

Metadata of the chapter that will be visualized online

Series Title	Methods in Molecular Biology	
Chapter Title	Nanofluidic Devices for Rapid Continuous-Flow Bioseparation	
Chapter SubTitle		
Copyright Year	2012	
Copyright Holder	Springer Science + Business Media, LLC	
Corresponding Author	Family Name	Fu
	Particle	
	Given Name	Jianping
	Suffix	
	Division	Departments of Mechanical Engineering and Biomedical Engineering
	Organization	University of Michigan
	Address	2276 G. G. Brown Building, 2350 Hayward Street, 48109-2125, Ann Arbor, MI, USA
	Email	jpfu@umich.edu
Author	Family Name	Mao
	Particle	
	Given Name	Pan
	Suffix	
	Division	
	Organization	
	Address	
	Email	
Abstract	Compared with conventional gel-based techniques, such as gel electrophoresis, which are routinely used for bioseparation in biology and biomedical laboratories, nanofluidic devices with regular engineered sieving structures offer the potential for faster separation, better resolution, higher throughput, and more convenient sample recovery. Here, we detail the fabrication process of a two-dimensional nanofluidic filter array device and its implementation for rapid continuous-flow separation of biomolecules such as proteins.	
Key words: (separated by ',')	Nanofluidics - Nanofilter - Bioseparation - Micro/nanofabrication - Continuous-flow - Molecular sieve	

Chapter 10

Nanofluidic Devices for Rapid Continuous-Flow Bioseparation

Pan Mao and Jianping Fu

Abstract

Compared with conventional gel-based techniques, such as gel electrophoresis, which are routinely used for bioseparation in biology and biomedical laboratories, nanofluidic devices with regular engineered sieving structures offer the potential for faster separation, better resolution, higher throughput, and more convenient sample recovery. Here, we detail the fabrication process of a two-dimensional nanofluidic filter array device and its implementation for rapid continuous-flow separation of biomolecules such as proteins.

Key words: Nanofluidics, Nanofilter, Bioseparation, Micro/nanofabrication, Continuous-flow, Molecular sieve

1. Introduction

The accurate and efficient separation and identification of different biomolecules, of varying biological properties from a complex biological sample, is critical to numerous areas of biological, medical, pharmaceutical, and food industry research (1, 2). Currently, bioseparation is routinely achieved with gel-based separation techniques, such as gel-exclusion chromatography and slab-gel electrophoresis. However, a common, underlying limitation of these gel-based techniques is a lack of engineering control in the molecular sieving and filtering process, which is a feature of the gel's intrinsic random nanoporous material properties. Moreover, these gel materials are difficult to integrate into automated multistep chip-based bioanalysis systems. Over the past decade, there has been a great interest in the development of regular sieving and filtering nanostructures to replace disordered porous gel media. This achieves a more

28 efficient separation than with the polymeric gels and fibrous
 29 membranes in terms of separation, speed, and resolution (3–5).
 30 So far, a number of regular sieving structure designs have been
 31 demonstrated with various degrees of success in bioseparation (6, 7).
 32 Recently, we introduced a unique, regular molecular sieving structure
 33 called the anisotropic nanofluidic-filter (nanofilter) array (ANA)
 34 and have used it for high-resolution continuous-flow separation
 35 for a wide range of DNA fragments between 50 and 23,000 bp and
 36 proteins between 11 and 400 kDa within a few minutes (8, 9).
 37 Here, we describe the fabrication procedure of the ANA device
 38 and its implementation to achieve continuous-flow separation of
 39 proteins.

40 The design of the ANA device consists of a two-dimensional
 41 periodic nanofilter array as shown (see Fig. 1a, b). Nanofilters,
 42 with a constriction size between 10 and 100 nm, are arranged in rows
 43 and separated by deep channels. Microfluidic channels surround
 44 the ANA, and they connect the ANA to fluid reservoirs, where
 45 external voltages are applied. The microfluidic channels provide
 46 sample injection and collection ports, and they also serve as elec-
 47 tric-current injectors to generate uniform electric fields over the
 48 entire ANA structure. When injected into the ANA, the initial
 49 sample mixture stream is fractionated into different streams, which
 50 are due to different passage rates of biomolecules through nano-
 51 filter regions, and are collected at intervals along the ANA opposite edge.

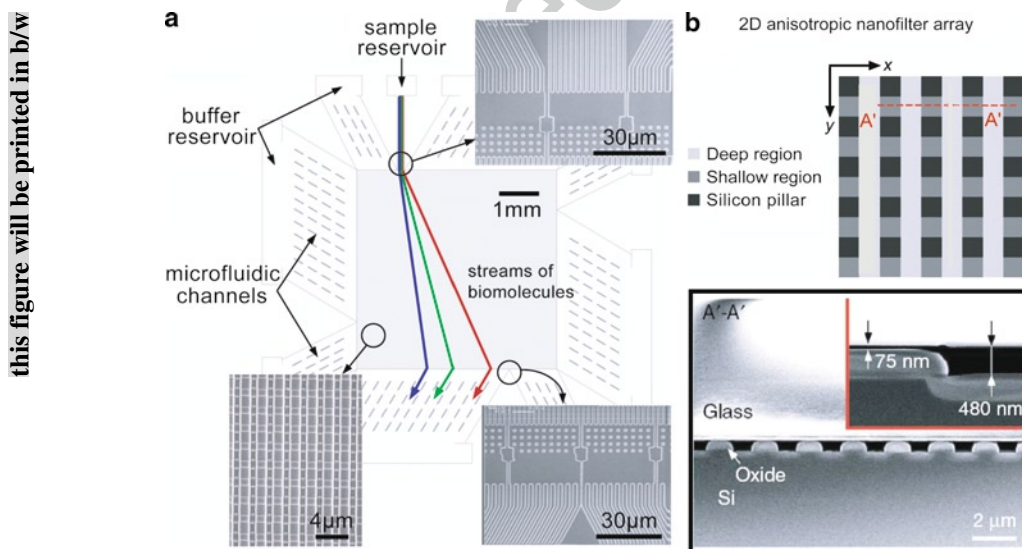


Fig. 1. Structure of the microfabricated device incorporating the ANA. (a) Schematic of the ANA device and scanning electron microscopy images shows the details of different device regions. The *inset* shows a photograph of the thumbnail-sized device. (b) Schematic and cross-sectional SEM image showing nanofilters arranged in rows, are separated by deep channels. (a) Is reprinted by permission from Macmillan Publishers Ltd: *Nature Nanotechnology*, copyright (2007). (b) Is reprinted by permission from Macmillan Publishers Ltd: *Nature Protocols*, copyright (2009).

Three different separation mechanisms (i.e., Ogston sieving, entropic trapping, and electrostatic sieving) have been applied in the ANA device to separate biomolecules of various sizes (5, 8). Generally, Ogston sieving and entropic trapping are most suitable for size-based separation of linear semiflexible or flexible biomolecules such as DNA and denatured proteins, while the electrostatic sieving works are best for native biomolecules such as globular proteins. Here, we describe the most useful application for implementation of the ANA for efficient fractionation of denatured proteins, which are based on the Ogston sieving mechanism. Ogston sieving is effective for biomolecules with sizes (e.g., radius of gyration) that are smaller than the nanofilter constriction size. Smaller molecules have a higher tendency to jump across the nanofilter and, therefore, assume a larger stream deflection angle in the ANA.

The ANA device offers several distinct advantages when compared with the conventional gel-based separation techniques in that it serves as a molecular sieving structure for an integrated bioanalysis microsystem (10, 11). These advantages include (1) the capability to continuously separate physiologically relevant molecules in a few minutes; (2) the versatile separation mechanisms that can take effect in the ANA to separate the biomolecules, which cover a wide range of biological sizes that are based on different molecular properties; (3) the continuous-flow operation of the ANA that facilitates the integration of the separation step with upstream or downstream analysis steps, thus, allowing bioseparation to be performed “in-line” with other continuous flow processes; and (4) the continuous-flow operation of the ANA for allowing downstream sampling, either of a detection signal or of the separated biomolecules themselves, to be time-integrated for improvement of the detection limit. (12).

2. Materials

In order to duplicate the protocol described below, the reader should have access to a microfabrication cleanroom and follow closely the microfabrication protocol described in this chapter to generate their own nanofluidic devices for continuous-flow bioseparation.

Prepare all solutions with purified deionized (DI) water to the desired concentration. Prepare and store all reagents at room temperature (unless indicated otherwise). Strictly follow all waste disposal regulations for waste materials.

2.1. Reagents for Device Fabrication

1. Hexamethyldisilazane (HMDS) (Sigma-Aldrich, St. Louis, MO).
2. Shipley SPR700-1.0 photoresist (Shipley Company, L.L.C., Marlborough, MA).

- 94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
3. Piranha solution: Add one part of the 30% hydrogen peroxide (Sigma-Aldrich) in the quartz tank. Add three parts of the 95% sulfuric acid (Sigma-Aldrich) to the tank. The actual volume of solutions can vary with the number of wafers to be cleaned. Prepare the solution immediately before use (see Note 1).
 4. 49% Hydrofluoric acid (HF) (Sigma-Aldrich).
 5. RCA-1: Add one part of the 27% ammonium hydroxide and one part of the 30% hydrogen peroxide (Sigma-Aldrich) to five parts of DI water in the quartz tank. Heat the solution with the hotplate to about 80°C, and maintain the temperature of the solution. Prepare the solution immediately before use.
 6. RCA-2: Add one part of the 27% hydrochloric acid (Sigma-Aldrich) and one part of the 30% hydrogen peroxide to six parts of DI water in the quartz tank. Heat the solution with the hotplate to about 80°C and maintain the temperature of the solution. Prepare the solution immediately before use (see Note 1).
 7. 20% (wt/vol) KOH solution: Add 3 L of DI water in the quartz tank. Weigh 600 mg of dry potassium hydroxide pellets (KOH) (Sigma-Aldrich) and add it into the tank. Heat the solution with water bath to 80°C, and maintain the temperature of the solution. Prepare the solution immediately before use (see Note 1).

117 **2.2. Reagents**
118 **for Separation**
119 **Experiment**

- 120
121
122
123
124
125
126
127
128
129
130
131
1. TBE 5× buffer: Tris–borate–EDTA. Dissolve TBE powder (Sigma-Aldrich) with DI water in a 1-L glass bottle to the desired working concentration. TBE 5× buffer contains 0.445 M Tris–borate and 10 mM EDTA, and the pH is about 8.3. Filter through a 0.2-μm syringe filter. Store at room temperature (see Note 1).
 2. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich): Dissolve 20 g of SDS powder in 100 mL of DI water to make 20% (wt/vol) SDS stock solution. Store at room temperature.
 3. Dithiothreitol (DTT) (Sigma-Aldrich): Dissolve 0.15425 g of SDS powder in 10 mL of DI water to make 1 M DTT stock solution. Store at room temperature.
 4. Alexa Fluor 488-conjugated cholera toxin subunit B and Alexa Fluor 488-conjugated β-galactosidase (Invitrogen, Carlsbad, CA). Store at –20°C (see Note 1).

132 **2.3. Equipment**
133 **for Fabrication**
134 **of the ANA Device**

- 135
136
137
1. Single-side polished silicon wafers (WaferNet Inc., San Jose, CA) and double-side polished Pyrex wafers (Sensor Prep Services, Inc., Elburn, IL).
 2. High-resolution chrome masks (Microtronics Inc., Newtown, PA) (see Note 1) Wet processing station (i.e., piranha solution tank, diluted HF solution tank, and wafer dump rinser)

(Semifab Inc., San Jose, CA) with wafer spin dryer (Verteq® Spin Rinser Dryer) (Verteq® Inc., Santa Ana, CA).	138 139
3. RCA wet processing station (with RCA-1 clean tank, RCA-2 clean tank, diluted HF solution tank, and wafer dump rinser) (Semifab Inc.).	140 141 142
4. KOH wet etching station with water bath.	143
5. Automated photoresist coat and develop system (Dual Track Spinner System).	144 145
6. Projection stepper (Nikon Precision Inc., Belmont, CA) or mask contact aligner (Electronic Visions, EV Group Inc., Phoenix, AZ).	146 147 148
7. Plasma photoresist stripper (plasma asher) (see Note 7).	149
8. Reactive-ion etcher (RIE) (Applied Materials Inc., Santa Clara, CA).	150 151
9. Deep reactive-ion etcher (DRIE) ICP Deep Trench Etching Systems (Surface Technology Systems, Birmingham, UK).	152 153
10. Vertical Thermal Reactor (VTR) Low Pressure Chemical Vapor Deposition (LPCVD) Furnace (Silicon Valley Group, Redwood City, CA).	154 155 156
11. Atmospheric thermal oxide furnace (Thermco 10K Furnace Systems) (Tetereon Technologies Limited, Horsham, UK).	157 158
12. Surface profilometer (KLA-Tencor Corp., San Jose, CA).	159
13. Spectroscopic ellipsometer (Prometrix) (KLA-Tencor Corp.).	160
14. Scanning electron microscopy (SEM, Zeiss SUPRA 40 High Throughput FESEM) (Carl Zeiss NTS GmbH, Oberkochen, Germany).	161 162 163
15. Wafer aligner and bonder (Electronic Visions, EV Group Inc.).	164 165
16. Wafer die saw (Disco Abrasive Systems K.K., Tokyo, Japan).	166
17. Inverted epi-fluorescence microscope (Inverted Microscope) (Olympus Imaging America Inc., Center Valley, PA).	167 168

3. Methods

169

3.1. Fabrication of Nanofluidic Devices

Carry out all the procedures in a clean room environment (i.e., class 100 or better), unless otherwise specified. The operational conditions of the microfabrication techniques, outlined below, depend strongly on the process parameters [e.g., pressure, temperature, gas flows, and radio frequency (RF) power] and the specific machines used. Therefore, the parameters listed are given only for guidance. These parameters are optimized empirically for specific application.

170
171
172
173
174
175
176
177

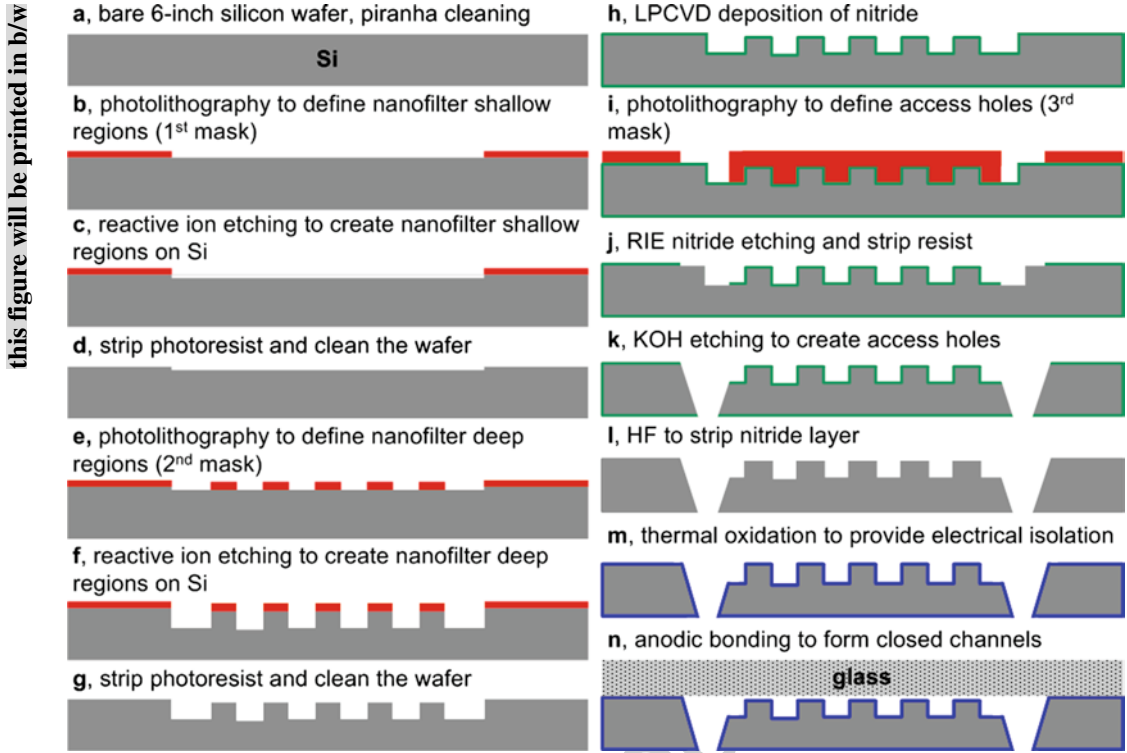


Fig. 2. Schematics of fabrication process of the ANA device. (a–d) Photolithography patterning and RIE etching of the nanofilter shallow regions. (e–g) Photolithography patterning and RIE etching of the nanofilter deep regions. (h–l) Photolithography patterning and KOH etching to create buffer access holes. (m) Growing thermal oxide to provide electrical isolation between silicon substrate and buffer solution. (n) Anodic bonding of silicon wafer with glass wafer to form sealed channels.

178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196

1. Start with a new 6-in. silicon wafer, immerse it in the Piranha solution for 10 min, then rinse it with DI water for 10 min, and dry the wafer with spin dryer (see Fig. 2a).

Prime coat the wafer with HMDS in a vacuum desiccator for 10 min. In the wafer spin dryer, dispense the Shipley Megaposit® SPR700-1.0 photoresist ($\mu \sim 5$ mL) (Rohm and Haas Electronic Materials LLC, Marlborough, MA) on the center of the wafer, spin at 500 revolutions per min (rpm) for 8 s (i.e., relative centrifugal force = $20.9625 \times g$) to spread the photoresist across the entire wafer, accelerate the wafer as quickly, as is practical, to a final spin speed of 4,000 rpm (i.e., relative centrifugal force = $1341.6 \times g$), and spin for 30 s. (RPM is quite commonly used for photoresist spinning.) Soft bake the wafer at 95°C for 60 s on a contact hotplate to drive off solvent from the spun-on photoresist. Expose the wafer with the first photomask using either a mask contact aligner or a projection stepper. Postexposure, bake the wafer at 115°C for 60 s on a contact hotplate. Develop the wafer with MEGAPOSIT®.

- MF CD-26 Developer (Rohm and Haas Electronic Materials LLC, Marlborough, MA) for 30 s, dump rinse the wafer with DI water for 2 min, and spin dry the wafer. Inspect the wafer under the optical microscope to determine if the desired patterns on the mask are successfully transferred to the photoresist. If not, strip the photoresist with the Piranha solution, and redo previous step 2 (see Fig. 2b and Note 8).
2. Hard bake the wafer at 130°C for 60 s on a contact hotplate. Measure the thickness of patterned photoresist film with the surface profilometer (see Note 9).
 3. Place the wafer in a reactive-ion etcher. Etch the wafer through the opened photoresist area by first, using CF_4 to remove native oxide; then, etch the silicon underneath to the desired depth (e.g., 55 nm) using Cl_2 and HBr plasma (see Fig. 2c). The etch rate is characterized empirically for an accurate control of the etch depth. Strip photoresist with the Piranha solution for 10 min, dump rinse the wafer with DI water for 10 min, and spin dry the wafer (see Fig. 2d). Measure the silicon trench depth with the surface profilometer, which is the thickness of the nanofilter shallow region (see Note 10).
 4. Repeat prior steps 2–4 in this section to fabricate the nanofilter deep regions, as well as, the microfluidic channels surrounding the ANA regions with the second photomask (see Fig. 2e–g and Note 11).
 5. Immerse the wafer in the RCA-1 solution at 80°C for 10 min, and dump rinse the wafer with DI water for 10 min. Transfer the still-wet wafer to the diluted HF solution (DI:HF=10:1) for 30 s to remove hydrous oxide film, and dump rinse the wafer with DI water for 10 min. Immerse the still-wet wafer into the RCA-2 solution at 80°C for 15 min, and dump rinse the wafer with DI water for 10 min. Then, spin dry the wafer.
 6. Deposit a thin layer of low-stress silicon nitride on both sides of the wafer using the VTR LPCVD furnace (see Fig. 2h). The thickness of the nitride film should be between 100 and 300 nm. Measure the nitride film thickness with the spectroscopic ellipsometer (see Note 12).
 7. Repeat prior step 2 of this section to pattern the photoresist for buffer access holes with the third photomask (see Fig. 2i).
 8. Etch the nitride layer through the opened photoresist area using CF_4 and O_2 plasma. Strip photoresist with the Piranha solution for 10 min; dump rinse the wafer with DI water for 10 min, and spin dry the wafer. Measure the nitride etch depth with the surface profilometer to ensure the nitride layer is etched through (see Fig. 2j and Note 13).
 9. Etch the buffer access holes through the silicon wafer with the KOH solution: 20% wt/vol at 80°C for about 8 h (see Fig. 2k).

243 The Si etch rate with the 20% KOH solution at 80°C is about
244 1.33 $\mu\text{m}/\text{min}$. Dump rinse the wafer with DI water for 10 min,
245 and spin dry the wafer. Immerse the wafer in the Piranha solu-
246 tion for 10 min, and dump rinse the wafer with DI water for
247 10 min. Immerse the still-wet wafer into the second Piranha
248 solution for 10 min, and dump rinse the wafer with DI water
249 for 10 min. Immerse the still-wet wafer into the diluted HF
250 solution (DI:HF = 50:1) for 30 s, dump rinse the wafer with
251 DI water for 10 min, and spin dry the wafer (see Note 14).

252 10. Immerse the wafer in a concentrated HF solution: 49% HF for
253 20–60 min (i.e., the etch rate of nitride in 49% HF is about
254 5 nm/min); dump rinse the wafer with DI water for 10 min,
255 and spin dry the wafer (see Fig. 2l and Note 15).

256 11. Repeat prior step 6 in this section to clean the wafer before
257 thermal oxidation. Thermally grow a thick layer of oxide, 300–
258 500 nm, with an atmosphere furnace to provide an electrical
259 isolation between the conductive Si substrate and buffer solu-
260 tion. Let the wafer cool down to room temperature. Measure
261 the oxide film thickness with the spectroscopic ellipsometer
262 (see Fig. 2m and Note 16).

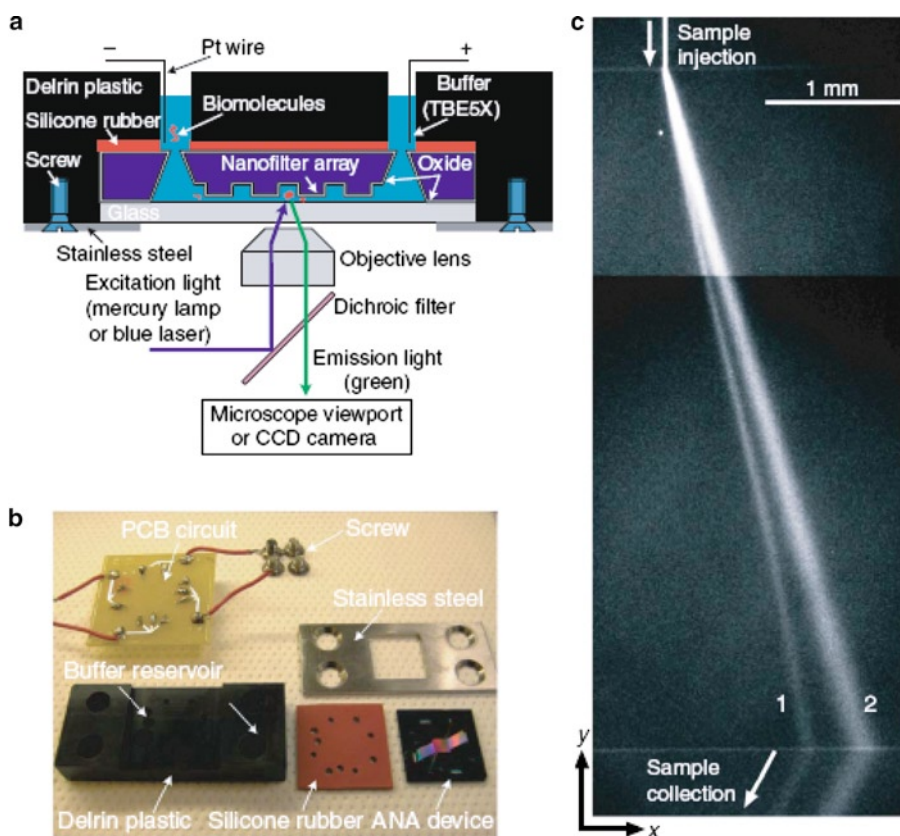
263 12. Immerse both the silicon wafer and Pyrex wafer in the Piranha
264 solution for 10 min; dump rinse both the wafers with DI water
265 for 10 min, and spin dry the wafers. Carefully place the Pyrex
266 wafer on top of the silicon wafer in the wafer bonder, and
267 anodically bond the two wafers at about 350°C and 800 V for
268 about 30 min (see Fig. 2n and Note 17).

269 13. Cut the bonded wafers with the die saw into individual ANA
270 devices. Each device has a dimension of about 26 mm by
271 26 mm (see Note 18).

272 **3.2. Bioseparation** 273 **with Nanofluidic** 274 **Device**

275 Carry out all the procedures at room temperature, unless other-
276 wise specified.

277 1. Assemble the ANA device with the in-house manufactured
278 Delrin gadget, as described in our previous work (9) (see
279 Fig. 3a, b). Fill the device with TBE 5 \times buffer (with additional
280 0.1% wt/vol SDS) through the buffer reservoirs on the Delrin
281 gadget (see Note 19). Since both the thermal oxide and glass
282 surface of the ANA device are hydrophilic, the TBE buffer can
283 fill the device spontaneously. Dip platinum wires into the buf-
284 fer reservoirs on the gadget, and connect them to an external
285 power supply through the custom-designed PCB. Run elec-
286 tro-osmosis through the device to remove air bubbles, if any,
287 for a few hours. The ANA device is now ready for biosepara-
tion experiments. Wrap the ANA device completely with para-
film to prevent buffer evaporation before storage at 4°C (see
Note 20).



this figure will be printed in b/w

Fig. 3. Experimental setup and results for the separation of proteins through the ANA device. (a) Schematic of the home-made Delrin plastic gadget holding the ANA device. The biomolecules in the buffer solution are driven by the applied vertical electric field to migrate across the nanofilter array. (b) Delrin gadget contains predrilled holes as buffer reservoirs that connect to the buffer access holes in the ANA device. The gadget also includes silicone rubber for sealing the ANA device. The Delrin gadget is connected to the external power supply through a custom-designed PCB and is mounted on the inverted epi-fluorescence microscope. (c) Composite fluorescent photograph of separation of proteins through the device under two orthogonal electric fields ($E_x = 75$ V/cm and $E_y = 50$ V/cm). Band assignments: (1) Alexa Fluor 488-conjugated cholera toxin subunit B (MW ~ 11.4 kDa) and (2) Alexa Fluor 488-conjugated β -galactosidase (MW ~ 116.3 kDa). (a) and (b) Are reprinted by permission from Macmillan Publishers Ltd: *Nature Protocols*, copyright (2009). (c) Is reprinted by permission from Macmillan Publishers Ltd: *Nature Nanotechnology*, copyright (2007).

2. Prepare labeled protein mixture. Mix 10 μ L of Alexa Fluor 488 conjugated cholera toxin subunit B and 10 μ L of β -galactosidase in a microcentrifuge vial. Add 2.5 μ L of 20% SDS stock solution and 2.5 μ L of 1 M DTT stock solution for denaturation. Place the SDS–DTT protein mixture in an 80°C water bath for 10 min. Add 450 μ L of TBE 5 \times buffer in the SDS–protein complex solutions. The final SDS–protein complex sample solution contains 15.1 μ g/mL cholera toxin subunit B, 90.9 μ g/mL β -galactosidase, 0.1% wt/vol SDS, and 5 μ M DTT. Prepare the protein sample immediately before use.
3. Load the biomolecule sample mixture into the sample reservoir of the Delrin gadget (see Note 19). Mount the Delrin

288
289
290
291
292
293
294
295
296
297
298
299

300 gadget by holding the device on an inverted epi-fluorescence
301 microscope. The external power supply is connected to the
302 gadget through a custom-made voltage divider box. The volt-
303 ages at the different buffer reservoirs of the ANA device are
304 adjusted continuously and independently to generate different
305 electric fields in the ANA device. During separation, the CCD
306 camera that is attached to the microscope is used to visualize
307 and record the fluorescence images of the fractionated biomol-
308 ecule streams in the ANA device. The voltages at the different
309 buffer reservoirs of the ANA device are finely tuned to achieve
310 the desired separation efficiency (see Fig. 3c and Note 21).

- 311 4. After separation, the fractionated biomolecules are collected
312 using a micropipette in the different reservoirs for downstream
313 analysis. Replace the buffer in the ANA device with fresh TBE
314 1× buffer, and run electro-osmosis through the ANA device
315 for at least 1 h to make sure that no biomolecule is left in the
316 ANA device. Replace the buffer in the device with fresh TBE
317 1× buffer. The ANA device is now ready for the next biosepa-
318 ration experiment or can be stored at 4°C for at least 6 months
319 (see Note 22).

320 4. Notes

- 321 1. When working with both sulfuric acid and hydrogen peroxide,
322 which are corrosive, wear appropriate safety attire (i.e., face
323 shield, apron, and chemical resistance gloves). Always add acid
324 to water when mixing. The mixing ratio of 3:1 for sulfuric acid
325 and hydrogen peroxide in the Piranha solution does not need to
326 be exact, and a rough measurement will work fine.
- 327 2. Wear appropriate safety attire when preparing both RCA-1 and
328 RCA-2 solutions.
- 329 3. During the KOH etch, it is important to place the quartz tank
330 in a water bath in order to maintain a uniform temperature in
331 the KOH solution to achieve uniform etch rate of silicon. Wear
332 appropriate safety attire.
- 333 4. Completely dissolve TBE powder using a magnetic stir bar.
334 Filter out TBE solution with a 0.2- μ m syringe filter. TBE 5×
335 buffer usually precipitates in about 2 months at room tempera-
336 ture. Prior to use, stir and filter TBE 5× solution to get rid of
337 precipitates. TBE precipitates can block or clog nanofluidic
338 channels in the ANA, and thus, affect separation efficiency.
- 339 5. Divide concentrated protein samples into aliquots, and store at
340 -20°C. Avoid repeated freezing and thawing of protein sam-
341 ples. For short-term usage, store samples at 4°C. This should
342 be stable for a few months. Protect samples from light.

6. First, photomasks are designed in-house, using computer-aided software, such as AutoCAD (Autodesk, San Diego, CA) or L-Edit (Tanner Research, Los Angeles, CA). For proper registration and alignment between different layers of photomask patterning, designs of alignment marks must be included into the photomasks. Photomasks are then fabricated by commercial shops (e.g., Microtronics, Newtown, PA) using direct electron-beam writing on a sensitized, chrome-coated soda-lime glass or quartz plate. The chrome film is sputtered or evaporated onto the plate and serves as an opaque layer to block ultraviolet (UV) light exposure. 343-353
7. Plasma asher uses oxygen plasma with controlled RF power to totally remove organic substances on the substrate. It is very similar to plasma etching-like reactive-ion etching (RIE), which has a much better control on etch rate and uniformity than plasma asher. 354-358
8. HMDS is toxic and flammable. Therefore, handle HMDS with care in a vented chemical hood. The wafer should be coated with photoresist, as quickly as possible, after the HMDS priming and wafer cools down. It is recommended that coating be performed no later than 60 min after completing the priming step. The photoresist is uniformly coated across the wafer. The exposure dose (or time) and development time are optimized by running a few dummy wafers before real wafers. The after-develop-inspection step is critical for monitoring the following: (1) if the correct mask is used; (2) if the quality of the photoresist film is acceptable; (3) if the critical dimensions are within the specified tolerances; and (4) if the registration or mask alignment is within specified limits. 359-371
9. The photoresist is hard baked to serve as the mask layer for RIE etching for a long period. The thickness of photoresist is monitored for consistency. The wafer, patterned with photoresist, is stored in a dry, clean environment at room temperature for at least 1 week. 372-376
10. It is critical to remove the native oxide layer before etching silicon substrate in order to achieve an accurate control of the silicon etch rate. Often, it is important to use oxygen plasma to clean the chamber of RIE machine. This greatly helps to reduce the risk of generating black silicon (grass) in the device area. 377-381
11. During the exposure step, align the features accurately on the second mask to those on the first mask using specific alignment marks. 382-384
12. Only the low-stress silicon nitride film serves as the KOH etch mask, since the conventional high-stress nitride film tends to possess pinholes and cracks that cause excessive Si etch during KOH etching. 385-388

- 389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
13. To ensure that the nitride layer is completely etched through, simply over-etch the nitride layer during the RIE process.
 14. The etch rate of silicon in KOH depends greatly on the crystallographic orientation of the etched Si surface as well as the temperature and concentration of the KOH solution. KOH etching of silicon (111) crystallographic planes is much slower (i.e., about hundreds of times slower) when compared with other planes such as (100) and (110). For example, KOH etching of (100) Si wafer produces characteristic V-shaped sidewalls that form a 54.7° angle with the surface. In order to etch through a 500- μm thick wafer, the dimension of the holes should not be smaller than 750 μm . Therefore, take into account the characteristics of KOH etching when designing the mask, such as the dimensions and geometries of buffer access holes. Reference (13) provides additional information. In addition, the wafer edge should be covered fully with photoresist during the RIE process, so that the intact silicon nitride layer on the wafer edge can protect the wafer edge that is etched during the KOH etching. Otherwise, the wafer will become very fragile and hard to handle. It is also important to remove KOH contaminants after KOH etching by performing double Piranha cleaning and one HF etch.
 15. We recommend a concentrated HF solution to strip the nitride layer, since HF does not attack silicon. Alternatively, a hot phosphate solution can be used to remove nitride, but it does etch silicon slowly. Therefore, the etch time in the phosphate solution needs to be carefully controlled. It is critical to remove silicon nitride completely for the subsequent oxidation process, because nitride can be a diffusion barrier for oxygen to reach the silicon surface underneath it.
 16. We often notice that a silicon wafer breaks after the thermal oxidation process. To avoid this problem, design buffer access holes to be as small as possible with the wafer edge as intact as possible. Cool the wafer down slowly after exiting from the oxidation furnace. The oxide thickness should be within a reasonable range of 300–500 nm. An oxide that is too thick will cause problems in the subsequent anodic bonding process, but the device that is passivated with a very thin layer of oxide cannot survive high external electric fields that are required for separation experiments.
 17. Surface cleanliness of the silicon and the Pyrex wafers is critical for their spontaneous hydrophilic bonding, which is important for successful anodic bonding. Choose Pyrex or Borofloat glass wafers with an excellent surface finish (i.e., small roughness). If needed, a slight increase of bonding temperature and voltage will help strengthen the bonding between two wafers.

18. During die saw cutting, the buffer access holes of the ANA device need to be properly sealed with a wafer dicing tape to prevent a water leak into the ANA device. Leaking water into the device will clog the nanochannels and ruin the device.
19. The Delrin gadget contains the following four machined parts: Delrin rectangular cuboid, stainless steel plate, silicone rubber gasket, and printed circuit board (PCB) with soldered Pt wires (see Fig. 3b). The Delrin cuboid has a square groove to embed the ANA device in the center and also contains ten drilled-through holes that can match and connect to the buffer access holes in the ANA device. The stainless steel plate and the Delrin cuboid are screwed together with the ANA devices and the silicone rubber gasket for tight assembly. Both the stainless steel plate and the silicone gasket have a square opening at the center to allow for observing bioseparation in the ANA using an inverted epi-fluorescence microscope. The Pt wires on the PCB serves as electrodes to provide an electric connection between the buffer solution and the external power supply.
20. The ANA device is filled with buffer solution immediately after the die saw cutting, since the hydrophilicity of both the thermal oxide and the glass surface in the ANA device deteriorates over time. It is important to remove any air bubbles that are generated during filling, because trapped bubbles will lead to a nonuniform electric field and even block sample injection. Low ionic strength buffer, such as TBE 0.5 \times , is more efficient to generate an electro-osmotic flow and remove air bubbles. Also, we usually fill the device with TBE 0.5 \times for long-term storage, since TBE 5 \times tends to precipitate shortly. To get the ANA device ready for separation experiments, we replace TBE 0.5 \times with fresh TBE 5 \times buffer in the reservoirs, and run electro-osmosis through the ANA device for a few hours or overnight. The current through the ANA device is monitored to ensure that the buffers are exchanged completely.
21. In order to generate independent voltages in different buffer reservoirs, we have designed a simple custom-built voltage divider box, instead of using multiple power supplies. Biomolecules are injected from the sample reservoir through the sample injection channel by only applying the vertical electric field (E_y) (see Fig. 1a). The horizontal electric field is set at zero or floating. The protein mixture flows into the nanofilter region as one single stream. Keep running the sample injection for a few minutes to reach a stable sample injection stream. Then, slowly increase the horizontal electric field to the desired value, and wait for a few minutes to stabilize the fractionated streams. Record the fluorescence images.

481 22. After separation, the biomolecules left in the ANA are depleted
 482 by running an electro-osmotic flow in the device. If the ANA
 483 device becomes dirty, a good way to recover the ANA device is
 484 to clean it with DI water, and bake it at 400–500°C in an oven
 485 or hotplate for a few hours. The organic contaminants inside
 486 the ANA device will burn out, but the device material (i.e., sili-
 487 con and glass) will survive. The ANA device can be refilled
 488 with TBE buffer for future use.

489 Acknowledgments

490 The authors wish to acknowledge financial support from the
 491 Department of Mechanical Engineering of the University of
 492 Michigan. The authors also extend their appreciation to J. Yoo for
 493 his contribution in the experimental setup and to H. Bow for help-
 494 ful discussions. The MIT Microsystems Technology Laboratories
 495 is acknowledged for support for the cleanroom microfabrication.

496 References

- 497 1. Scopes, R.K. (1993) Protein Purification, Principles and Practice. Springer-Verlag, New York. 520
- 498 2. Goodsell, D.S. (2004) Bionanotechnology, Lessons from Nature. Wiley-Liss, Hoboken, New Jersey. 521
- 500 3. Tegenfeldt, J.O., Prinz, C., Cao, H., et al. (2004) Micro- and nanofluidics for DNA analysis. *Anal Bioanal Chem* 378, 1678–1692. 522
- 501 4. Han, J., Fu, J., Schoch, R.B. (2008) Molecular Sieving Using Nanofilters: Past, Present and Future. *Lab Chip* 8, 23–33. 523
- 502 5. Fu, J., Mao, P., Han, J. (2008) Artificial molecular sieves and filters: a new paradigm for biomolecule separation. *Trends Biotechnol* 26, 311–320. 524
- 503 6. Han, J., Craighead, H.G. (2000) Separation of Long DNA Molecules in a Microfabricated Entropic Trap Array. *Science* 288, 1026–1029. 525
- 504 7. Huang, L.R., Tegenfeldt, J.O., Kraeft, J.J., et al. (2002) A DNA prism for high-speed continuous fractionation of large DNA molecules. *Nat Biotechnol* 20, 1048–1051. 526
- 505 8. Fu, J., Schoch, R.B., Stevens, A.L., et al. (2007) A patterned anisotropic nanofluidic sieving structure for continuous-flow separation of DNA and proteins. *Nat Nanotechnol* 2, 121–128. 527
- 506 9. Fu, J., Mao, P., Han, J. (2009) Continuous-flow bioseparation using microfabricated anisotropic nanofluidic sieving structures. *Nat Protoc* 4, 1681–1698. 528
- 507 10. Eijkel, J.C.T., van den Berg, A. (2006) Nanotechnology for membranes, filters and sieves: A series of mini-reviews covering new trends in fundamental and applied research, and potential applications of miniaturised technologies. *Lab Chip* 6, 19–23. 529
- 508 11. Pamme, N. (2007) Continuous flow separations in microfluidic devices. *Lab Chip* 7, 1644–1659. 530
- 509 12. Righetti, P.G., Castagna, A., Herbert, B., et al. (2003) Prefractionation techniques in proteome analysis. *Proteomics* 3, 1397–1407. 531
- 510 13. Madou, M. (1997) *Fundamentals of microfabrication*. CRC Press, Boca Raton, Florida. 532
- 511 533
- 512 534
- 513 535
- 514 536
- 515 537
- 516 538
- 517 539
- 518 540
- 519 541
- 542