Electronic Supplementary Material (ESI) for Lab on a Chip
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Electronic Supplementary Information:

Biomolecule patterning of cell-instructive hydrogels by hydrodynamic flow focusing

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1. Modular device assembly & manipulation

PDMS injection molding

The modular microfluidic device we designed allows PDMS injection molding, can be seen in Fig. S1b-c. This module comprises; screw nuts (4), steel bars (10), a PMMA top piece, a spacer with an insert, a spacer and a PMMA bottom piece with the silicon chip.

For PDMS injection molding, the module is assembled as follows: *(i)* the silicon chip is placed onto the PMMA bottom piece (part #5) within the small-engraved recess (500 µm deep). *(ii)* The spacer (part #4, 0.5 cm high) is stacked onto the bottom piece. Note that screws are inserted in the PMMA bottom piece. *(iii)* The spacer with the insert (part #3, 0.5 cm high) is stacked on top. *(iv)* The PMMA top piece (part #2) is stacked (and orientated to match the through holes with the microstructures on the silicon chip) and tightened with screw nuts. *(v)* The steel rods (1.5 cm high, Ø = 0.16 cm) are inserted into the through holes of the top PMMA piece and pressed onto the silicon chip. The assembled PDMS injection module can be seen in Fig. S1c.

Afterwards, PDMS is injected with a syringe into the inlets of the PMMA top piece, excess PDMS is collected in a second syringe (remove the plunger) inserted in the second PMDS inlet, as seen in Fig. S1c. The module is then placed in a hoven (60°C) for 4-8 hours. Upon disassembly the PDMS chip maintained within the spacer with the insert (part #7) is recovered.

Microfluidic module assembly

The second module for microfluidics can be seen in Fig. S1b(right). The module comprises; screw nuts, the PDMS chip in its PMMA holder, a PMMA coverslip holder, a rubber O-ring and a PMMA bottom piece.

For gradient patterning, the module is assembled as follows: *(i)* the coverslip bearing the hydrogel is placed onto the PMMA bottom piece (part #10). The cover slip is manually aligned in the centre. *(ii)* The rubber O-ring and the coverslip holder (part #9, 0.3 cm high with an indent of 0.1 cm for the rubber O-ring) are stacked onto the PMMA bottom piece and tightened. *(iii)* The PDMS chip in its spacer (part #7) is stacked, pressed onto the coverslip and tightened.

Afterwards, the microfluidic module could be connected to a syringe pump for patterning, as seen in Fig. S1d.

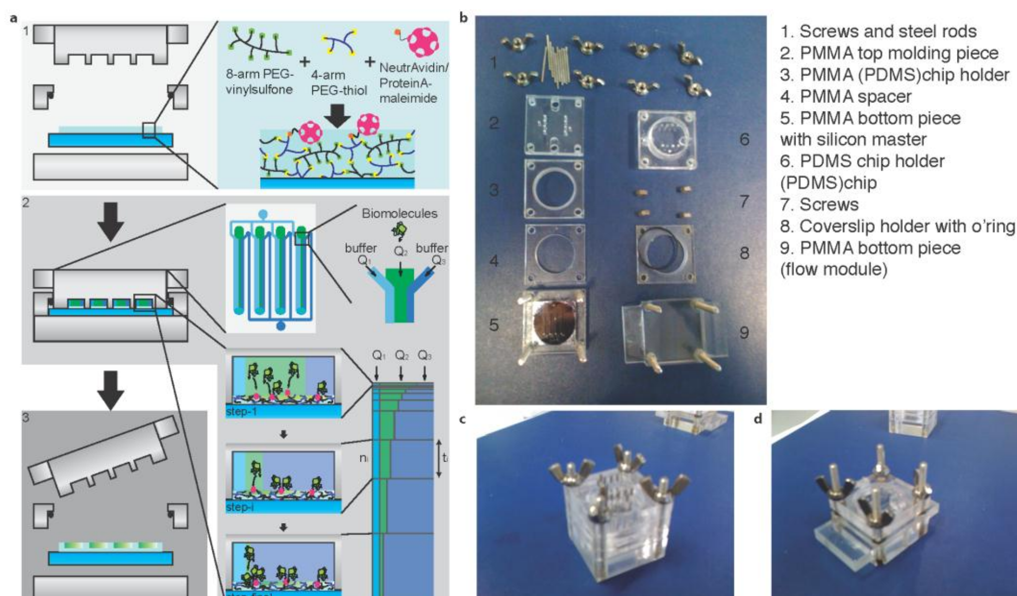

Fig. S1: Modular Microfluidic device assembly for injection molding and microfluidics patterning of four parallel biomolecular gradients on hydrogel. a, Microfluidic patterning of four parallel gradients. *Step 1.* Device assembly and hydrogel formulation. Hydrogels functionalized with NeutrAvidin and/or ProteinA are casted on round coverslips (right box). These hydrogels are then placed onto the bottom piece. An O-ring and a coverslip holder are tightened to prevent the hydrogel to slide. The PDMS chip enclosed in the holder is pressed onto the construct and tightened to ensure proper sealing of the microchannels. *Step 2.* Protein patterning by hydrodynamic flow focusing. The microfluidic layout comprises four parallel flow focusing units. Linear gradients of tethered proteins are obtained by hydrodynamic flow focusing. Control of the buffer (Q_1 and Q_3) and protein solution (Q_2) flow rates enables to sequentially reduce, in several steps (n_i), the protein stream width (green) from right-to-left. NeutrAvidin and/or ProteinA grafted to the hydrogel readily capture (black arrows) tagged proteins from solution. The duration of individual steps (t_i) dictates the amount of protein being locally captured on the hydrogel surface. This allows the generation of tethered protein, for example in the form of a linear gradient. *Step 3.* Recovery of the patterned gradient tethered to a hydrogel surface. The device is disassembled and the coverslip recovered for further imaging and cell experiments. **b**, Moulding module (left) and Microfluidic module (right). The different pieces required for PDMS molding are depicted and labelled. The different pieces required for microfluidics are depicted and labelled. **c**, Assembled module for PDMS injection moulding. **d**, Assembled microfluidic device.

2. Characterization of longitudinal stability of patterned gradients

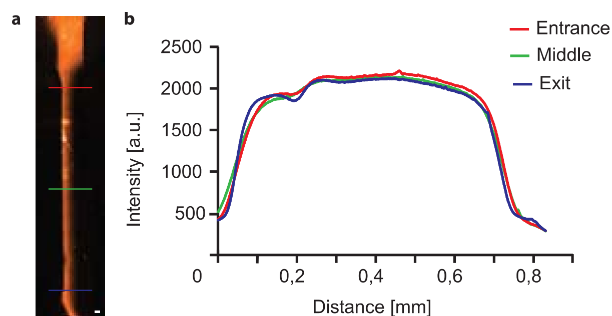

Fig. S2 Characterization of longitudinal stability of patterned gradients. a, Stitched fluorescent micrographs of a stripe pattern by HFF on hydrogel. (Scale bar = 200 µm) **b**, Graphical representation of the intensity profile measured at, respectively, the entrance (red line), the mid section (green line) and the end (blue line) of patterned stripe shown in **a**.

3. Complex gradient profiles of biologically relevant proteins

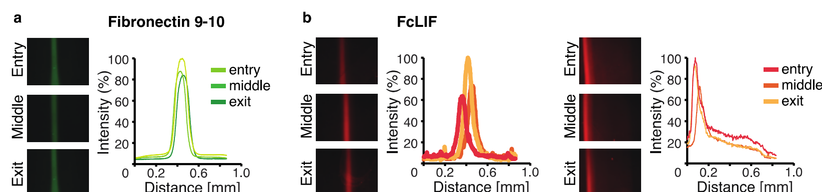

Fig. S3: HFF-based patterning of more complex gradient profiles for biologically relevant proteins. a, Micrographs and intensity profile plots of FITC-FN III9-10-biotin on NeutrAvidin-functionalized PEG hydrogels. Gaussian gradient profiles was obtained. **b**, Micrographs and intensity profile plots of DsRED-FcLIF on ProteinA-functionalized PEG hydrogels. Exponential and Gaussian gradient profiles were obtained.

4. ESC cultured on tethered FcLIF gradients and controls

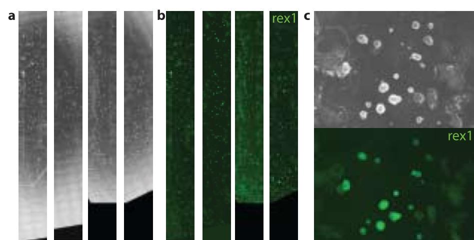

Fig. S4: ESCs cultured on tethered FcLIF gradients. a, Stitched brightfield micrographs of ESCs cultured on tethered FcLIF gradients. **b**, Stitched fluorescent micrographs of ESCs cultured on tethered FcLIF gradients. **c**, Micrographs brightfield and fluorescent of ESCs culture on adherent hydrogel in presence of soluble FcLIF.

5. Summary of programming parameters

Table S1 Program parameters for linear gradient patterning/effect of discrete step number

NeutrAvidin/BSA-biotin												
	5 steps				10 steps				20 steps			
step	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]
1	0.1	12	10	3	0.2	12	10	3	0.1	12	10	3
2	54	14	8	3	22.8	13	9	3	10.8	12.5	9.5	3
3	60	16	6	3	24.6	14	8	3	10.8	13	9	3
4	144.6	18	4	3	25.2	15	7	3	11.4	13.5	8.5	3
5	941.4	20	2	3	25.8	16	6	3	12	14	8	3
6					39	17	5	3	12	14.5	7.5	3
7					59.4	18	4	3	12	15	7	3
8					87.6	19	3	3	11.4	15.5	6.5	3
9					128.4	20	2	3	12	16	6	3
10					787.2	21	1	3	13.8	16.5	5.5	3
11									18.6	17	5	3
12									23.4	17.5	4.5	3
13									27.6	18	4	3
14									31.8	18.5	3.5	3
15									39.6	19	3	3
16									49.8	19.5	2.5	3
17									58.8	20	2	3
18									64.2	20.5	1.5	3
19									82.8	21	1	3
20									697.2	21.5	0.5	3

Table S2 Program parameters for linear gradient patterning/ effect of flow rate

NeutrAvidin/BSA-biotin										
		linear 10 µl/min			linear 15 µl/min			linear 20 µl/min		
step	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]
1	0.2	12	10	3	7	15	3	2	20	3

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2	22.8	13	9	3	8.5	13.5	3	4	18	3
3	24.6	14	8	3	10	12	3	6	16	3
4	25.2	15	7	3	11.5	10.5	3	8	14	3
5	25.8	16	6	3	13	9	3	10	12	3
6	39	17	5	3	14.5	7.5	3	12	10	3
7	59.4	18	4	3	16	6	3	14	8	3
8	87.6	19	3	3	17.5	4.5	3	16	6	3
9	128.4	20	2	3	19	3	3	18	4	3
10	787.2	21	1	3	20.5	1.5	3	20	2	3

Table S3 Program parameters for complex gradient patterning

NeutrAvidin/BSA-biotin								
			Exponential			Gaussian		
step	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]
1	0.1	12	10	3	0.1	7.5	10	7.5
2	0.6	13	9	3	0.6	8	9	8
3	0.6	14	8	3	1.8	8.5	8	8.5
4	0.6	15	7	3	4.8	9	7	9
5	2.4	16	6	3	10.8	9.5	6	9.5
6	5.4	17	5	3	21.6	10	5	10
7	11.4	18	4	3	37.2	10.5	4	10.5
8	25.2	19	3	3	68.4	11	3	11
9	57	20	2	3	175.2	11.5	2	11.5
10	1096.8	21	1	3	879.6	12	1	12

Table S4 Program parameters for gradient patterning

Protein A/IgG												
	linear				exponential				Gaussian			
step	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]	time [s]	Q1 [µl/min]	Q2 [µl/min]	Q3 [µl/min]
1	0.2	12	10	3	3.6	7	15	3	0	5	15	5
2	27	13	9	3	2.4	8.5	13.5	3	0.6	5.75	13.5	5.75
3	39	14	8	3	3	10	12	3	0	6.5	12	6.5
4	72	15	7	3	6	11.5	10.5	3	1.8	7.25	10.5	7.25
5	71.4	16	6	3	9.6	13	9	3	6.6	8	9	8
6	82.2	17	5	3	16.2	14.5	7.5	3	17.4	8.75	7.5	8.75
7	105.6	18	4	3	42	16	6	3	54	9.5	6	9.5
8	130.2	19	3	3	96.6	17.5	4.5	3	140	10.25	4.5	10.25
9	225	20	2	3	182.4	19	3	3	268.2	11	3	11
10	447.6	21	1	3	838.2	20.5	1.5	3	701.4	11.75	1.5	11.75