Electronic Supplementary Information

COvalent monolayer patterns in Microfluidics by PLasma etching Open Technology - COMPLOT

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Experimental section

General. Chemicals were purchased from Sigma Aldrich/Merck unless mentioned otherwise. Menzel-Gläser borosilicate glass microscope slides were used to fabricate the microfluidic device and were purchased from Thermo Scientific. Hyper pure polished silicon wafers (76.2 mm diameter, purchased from University Wafer, USA) were used as silicon masters. A photomask template containing patterns, with different feature and spacing sizes, was designed and ordered from JD photo data (UK). Deionized (DI) water was used in all the experiments. Cyanine 5-diadamantane (Cy5-Ad_2) was synthesized by Dr. Mark Rood from the Interventional Molecular Imaging lab at the Leiden University Medical Centre (LUMC, Leiden, the Netherlands).[1]

FIJI/ImageJ data analysis. The results were analysed with FIJI software (ImageJ).[2] The images were rotated in such a way that the patterns were positioned vertically and the gray value intensity range was set to 0-3000 a.u. Then, a square was drawn over the patterns and the averaged gray values were collected within that square. The gray values (a.u.) were then plotted against the distance (μm) to make a profile plot of the image. For calculating normalized fluorescent intensity of patterns and spacing, the raw values were normalized to the minimal value of the data set. For creating a live profile plot video in FIJI software, a script was obtained from image.sc forum (https://forum.image.sc/t/save-real-time-plot/7237) posted and created by Nicolas Chiaruttini aka 'NicoKiaru' (Script S1).[3] The sequence of images from the time lapse experiment was opened and the maximum brightness was adjusted to 3000 a.u. (gray value). A 'live' profile plot was created as before by plotting the gray values against the distance, but also selecting **live** on the profile plot window. The range for the y-axis (gray values) was then set to a maximum value of 3000 a.u.. The script S1 was then copied into the 'Script' function (from main menu File -> New) and then run with the opened sequence of images and the live profile plot. This creates a video of the profile plot window which was then combined

'vertically' with the sequence of images using the 'Combine' function (from main menu Image -> Stacks -> Tools). The video was saved at 7 frames per second.

Glass surface functionalization. The general glass surface functionalization with β-cyclodextrin was performed as described by Onclin *et al*.[4] and creating patterns was carried out through plasma microcontact patterning (PμCP).[5] In short, glass microscope slides of size 76 x 26 mm were cleaned and oxidized with piranha solution (H_2SO_4 (95-98%)/ H_2O_2 (35%), 3.33:1 v/v; *Warning! Piranha solutions must be handled with caution as they may unexpectedly detonate*) for 45 minutes, rinsed with large amounts DI water, and dried under N_2. The glass slides were placed in a high vacuum, pre-heated desiccator together with a glass vial containing 1 mL of 3-aminopropyltriethoxysilane (APTES, 99%) and placed in an oven at 70° C overnight for chemical vapor deposition (CVD) of APTES. An RC6 Chemistry Hybrid Pump from Vacuubrand (vacuum of 2 x 10^{-3} mbar) was used for applying high vacuum condition in the desiccator. Following amine monolayer formation, the glass slides were removed from the desiccator and rinsed with toluene (HPLC grade, VWR, the Netherlands) and dichloromethane (DCM, VWR). The glass slides were then cured for at least 1 hour in the oven at 70°C. Next, the glass slides were immersed in 0.1 M 1,4-phenylene diisothiocyanate (PDITC, TCI Chemicals Belgium) in anhydrous toluene (max 0.002% H_2O, VWR) for 2 hours under argon atmosphere to yield isothiocyanate bearing layers. Following the immersion, the surfaces were rinsed with toluene and DCM and incubated in 0.72 mM heptakis amino β-cyclodextrin (β-CD, Cyclodextrin Shop, the Netherlands) in aqueous solution at pH 8.0 (reached by adding small amounts of 1 M NaOH). This incubation was carried out for at least 2 hours. Surfaces were then rinsed with DI water and dried with nitrogen.

PDMS replica fabrication (PDMS microchannel features). A 3D mold with a ridge of dimensions of 20 x 2 x 0.5 mm was designed in SketchUP (Trimble) and printed with an LCD 3D printer (Photon Anycubic) using Anycubic 405nm UV Clear Resin. This will form a

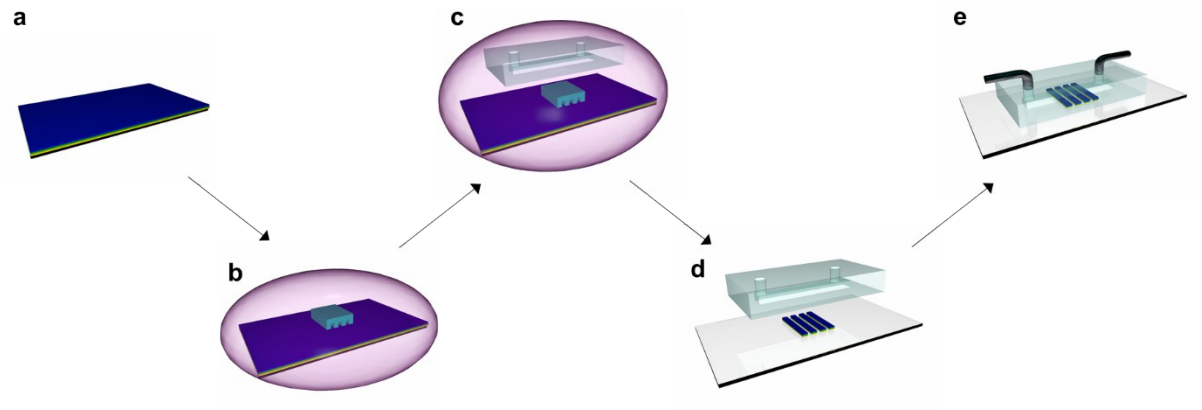
microchannel of volume 20 µL over a glass surface. After 3D printing, the mold was washed with ethanol and isopropanol and then allowed to dry. For the microfluidic device containing 5 channels, the same channel dimensions of 20 x 2 x 0.5 mm were used with a spacing of 2 mm between the channels. For an easy peeling of the PDMS, the 3D printed molds were coated with trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (PFOTS, 97%) using chemical vapor deposition (CVD) in a vacuum desiccator; The 3D printed structure was air plasma activated for 30s, then it was placed in a desiccator with a vial of 100 µL of PFOTS and high vacuum was applied using the pump. For the plasma cleaning process, an Inseto Plasma Etch, Inc. PE-25 benchtop air plasma cleaner was used at its maximum RF plasma power of 100 W with an air flow of ~10 cc/min, which allowed for a vacuum pressure of 200-250 mTorr within the chamber during plasma treatment.†

The desiccator was then left under static vacuum for overnight CVD of PFOTS. After deposition, the 3D printed structure was removed from the desiccator and left in the oven at 70 °C for 1 h, then it was washed with ethanol and isopropanol. PDMS replicas were prepared by pouring PDMS mixture (10:1 PDMS/curing agent) on the 3D printed PFOTS coated mold. After overnight curing at 70 °C, the PDMS was cut out the mold and sonicated in ethanol for 4 minutes for removing the small molecular weight and unreacted PDMS chains. The PDMS replica was then dried with nitrogen. An inlet and outlet were then created on the edges of the microchannel features by puncturing through the PDMS with a 1.5 mm Ø punch (KAI, Japan).

Plasma microcontact patterning (PµCP). PDMS stamps for PµCP were fabricated using the standard technique from Whitesides.[6] A silicon master was first fabricated by spin-coating at 1500 rpm with SU-8 2025 photoresist (Microchem, USA) to yield a 50 µm thick photoresist layer and then treated using standard UV photolithography setup with a photomask containing 150 µm patterns and 50 µm pattern spacing.‡ The UV lamp irradiated silicon wafers at λ = 350 nm at 50% intensity. After UV photolithography, the silicon master and a separate

glass vial containing 100 µL of trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (PFOTS, 97%) were introduced into a desiccator under vacuum for overnight CVD. After incubation with PFOTS, the wafer was cleaned with isopropanol and dried with nitrogen. Stamps were prepared by casting a 10:1 (w/w) mixture of poly(dimethylsiloxane) (PDMS) and curing agent (Sylgard 184, Dow Corning) onto the silicon master with 150 µm patterns and 50 µm pattern spacing. After overnight curing at 70 °C, the PDMS stamps were cut out the master to ca 0.75 cm^2 and sonicated in ethanol to remove low molecular weight PDMS. The PDMS stamps were then brought into conformal contact with the freshly prepared functionalized glass surfaces. For multichannel devices, multiple strips of PDMS stamps were cut approximately to the same width (2 mm) of a microchannel. The β-CD glass slide was then placed on top of a template PDMS replica with multiple channels to help align the cut PDMS stamps on top of the glass surface. β-CD covered slides were placed in the plasma cleaner for 4 cycles of 1 minute each. Again here, the Inseto Plasma Etch, Inc. PE-25 benchtop air plasma cleaner was used at its maximum RF plasma power of 100 W and air flow of ~10 cc/min, which allowed for a vacuum pressure of 200-250 mTorr within the chamber.

Microfluidic channel fabrication. The PDMS replica was placed facing upwards in the plasma oven along with the PµCP-β-CD surface for the 4^{th} plasma cycle. The PDMS stamp was removed from the patterned β-CD surface and the PDMS replica was firmly placed on top



Scheme S1: Schematic showing more detailed sequence of steps for microfluidic device fabrication with β-CD patterns. a) Surface functionalized with β-CD monolayers on glass surface. b) PµCP cycles 1-3 of β-CD surface. c) 4^{th} plasma cycle including PDMS replica with PµCP β-CD surface. d) Placing of PDMS replica on patterned surface after removal of PDMS stamp. e) Plasma bonded PDMS/glass hybrid microfluidic device with inlet and outlet tubing.

of the patterned glass slide, with the microchannel facing downwards. A piece of paper was then placed on top of the PDMS replica, followed by a non-functionalized microscope glass slide. Clamps were then used to keep the non-functionalized microscope glass slide and PDMS replica firmly pressed against the functionalized glass surface (as shown in Figure S2) and microfluidic device was incubated in the oven at 70 °C for 1 hour. The holders were then removed from the microfluidic device and tubing was connected to the inlet and outlet of the channel. The inlet tubing was then connected to a syringe pump and the outlet tubing to a waste vial. The steps for PμCP on cyclodextrin modified glass surface and subsequent PDMS/glass microfluidic device fabrication are shown in more detail in Scheme S1.

Flow experiments. Before using the β-CD functionalized microfluidic device for immobilization of Cy5-Ad_2, the channel and surface was thoroughly washed with DI water at 800 μL/min for 15 mins in order to clean the channel before use. Then, 0.23 μM of Cy5-Ad_2 in phosphate buffered saline (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was flowed over β-CD functionalized microfluidic device at a flow rate of 50 μL/min for 15 mins. The functionalized glass surfaces were imaged using a Leica DMi8 epifluorescence microscope with 10X or 5X magnification objective lens and beam intensity of 100%. For 10X objective images, exposure time was set to 278 ms and for 5X objective images to 500 ms. Cy5-Ad_2 was excited at a wavelength (λ_{ex}) of 590-650 nm and emission (λ_{em}) was collected at 662-738 nm (using the Y5 filter cube). The surface was imaged using the Cy5 filter on the microscope before and during addition. After addition of Cy5-Ad_2, the surface was first washed with a flow of PBS at 200 μL/min for 5 mins and then DI water also at 200 μL/min and for 5 mins. Again, images of the surface were captured with Cy5 filter on the microscope. To remove immobilized Cy5-Ad_2, the surface was washed for several cycles with an aqueous solution of 10 mM β-CD, pH 10 at 800 μL/min for 10 mins per cycle. In between cycles, syringes were refilled with β-CD solution as necessary, and a flow of with β-

CD solution was applied again. When Cy5-Ad_2 was sufficiently removed from the surface according to fluorescence imaging, the microfluidic device was washed with a flow of DI water at 200 μL/min for 5 mins to remove remaining β-CD from the channel and surface. Then, a flow of Cy5-Ad_2 was re-applied at the same flow rate and duration, and the surface was washed with a flow PBS and DI water. The re-usability of the β-CD functionalized microfluidic device was repeated for a total of 4 uses. For determining the binding kinetics of Cy5-Ad_2 to β-CD, a 5x serial dilution of 0.23 μM Cy5-Ad_2 was first obtained by diluting from a concentration of 0.23 μM until 0.36 nM in PBS. The different concentrations of Cy5-Ad_2 were sequentially flowed over different channels in the multi-channel microfluidic devices at a flow rate of 50 μL/min for 15 mins (then washed with PBS at 200 μL/min for 5 mins and DI water at 200 μL/min for 5 mins). For the time lapse experiment on the microscope (Video S1), addition of Cy5-Ad_2 over β-CD patterns was carried out at 50 μL/min for 1 minute and images were captured every second. After 1 minute, the flow was stopped and the Cy5-Ad_2 syringe tube was switched with a syringe tube filled with PBS and a flow of 200 μL/min was applied. Images were then captured every second for 2 minutes until the flow was stopped.

Supplementary Figures

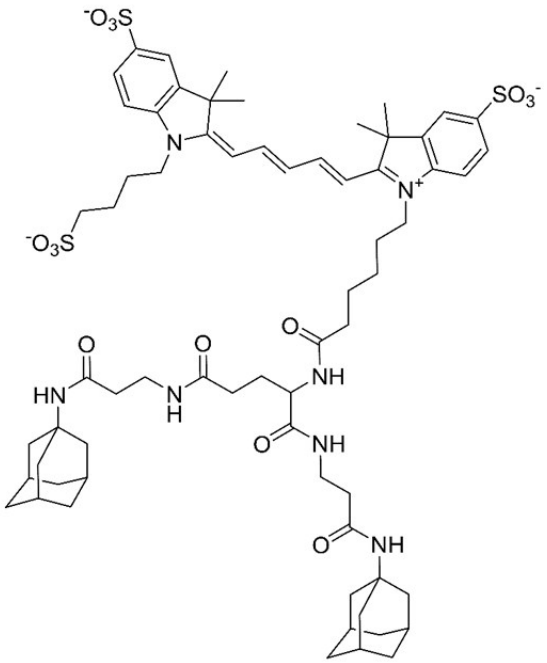


Fig. S1: Structure of diamantane functionalized Cyaning 5 dye (Cy5-Ad_2) used for binding experiments with β-CD functionalized surfaces.

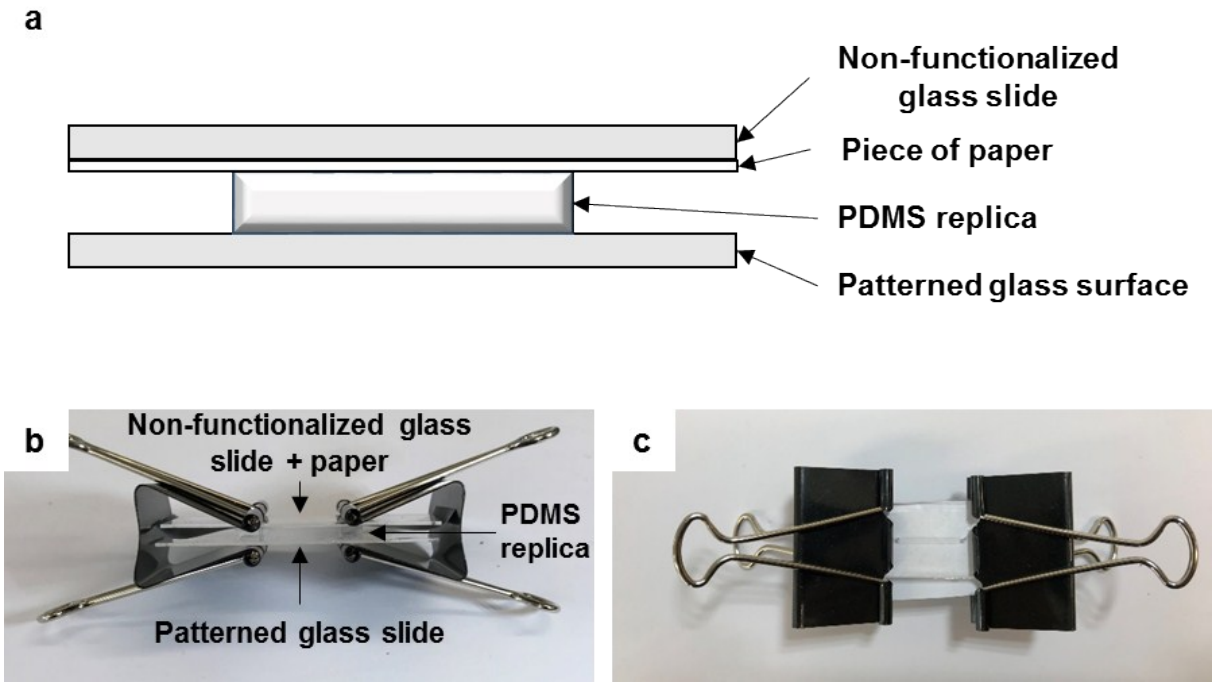


Fig. S2: Scheme showing setup of microfluidic with empty glass slide and piece of paper before attaching clamps (a). Photographs of microfluidic device being held in place by clamps before curing in the oven (b). Microfluidic device with clamp turned upside down to show functionalized glass surface and channel (c).

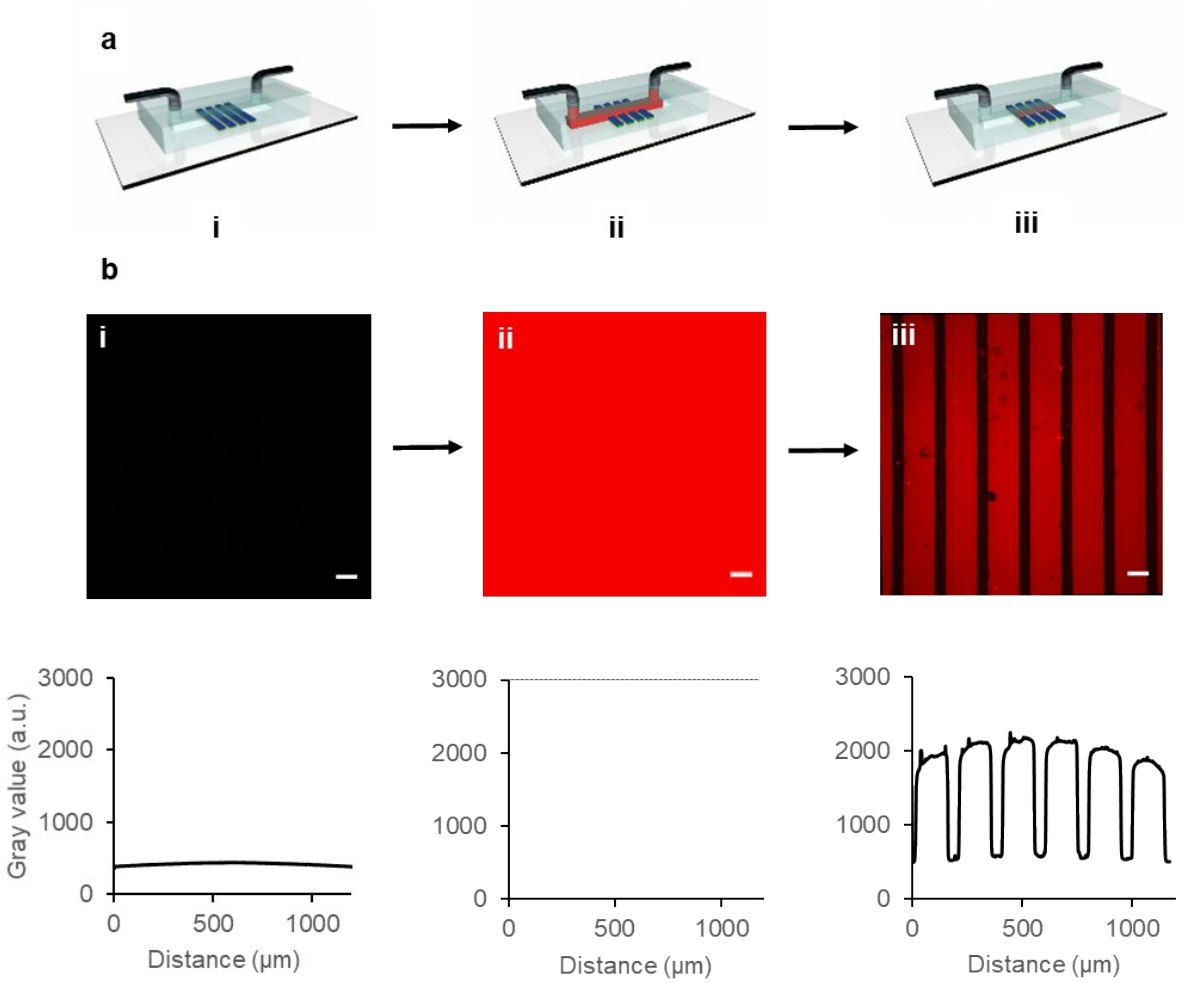


Fig. S3: a) Scheme depicting events for Cy5-Ad_2 immobilization within microfluidic channels: (i) β-CD patterned microfluidic device before addition of Cy5-Ad_2, (ii) Cy5-Ad_2 addition over surface in device, and (iii) specifically immobilized Cy5-Ad_2 on β-CD patterns. b) Microscopy images and constituent profile plots of (i) β-CD patterned microfluidic device before addition of Cy5-Ad_2, (ii) during addition of Cy5-Ad_2 (gray values on profile plot is above 3000 a.u. due to saturation) and (iii) after washing the surface with PBS and DI water. Scale bars are 100 μm.

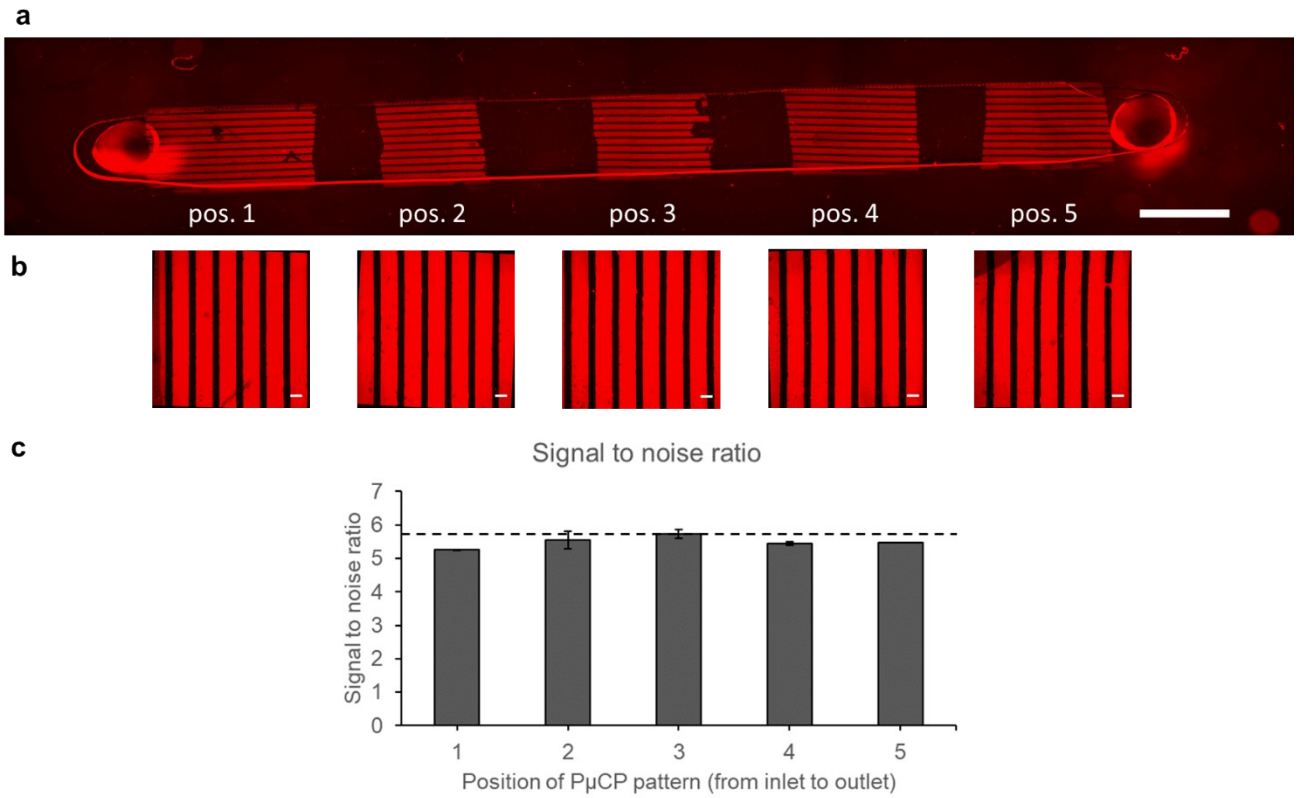


Fig. S4: Control experiment to test the effect of using multiple strips of a PDMS stamp for PμCP at different locations on one β-CD functionalized surface. a) Overview of the single channel after addition of 0.23 μM Cy5-Ad_2. Different positions of β-CD patterns in the channels are marked. Image was captured with 5x objective using 6x1 tilescan settings and scale bar is 2 mm. b) 10x objective images of the separate positions after 90˚ rotation which were used for carrying out profile plot analysis and determine signal to noise ratio of fluorescent patterns. c) Graph showing the average signal to noise ratio of patterns in different positions.

Script S1: FIJI Script for creating live profile plot videos (LiveGraphtoStack)

```
import ij.ImageStack
import ij.ImagePlus
#@ImagePlus(label="your image containing the ROI", description="your image") myImage
#@Integer (label="initial slice") iniSlice
#@Integer (label="final slice") endSlice
#@Integer (label="step slice", value=1) stepSlice
#@ImagePlus(label="your live graph", description="live graph") myGraph
#@Integer (label="delay for GUI update (ms)", value=100) delayInMs
#@output ImagePlus outputGraph

// Initializes new stack
stack = new ImageStack(myGraph.width, myGraph.height)

for (slice=iniSlice;slice<=endSlice;slice=slice+stepSlice) {
        // sets position of input image
        myImage.setSlice(slice)
        // waits for live graph update...
        Thread.sleep(delayInMs)
        // copy and adds slice to new stack
        stack.addSlice(null, myGraph.getProcessor().clone())
}

// builds output ImagePlus
outputGraph = new ImagePlus(myGraph.getTitle()+"-stack", stack)
```

Notes and references

† Air plasma works similar to oxygen plasma and the surface cleaning is based on chemical, isotropic etching with highly reactive oxygen radicals.[7]

‡ The 50 µm thickness of the negative photoresist was chosen to have a height difference of features and spacing of 50 µm on the resulting PDMS stamp after molding. This was to make sure that the aspect ratio of the stamps (height divided by width of features) was neither too low or too high for the 150 x 50 µm feature sizes used. This avoids complications with the stamp during patterning, such as 'sagging' of the spacing and 'collapsing' of features.[8]

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