D)**.

669

670 **Figure 3: Delayed arrival of solutions from the reservoirs to the PDMS chamber and rapid**
 671 **change of biochemical conditions. (A–C)** Delayed arrival of solutions from the reservoirs to the
 672 PDMS chamber. (**A**) Depending on the chamber geometry, the tube length, and the applied
 673 pressure at the inlet(s), the replacement of one solution by another is not instantaneous. After
 674 changing the reservoir tube to one containing a fluorescent solution (0 min), the solution
 675 progressively fills in the tubing (0.4 min) and the PDMS chamber (1–2 min). Indicative timing is
 676 given for a 150 mbar applied pressure, 80 cm PEEK tubing, and a 1600 μm wide, 20 μm high
 677 PDMS chamber. (**B**) The parabolic flow profile inside the PEEK tubing generates an effective
 678 gradient of fluorescence along the tubing radial profile and inside the chamber (see also **Figure**
 679 **1B**). (**C**) Delayed arrival of solutions can be quantified by measuring the background
 680 epifluorescence signal in the chamber as a function of time. Experimental conditions: 0.5 μM
 681 10% Alexa-568-labeled G-actin is injected with 150 mbar through a flow meter and 80 cm PEEK
 682 tubing. (**D,E**) Rapid change of biochemical conditions. (**D**) Pattern of incoming solutions in two
 683 **Mid Flow** conditions. (**E**) Increase in background fluorescence as a readout of actin
 684 concentration. Time $t = 0$ is set as the onset of fluorescence increases. Solution 1: 0.5 μM 10%
 685 Alexa-488-labeled G-actin, solution 2: F-buffer. (**C,E**) PDMS chamber: 20 μm high and 1600 μm
 686 wide. The epifluorescence intensity, $\sim 2 \mu\text{m}$ above the surface, was quantified by averaging the
 687 signal over the full field of view, normalized to 0 in the absence of fluorophore and 1 at
 688 maximum intensity.

689

690 **Figure 4: Representative results. Typical experiments in which actin filaments are polymerized**
 691 **from spectrin-actin seeds and exposed to different ABPs.** For the sake of clarity, only a fraction
 692 of the field of view is shown. (**A**) Outcome from the basic polymerization-depolymerization
 693 experiment (section 8). Filaments are polymerized with a solution of 0.8 μM 10% Alexa-488
 694 labeled G-actin, aged for 15 min to convert all subunits into ADP-actin (not shown), and
 695 depolymerized when exposed to F-buffer only. Bottom: kymographs used to quantify the
 696 polymerization and depolymerization rates. Acquired at 1 frame/5 s, 200 ms exposure time, 150
 697 mW 488 nm laser at 9% power, TIRF (laser penetration depth 250 nm). (**B**) Fragmentation of
 698 single actin filaments by 500 nM mCherry-cofilin-1. Actin is labeled with ATP-ATTO488²²
 699 (yellow) and cofilin-1 is fused to mCherry (blue). Top: fraction of a field of view. Note: protein

700 aggregates on the surface. Bottom: kymograph showing the binding of cofilin-1 to a filament
701 (arrowheads show cofilin-1 domain nucleation events), leading to a fragmentation event
702 (lightning symbol). Acquired at 1 frame/4 s, 200 ms exposure, 150 mW 488 nm laser at 16% and
703 100 mW 561 nm laser at 12% power, epifluorescence. (C) Bundling of actin filaments by fascin
704 (section 10.5). Filaments were first polymerized with 0.8 μ M 5% Alexa-488 labeled G-actin and
705 bundled with 200 nM fascin. Compared with single filaments, filament bundles appear two- to
706 threefold brighter and not perfectly aligned with the flow. Acquired at 1 frame/10 s, 200 ms
707 exposure, 20% 200 W Mercury lamp intensity, epifluorescence. (A–C) The background was
708 subtracted with ImageJ's ad hoc function.

709

710 **Table 1: Actin and actin-binding proteins**^{23–30}

711

712 **Table 2: F-buffer composition.** DABCO and a relatively high concentration of DTT are used to
713 limit photo-induced damage to filaments due to light exposure during fluorescence microscopy
714 experiments.

715

716 **Table 3: Correspondence between applied pressures and measured flow rates.** The resulting
717 flow rates highly depend on the experimental setup. Values are given for a microfluidic
718 chamber with a 1 cm long main channel of cross-section 20 μ m x 800 μ m (height x width),
719 connected to each reservoir with 80 cm long PEEK tubing.

720

721 **Supplementary File 1: Classical issues, causes, and solutions.** They commonly encountered
722 issues when working with microfluidics and/or single actin filaments.

723

724 **DISCUSSION:**

725 Compared to standard single-filament methods where actin filaments are anchored to the
726 surface by multiple points along their length or maintained close to it by a crowding agent such
727 as methylcellulose, microfluidics offers a number of advantages. As interactions with the
728 surface are minimal, the artificial pauses these interactions can induce during both elongation
729 and depolymerization are avoided. The filaments are aligned by the flow, parallel to each other,
730 easing their monitoring and the measurement of their lengths. The solution around the
731 filaments is constantly renewed, exposing them to constant protein concentrations. Being able
732 to rapidly (<1 s, **Figure 3D,E**) switch between different protein solutions to which the filaments
733 are exposed allows one to perform time-controlled sequential experiments, which are often
734 instrumental for kinetic studies. Finally, the viscous drag exerted by the flowing solution on the
735 filaments can be exploited to apply controlled mechanical stress on the filaments
736 (Representative Results section). One should note that moderate fluid flows (**Mid Flow** pressure
737 settings) bring filaments close enough to the surface (~250 nm) to efficiently image them with
738 TIRF while generating minimal tension (<1 pN)¹⁴.

739

740 Compared to classical single-filament assays, however, microfluidics will require larger volumes
741 of protein solutions: typically a few 100 μ L, when a standard experiment could be done with
742 less than 10 μ L. This can be a limitation when using precious proteins. Classical experiments can
743 be used to help establish the relevant experimental conditions (e.g., absolute or relative

744 concentrations of different proteins) before starting a series of microfluidic experiments.
745 Another limitation, as for any other single-filament techniques *in vitro*, comes from the
746 imperfect passivation of the coverslip surface. Reproducibility in coverslip cleaning and binding
747 of the passivation layer (BSA, PEGylation, etc.) is always hard to control. A one-step passivation
748 technique based on PEG-silane surface treatment has become the technique of choice in many
749 laboratories^{7,15}. As such, the effective density of filament seeds may vary between experiments
750 by roughly twofold, even when repeated as accurately as possible. One should aim for a
751 satisfactory range of filament surface density and be prepared to repeat the experiment if
752 needed. Commonly encountered issues when working with microfluidics and/or single actin
753 filaments are discussed in **Supplementary File 1**.

754
755 For the basic protocol presented here, one should note that spectrin-actin seeds, which can be
756 viewed as short stabilized filaments, are randomly oriented as they stick to the surface. As a
757 consequence, as the filaments grown from these seeds align with the flow, their portion closest
758 to the seed will be sharply bent, each with its own angle. The length over which filaments are
759 bent is usually very small when filaments are exposed to mid or high flows. In fact, this length
760 will generally be smaller than the diffraction limit (~200 nm) and will thus not be easily
761 detected. Importantly, the ABPs that are sensitive to filament curvature will bind and function
762 differently in this highly bent region. To avoid biasing results, the simplest is to exclude this
763 region from the analysis²¹.

764
765 Before we started using microfluidics to manipulate and visualize single actin filaments, it had
766 already been used to study single DNA filaments³, which are far more flexible. This can give rise
767 to notable differences, as the flow can dramatically unwind DNA and change its apparent length
768 dramatically. Microfluidics can also be used, very similarly to the method presented here, to
769 study microtubules; these are much stiffer but can nonetheless be made to align with the flow
770 in order to measure their elongation and depolymerization, taking advantage of the rapid
771 switch of conditions^{31,32}, or be bent by a perpendicular flow to measure microtubule plasticity⁷.

772
773 We have presented here the protocol for the basic experiment, where filaments are anchored
774 by one end only and where the flow direction in the field of view is the same throughout the
775 experiment. These two characteristics can be varied. For example, filaments can be anchored
776 by multiple points in order to generate a different force profile along the filament. Likewise, the
777 flow direction can be varied (in the vicinity of the junction between the entry channels, in the
778 flow chamber) to locally bend filaments, as the unanchored part will be pointing in a different
779 direction than the anchored filament segment²¹. Filaments elongating from randomly anchored
780 spectrin-actin seeds can also be exposed to cross-linking proteins to form bundles³³ (see section
781 10). By combining microfluidics with other techniques (micropatterning, optical tweezers, etc.)
782 or designing microfluidic chambers with compartments to modify flow lines, multiple
783 configurations can be created to study specific ABP activity onto single filaments or to form
784 small actin networks³⁴. The number of combinations, together with the advantage and
785 versatility of microfluidics, offers many tools to researchers in order to decipher the spatio-
786 temporal regulation of actin networks at the molecular scale.

787

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794

795 **DISCLOSURES:**

796 The authors declare no conflicts of interest.

797

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