

Patterning of cell-instructive hydrogels by hydrodynamic flow focusing†

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Microfluidic gradient systems offer a very precise means to probe the response of cells to graded biomolecular signals *in vitro*, for example to model how morphogen proteins affect cell fate during developmental processes. However, existing gradient makers are designed for non-physiological plastic or glass cell culture substrates that are often limited in maintaining the phenotype and function of difficult-to-culture mammalian cell types, such as stem cells. To address this bottleneck, we combine hydrogel engineering and microfluidics to generate tethered protein gradients on the surface of biomimetic poly(ethylene glycol) (PEG) hydrogels. Here we used software-assisted hydrodynamic flow focusing for exposing and rapidly capturing tagged proteins to gels in a step-wise fashion, resulting in immobilized gradients of virtually any desired shape and composition. To render our strategy amenable for high-throughput screening of multifactorial artificial cellular microenvironments, a dedicated microfluidic chip was devised for parallelization and multiplexing, yielding arrays of orthogonally overlapping gradients of up to 4×4 proteins. To illustrate the power of the platform for stem cell biology, we assessed how gradients of tethered leukemia inhibitory factor (LIF) influence embryonic stem cell (ESC) behavior. ESC responded to LIF gradients in a binary manner, maintaining the pluripotency marker Rex1/Zfp42 and forming self-renewing colonies above a threshold concentration of 85 ng cm^{-2} . Our concept should be broadly applicable to probe how complex signaling microenvironments influence stem cell fate in culture.

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Introduction

Protein-mediated signaling is of utmost importance in governing cellular behavior, for example in the patterning of a developing embryo by morphogen proteins,¹ such as Activin and Nodal directing left/right asymmetry,² or the differentiation into mesodermal/endodermal lineages.³ Notably, morphogens are often displayed in a combinatorial and graded fashion,⁴ and they are frequently tethered to the extracellular matrix (ECM).⁵

Microtechnologies offer unprecedented means to generate precise gradients of soluble and surface-tethered biomolecules, opening up exciting applications in (stem) cell biology.^{6–8} For instance, microfluidic systems have been used to establish cytokine gradients to control human neural progenitor cell differentiation,⁹ or to investigate the role of autocrine and paracrine signaling in regulating ESC self-renewal.¹⁰

However, despite these exciting early examples, microfluidic gradient systems have not been widely used to address

pertinent questions in stem cell biology. We postulate that this is due to some shortcomings of microfluidic cell culture platforms: First, these systems are built on non-physiological plastic or glass cell culture substrates that may negatively impact cell fate. Secondly, microfluidic systems are not well suited for long-term stem cell culture due to the limited space available for cell growth and the difficulties to manipulate cells in microchannels.^{11–13} Thirdly, the continuous perfusion of microfluidic systems may expose cultured cells to aberrant shear stresses.¹¹ Finally, microfluidic gradient systems may have a relatively limited throughput compared to other approaches, such as protein microarrays used for cell phenotypic screenings.^{14,15}

To address these issues, we have been developing microfluidic approaches to pattern protein gradients on the surface of soft and biomimetic PEG hydrogels.^{16,17} We recently reported proof-of-principle experiments on the protein gradient patterning of gels using software-controlled hydrodynamic flow focusing (HFF).¹⁷ Here we aimed at fully characterizing this versatile method, as well as expanding its usefulness towards high-throughput screening experiments. We performed a parametric analysis of the effect of several HFF parameters and hydrogel properties, generating high-resolution protein gradients of virtually any shape. Furthermore, we designed a microfluidic device to parallelize gradient patterning, producing arrays of overlapping gradients as a means to

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rationally screen stem cell culture microenvironments. Finally, we validated this system by probing the effect of tethered LIF gradients on the behavior of mouse ESC, identifying a threshold concentration of immobilized LIF that is necessary for the maintenance of pluripotency on soft hydrogel substrates.

Methods

Protein labeling

Recombinant ProteinA (BioVision) was covalently modified with a heterofunctional *N*-hydroxysuccinimide (NHS)–PEG–maleimide (PEG MW 3500, JenKem Technology) to facilitate covalent incorporation into a PEG hydrogel network.¹⁶ Biotin was attached to bovine serum albumin (BSA, Invitrogen) using the NHS-EZ-link biotinylation kit (Pierce) according to the manufacturer's instruction. To visualize protein tethering on biofunctional hydrogels, BSA–biotin, human IgG (hIgG, Invitrogen) and Fc-chimeric leukemia inhibitory factor (FcLIF, generously provided by the Protein Expression Core Facility at EPF Lausanne) were fluorescently labeled with Alexa488-NHS (Invitrogen) or DsRed-NHS (Invitrogen), following the manufacturer's instructions. Fibronectin fragment 9–10 (FN III9–10, 21 kDa; a generous gift from Hubbell and Martino¹⁸), comprising of a RGD sequence and a free N-terminal cysteine was biotinylated and fluorescently labeled. Briefly, FN III9–10 was first reduced using a tris(2-carboxyethyl) phosphine hydrochloride (TCEP) gel (Pierce) and biotinylated using EZ-link maleimide–PEG₂–biotin (Pierce) following the manufacturer's instructions. The biotinylated protein was labeled with fluoresceine isothiocyanate (EZ-label FITC protein label kit, Pierce). Finally, the solution was dialyzed (Slide-A-Lyzer Mini Dialysis Unit, 10 kDa, Thermo Scientific) against phosphate buffered saline (PBS) overnight to remove unreacted compounds.

Formation of thin biofunctional hydrogels

PEG-based hydrogels containing NeutrAvidin and/or ProteinA were cast onto round silanized (mercaptopropyltrimethoxysilane, MPS, Falcon) glass slides or coverslips ($\varnothing = 20$ mm) as described.^{16,17} Briefly, hydrogels (5% w/v) were formed *via* Michael-type addition by mixing two aqueous precursors containing 8arm-PEG-vinylsulfone (VS)¹⁹ (mol. weight: 10 kDa, buffer: 0.3 M triethanolamine at pH 8) and 4arm-PEG-thiol (SH) (10 kDa, NOF, Japan) at equal stoichiometry of the functional groups. Crosslinking was conducted for 30 min at 37° Celsius. PEG-tethered NeutrAvidin or ProteinA was added to the precursor solution at a concentration of 3.36 mg mL⁻¹. To fabricate thin hydrogel films (thickness: 25 μ m), the precursor solution was cast between a silanized round glass coverslip and a hydrophobic (Sigmacoat, Sigma-Aldrich) glass slide. After removing the hydrophobic glass slide, covalently attached hydrogel films were extensively washed in PBS and stored at 4 °C for at least eight hours before protein patterning by HFF.

Fabrication of microfluidic chips for hydrodynamic flow focusing

Standard photolithography (SU8 on silicon) and soft lithography were used to produce gradient-generating networks of microchannels as described.^{16,17} Chips were fabricated by poly(dimethylsiloxane) (PDMS) injection molding. Details of the fabrication can be found in the electronic supplementary information (ESI)†.

Microfluidic set-up and device assembly

A dedicated microfluidic device was designed and built for gradient array generation (Fig. S1, ESI†). The assembly of the microfluidic device is shown in Fig. S1a, b (ESI†). Briefly, hydrogel-coated coverslips were placed at the bottom piece of the microfluidic device. To prevent the coverslips from moving around, slight pressure was exerted onto the bottom piece using an O-ring and a PMMA coverslip holder (Fig. S1a, b, ESI†). The PDMS chip was then pressed onto the hydrogel and fixed to maintain constant sealing (Fig. S1a, scheme 2, ESI†). The assembled microfluidic device was primed with PBS using a Pasteur pipette and immersed in PBS under a vacuum for 30 min to remove trapped air bubbles. Syringes were filled with PBS (two inlets) or a protein solution (four inlets, each filled with a protein solution of interest at 0.1 mg mL⁻¹). Finally, the syringes were mounted onto the programmable syringe pump (NEMEsys, Certoni) and Tygon tubings were connected to the inlets for HFF patterning. All manipulations were performed in a cell culture hood to prevent contamination.

Characterization of protein patterns generated by HFF

Protein patterning was assessed by fluorescent microscopy (Leica DMI4000 or Zeiss Axio Observer). The multichannel scanning and scan reconstruction functions of the Metamorph software were used to stitch individual images to reconstruct entire gradient patterns. Intensity profiles of the fluorescent protein patterns were measured by image analysis using ImageJ.

Mouse Rex1-GFP ESC culture

A Rex1-GFP reporter mouse ESC line²⁰ (generously provided by Austin Smith, University of Cambridge) was used to probe the maintenance of pluripotency and colony formation in response to tethered biomolecule gradients on hydrogels. Rex1 (*zfp42*) is a zinc finger protein that is expressed selectively in naïve ESCs, and thus a very good reporter for the *in vitro* maintenance of these cells (Austin Smith, personnel communication). ESC were expanded on gelatin-coated plastic dishes (Fluka) in DMEM (glutamax, GIBCO) medium supplemented with non-essential amino acids (0.1 mM, Invitrogen), sodium pyruvate (1 mM, Invitrogen), beta-mercaptoethanol (0.1 mM), 15% fetal bovine serum (FBS, Hyclone), 1% pen/strep, L-glutamine (0.5 mL) and leukemia inhibitory factor (LIF, 1 U mL⁻¹, Millipore).

Generation of PEG hydrogel formulations for adherent ESC culture

Biofunctional hydrogels for ESC-based assays were fabricated as mentioned above followed by immersion for one hour at 37

°C in a solution containing 0.2% w/v thiolated-gelatin (Gelin-S, Glycosan Biosystems).

Quantification of tethered FcLIF concentration

ProteinA-functionalized hydrogels were exposed to fluorescently tagged FcLIF (DsRED-FcLIF) solutions of defined concentrations for one hour at room temperature. The gels were washed three times for 30 min with PBS before imaging (Axiovert Observer, Zeiss). A standard curve was generated based on the measured fluorescent intensities for each concentration.

Culture of ESC on gel-tethered FcLIF gradients

Functionalized hydrogels were prepared as described above. After HFF patterning, hydrogel substrates were washed thoroughly with PBS and placed in a 12 well plate. 15 000 ESCs were seeded on the patterned gels in standard culture media that was depleted of soluble LIF and cultured for three days in a humidified incubator (at 37 °C). Cells on arrayed gradients and controls were scanned by automated live microscopy (Axiovert Observer, Zeiss, Metamorph software). Image processing and analysis was performed using Metamorph. Non-patterned hydrogels were used as experimental controls where ESCs were cultured in the presence or absence of soluble LIF.

Results

Engineering hydrogel substrates for protein capture

Our method of gradient making by HFF relies on a succession of discrete patterning steps where, for a given flow rate, the duration of each step is predetermined by the immobilization kinetics of a tagged protein on the PEG gel substrate (Fig. 1). Consequently, we first determined the binding kinetics of the two fluorescent model proteins Alexa488-BSA-biotin and Alexa488-hIgG. To this end, thin NeutrAvidin- or ProteinA-functionalized hydrogels (Fig. 1a) were exposed to a focused protein stream for variable durations (Fig. 1b). Identical assays were performed for three different flow rates of the protein stream to assess its effect on the patterning process. To avoid a significant widening of the focused protein stream along the entire length of the microchannels, a range of flow rates was chosen that resulted in minimal lateral biomolecule diffusion (not shown). Indeed, the cross-sectional profile of the protein pattern is indistinguishable from the channel beginning, to its end, that is, over *ca.* one centimeter (Fig. S2, ESI†). The intensities of the resulting protein patterns (Fig. 1b) were plotted against time to yield immobilization kinetics for both binding schemes (Fig. 1c, d). In all cases, increasing protein amounts were captured with increasing exposure times until saturation was obtained. This indicates that a rather large concentration (max. 200 ng cm⁻² for both model proteins¹⁶) can be immobilized on these PEG hydrogels.

Biomolecule gradient patterning of gels by HFF

We next utilized our knowledge on biomolecule immobilization kinetics to pattern protein gradients by HFF (Fig. 2a, b). Patterns were generated at variable flow rates using an

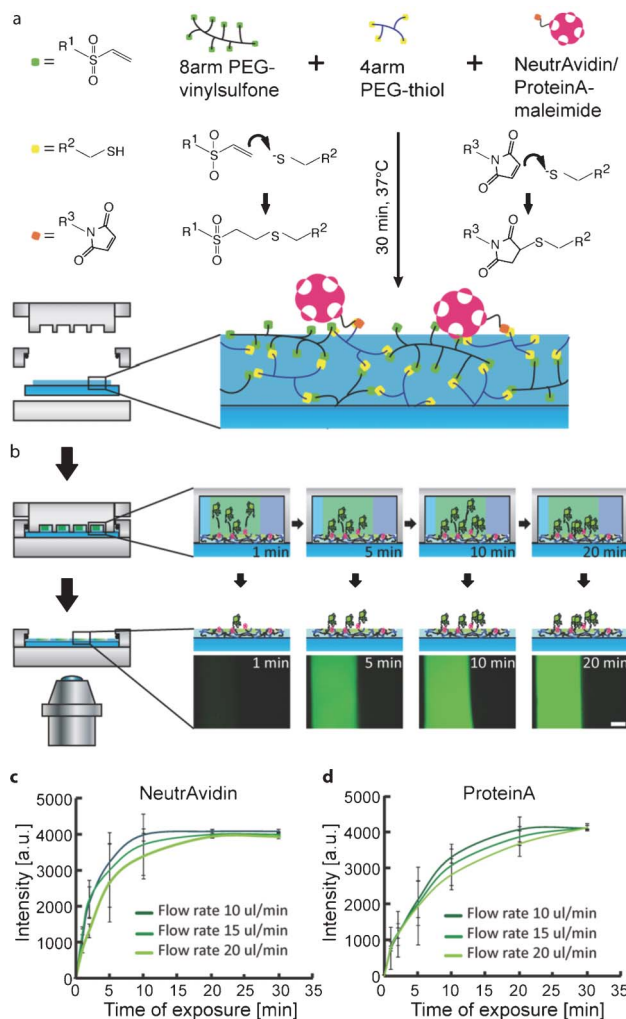


Fig. 1 Hydrogel engineering and protein capture by flow focusing. (a) A schematic representation of hydrogel formation and bioconjugation NeutrAvidin and/or ProteinA. (b) Determination of protein immobilization kinetics for Alexa488-BSA-biotin captured on gel-displaying NeutrAvidin. Fluorescent micrographs of the resulting patterned protein stripes after various exposure times are shown (scale bar = 100 μ m). (c) BSA-biotin immobilization curves for variable flow rates. (d) hlgG immobilization curves for variable flow rates.

increasing number of steps (Fig. 2c, programming parameters are listed in Table S1-2, ESI†), resulting in a transition from a step-wise to a smooth gradient profile (Fig. 2c). Statistical analysis by Matlab (*polyfit* and *polyval* functions setting the grade to one) of the comparison between the angular coefficient of the theoretical and the interpolated line of the pattern intensity profile demonstrated a decreasing error percentage with an increasing number of steps (Table 1). Furthermore, in accordance with our calculations, variations of the flow rates resulted in a widening of linear gradients (Fig. 2d). Further statistical analysis demonstrated an optimal equivalence between the model and the resulting pattern profile widths using an intermediate flow rate (Table 1). Overall, these data show that highly controlled gradient patterning can be achieved using optimized HFF parameters.

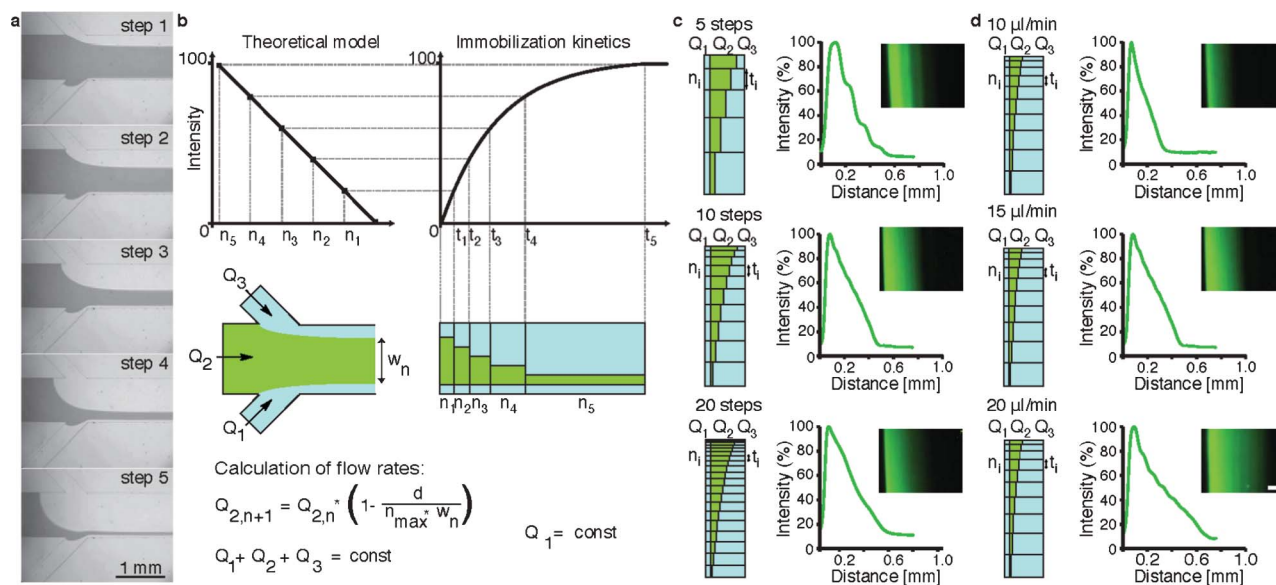


Fig. 2 Protein gradient patterning on hydrogels by HFF; example of a linear gradient. (a) Selected micrographs from a time series of HFF patterning. At each step, the central stream (in grey) is narrowed (scale bar = 1 mm). (b) Implementation of the correlation between the theoretical model and empiric data to yield a linear gradient pattern. Buffer and protein solution flow rate (Q_1 , Q_3 and Q_2) are calculated to sequentially narrow the protein stream (green, w_n), where d is the width of the main channel (ca. 900 μm). Note that for all experiments, the total flow rate was maintained at 25 $\mu\text{l}/\text{min}$. Determination of the duration of each step (t_n) is obtained by correlating the mathematical model, here a straight line, to the measured immobilization kinetic curve. (c) Micrographs and intensity plots of the linear gradient obtained with a variable step number. (d) Micrographs and intensity plots of the linear gradient obtained with a variable flow rate. (Scale bar = 100 μm).

Programmable patterning of more complex gradient shapes

Next, we sought to use HFF for patterning of gradients with fully user-defined profiles. To demonstrate this, we chose to pattern exponential and Gaussian gradients using NeutrAvidin/Biotin and ProteinA/Fc affinity binding strategies (Table S3–4, ESI†). The resulting patterns showed a very good agreement with the programmed intensity profiles (Fig. 3a, b). Moreover, a measurement of fluorescent intensity profiles every three millimeters along the entire gradient length showed a very good cross-sectional profile stability of the patterns (Fig. 3 and Fig. S2, ESI†).

Finally, we used HFF to pattern the gradients of molecules that have a biological function. To this end, we successfully patterned biotinylated recombinant fibronectin fragment III9-10 and Fc-chimeric leukemia inhibitory factor (FcLIF) as linear (Fig. 3c, d) and more complex gradient patterns (Fig. S3, ESI†).

Patterning of protein gradient arrays

We next sought to employ HFF to generate arrayed protein gradients that might be powerful tools for high-throughput screening of multifactorial artificial stem cell microenvironments. A dedicated microfluidic PDMS chip was designed consisting of a channel system with four parallel flow-focusing units (1200 $\mu\text{m} \times 900 \mu\text{m} \times 100 \mu\text{m}$) (Fig. S1a, ESI†). To minimize the number of inlets and outlets for patterning of multiple gradients in one step, the buffer inlets of each unit are coupled together, while each unit has individual inlets for protein solutions. The width of the connecting microchannels is 200 μm at the flow focusing regions with an intersection angle of 45°. The width from inlet to the intersection was calculated to yield equal fluidic resistance. As a result, software-controlled adjustment of the flow rates of individual liquid streams allows dynamic control of the width of the

Table 1 Statistical analysis of the patterning resolution optimization

Step number	Angular coefficient interpolated curve	Angular coefficient theoretical curve	Error (%)
5	-7.9×10^3	-6.66×10^3	15.55
10	-6.96×10^3	-7.44×10^3	6.97
20	-4.19×10^3	-4.19×10^3	1.73
Flow rate ($\mu\text{L}/\text{min}^{-1}$)	Pattern width theoretical (μm)	Pattern width measured (μm)	Error (%)
10	360	320.7	10.91
15	540	513.9	4.83
20	720	638.9	28.62

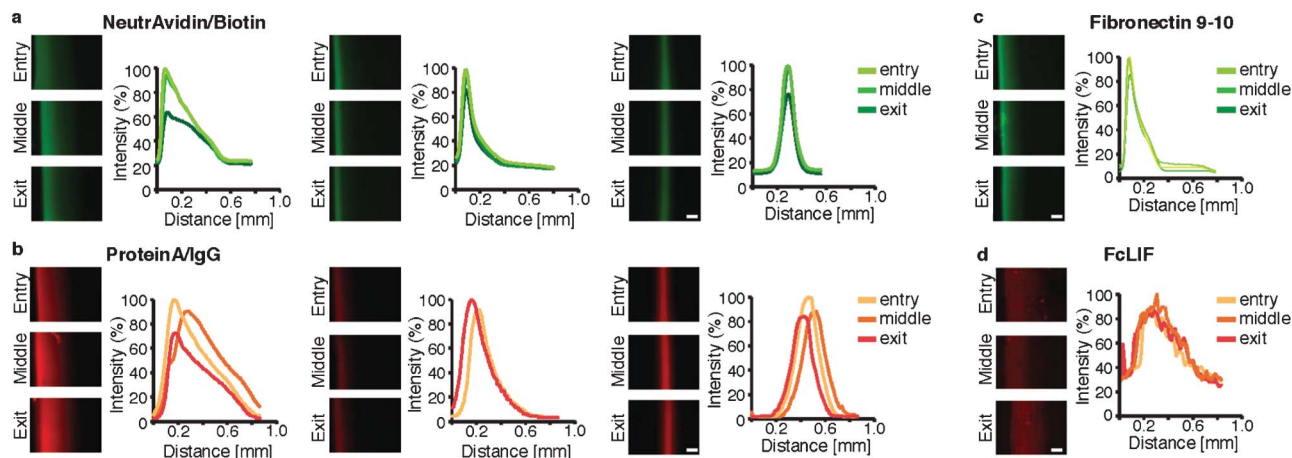


Fig. 3 HFF-based patterning of more complex gradient profiles. (a) Micrographs and intensity profile plots of Alexa488–BSA–biotin on NeutrAvidin-functionalized PEG hydrogels. Linear, exponential and Gaussian gradient profiles were obtained. (b) Micrographs and intensity profile plots of DsRED–hIgG on ProteinA-functionalized hydrogels. Linear, exponential and Gaussian gradient profiles were obtained. (c) Micrographs and intensity profile plots of a linear FITC–FN III9–10–biotin gradient on NeutrAvidin-functionalized gels. (d) Micrographs and intensity profile plots of a linear DsRED–FcLIF gradient on ProteinA-functionalized gels.

protein streams simultaneously in each of the flow focusing units.

This device was used to pattern four parallel linear gradients of Alexa488–hIgG on ProteinA-modified PEG gels (Fig. 4). The resulting patterns on hydrogels obtained after device disassembly and washing are shown in Fig. 4a. Quantification of the intensities across the four parallel gradients showed a highly similar linear profile, demonstrating that simultaneous patterning by flow focusing is possible over long distances (Fig. 4b).

Patterning of overlapping protein gradient arrays

Taking advantage of an the orthogonal protein capture scheme, we next aimed at generating arrays of orthogonally overlapping gradients using a two-step patterning process (Fig. 4c). To this end, the microfluidic device (Fig. S1, ESI†) was further augmented to enable accurate orthogonal alignment of the PDMS chip to the previous pattern.

The first set of four parallel gradients of fluorescent-BSA–biotin was patterned as described above (Table S3, ESI†). The PDMS chip was then turned by 90° for a second patterning step of parallel gradients of fluorescent-hIgG (Table S4, ESI†). Stitched fluorescent micrographs of the resulting overlapping gradient arrays on a hydrogel are depicted in Fig. 4d. Quantification of fluorescent intensities show a linear profile for both single and overlapping gradients (Fig. 4e).

Controlling ESC fate by tethered LIF gradients

To validate our platform, we chose to probe the effect of tethered LIF on mouse ESC self-renewal. LIF, *via* a signal transducer and activator of transcription 3 (STAT3), is a key regulator of ESC pluripotency and an essential component in maintaining ESCs in feeder-free cultures.²¹ Immobilization of LIF on poly(octadecene-*alt*-maleic anhydride) substrates *via* a flexible PEG linker was previously shown to allow the maintenance of ESC over extended periods of time.²²

To facilitate ESC adhesion, PEG hydrogels were first modified with gelatin. In the presence of soluble LIF, these substrates sustained efficient colony formation of self-renewing (*i.e.* Rex1 positive) ESC colonies for extended culture periods (Fig. 5a, b), and can also be used to induce and study differentiation.

Hydrogel films were patterned by HFF to generate arrays of linear gradients of immobilized FcLIF (Fig. 3d). Strikingly, colony size and morphology was strongly dependent on FcLIF concentrations; compact and round colonies were observed within regions of higher concentration, whereas they were flat and more spread out at a lower concentration and outside of the patterned area (Fig. 5c and Fig. S4a, ESI†). This morphological difference suggested that ESCs differentiate when not exposed to tethered FcLIF above a certain concentration. Indeed, expression of the pluripotency marker Rex1 was found to be clearly higher within regions of higher concentration compared to un-patterned areas (Fig. 5d and Fig. S4b, c, ESI†).

The distribution of GFP intensities of individual colonies (Fig. 5e) and the colony area (Fig. 5f) across tethered FcLIF gradients revealed a binary behavior. GFP intensities were significantly ($p < 0.001$) higher at concentrations above *ca.* 85 ng cm⁻² of tethered FcLIF (Fig. 5g). Similarly, colony areas were found to be significantly ($p < 0.001$) smaller above this threshold concentration (Fig. 5h). Therefore, a minimal concentration of tethered FcLIF is required to sustain ESC self-renewal on these soft PEG hydrogels. Interestingly, the threshold value of ~ 85 ng cm⁻² is in good agreement with a previous report,²² in which ESC pluripotency was assessed on immobilized LIF based on the expression of the transcription factor Oct4.

Conclusions

Here we used software-assisted hydrodynamic flow focusing to modify engineered hydrogels with graded protein patterns.

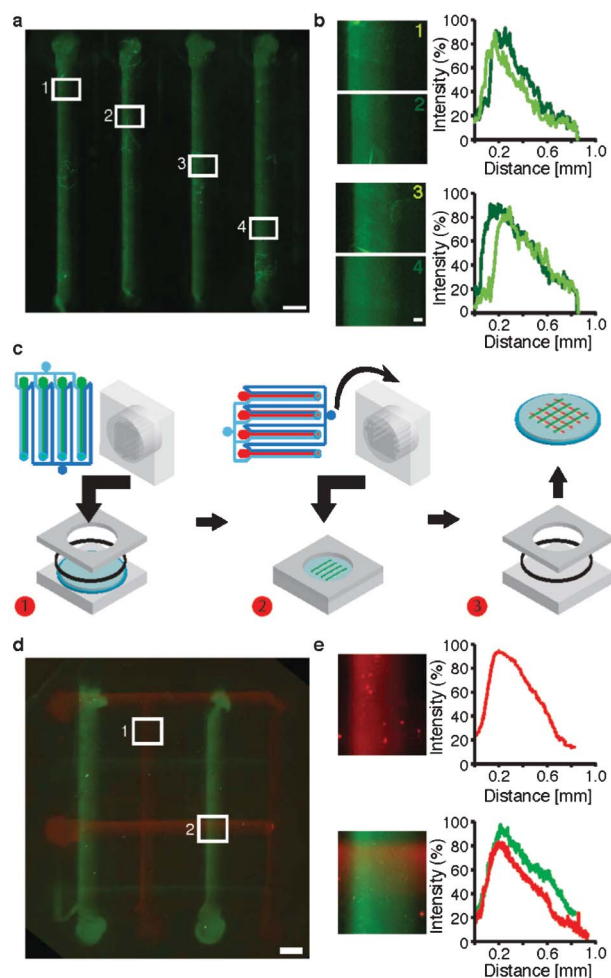


Fig. 4 Patterning of arrayed protein gradients. (a) Stitched micrographs of four parallel Alexa488-hlgG gradients patterned on PEG gel using HFF. (b) Micrographs showing the magnification of individual gradients (white frames) and a graphical representation of their respective intensity profiles (scale bar = 100 μm). (c) A scheme showing the patterning of arrays of overlapping gradients. Step 1: The microfluidic device is assembled and the first set of four gradients is patterned. Step 2: The microfluidic device is partially disassembled with the patterned hydrogel remaining fixed to ensure good alignment. The microfluidic chip is turned by 90° and the second set of parallel gradients is patterned. Step 3: The patterned hydrogel is recovered and used for experiments. (d) Stitched micrographs of a four-by-four gradient array of fluorescent-BSA-biotin (vertically) and fluorescent-hlgG (horizontally). (Scale bar = 900 μm). (e) Micrographs of regions of interest of the gradient array (white frames) and a graphical representation of corresponding intensity profiles.

Our method is amenable to generate gradients of virtually any given shape and composition. By using more sophisticated microfluidic approaches, gradient patterning could be parallelized to obtain arrays of orthogonally overlapping gradients. Because our method combines spatial patterning by microfluidics with a macro-scale cell culture on biomimetic gel substrates, we believe it should be useful for studying dynamic cell behavior, such as cell migration, axonal growth and, perhaps even more excitingly, the biology of pluripotent stem cells.

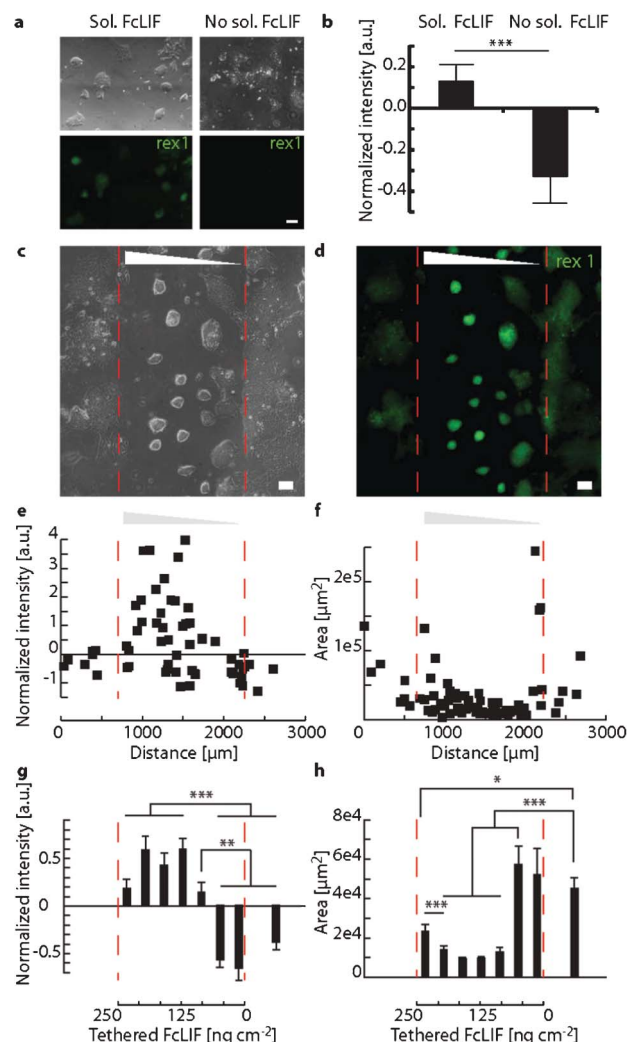


Fig. 5 Influence of tethered LIF gradients on ESC behavior. (a) Bright-field and fluorescent micrographs of ESC cultured for 3 days on gelatin-modified PEG hydrogels in the presence or absence of soluble LIF. (b) Analysis of the GFP (Rex1) signal. Normalization by the standard score, average area (s.e.m.). (c) A bright-field micrograph of ESC cultured on gel displaying tethered FcLIF gradients. Dashed red lines show the pattern area. (d) The GFP signal on the same area. (e) Quantification of the GFP signal (Rex1) of individual ESC colonies across a FcLIF gradient. (f) Quantification of the colony area across a FcLIF gradient. (g) Bar plot of the GFP signal (Rex1) of individual ESC colonies across FcLIF gradients and outside of the pattern area. Normalization by the standard score, average area (s.e.m.). (h) Bar plot of the colony area across FcLIF gradients and outside of the pattern area. Normalization by the standard score, average area (s.e.m.). (Scale bar = 100 μm). Comparison by *t*-test, Bonferroni corrections for multiple comparisons. **p* < 0.05, ***p* < 0.005, ****p* < 0.001.

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