Protein patterning on hydrogels by direct microcontact printing: application to cardiac differentiation

A. G. Castaño, V. Hortigüela, A. Lagunas, C. Cortina, N. Montserrat, J. Samitier and E. Martínez.

SUPPLEMTARY INFORMATION

1. Preparation of substrates.

Glass slides (Corning, USA) were cleaned in piranha acid (7:3 v/v solution of H_2SO_4 and H_2O_2) for 30 min to increase their hydrophilicity and then rinsed thoroughly with Milli-Q water. *Caution: piranha acid is a strong oxidizer and a strong acid. It should be handled with extreme care, as it reacts violently with most organic materials*. Afterwards, they were mounted on ProPlate® 4-well microarray system (Grace Bio-Labs, USA). Matrigel™ (BD Biosciences) was thawed and diluted from 1/10 v/v to 1/40 v/v in DMEM (Gibco 11960, Spain), and gelatin from porcine skin (Sigma G2500, Spain) was dissolved in Milli-Q water at 56ºC from 0.05% to 0.5% w/v. The solutions were poured into predefined wells on the glass slides and, after the gelation process for 2 h at 37ºC, the excess of solution was removed. Matrigel™ substrates were rinsed with 1X PBS and then with Milli-Q water. Subsequently, for the freeze-drying procedure, glass slides were immersed in liquid nitrogen and freeze-dried for 24 hours at -50ºC and 0.06 mbar of pressure (Christ Alpha 1-4 SC, SciQuip, UK). After freeze-drying, hydrogels were characterized by ultra-high resolution scanning electron microscopy (Nova™ NanoSEM 230, FEI Company, The Netherlands) working in low vacuum mode, at 0.5 mbar of water vapor pressure.

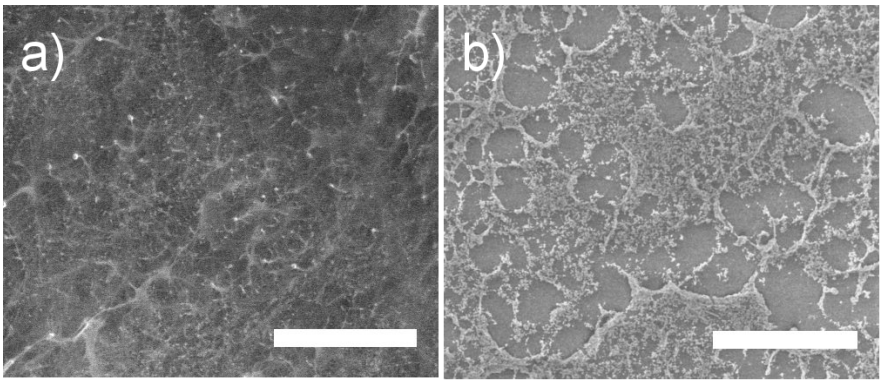

Fig. S1 Scanning Electron Microscopy (SEM) images of (a) freeze-dried gelatin 0.1% w/v and (b) Matrigel™ 1/40 v/v.

PDMS stamps were fabricated by casting a 10:1 (w/w) mixture of PDMS and curing agent (Sylgard 184, Dow Corning) on a microstructured silicon master fabricated by standard UV photolithography. Stamps were cured overnight at 60ºC. Prior to their use, stamps were cleaned with ethanol in an ultrasound bath for 5 min. After that, stamps were inked with 100 µL of streptavidin, Texas Red® conjugate (Invitrogen S872, Spain), human fibronectin (Sigma **F2518, Spain) or human laminin** (Sigma **L6274, Spain),** diluted in 1X PBS (100 µg mL^{-1}). Then, they **were incubated for 45 min, followed by washing with 1X PBS and Milli-Q water, and dried under nitrogen flow. For the printing procedure, stamps were placed in contact with the freeze-dried hydrogels for 10 min. Characterization of the patterned surfaces was performed by fluorescence microscopy prior and 24 hours after reconstitution.**

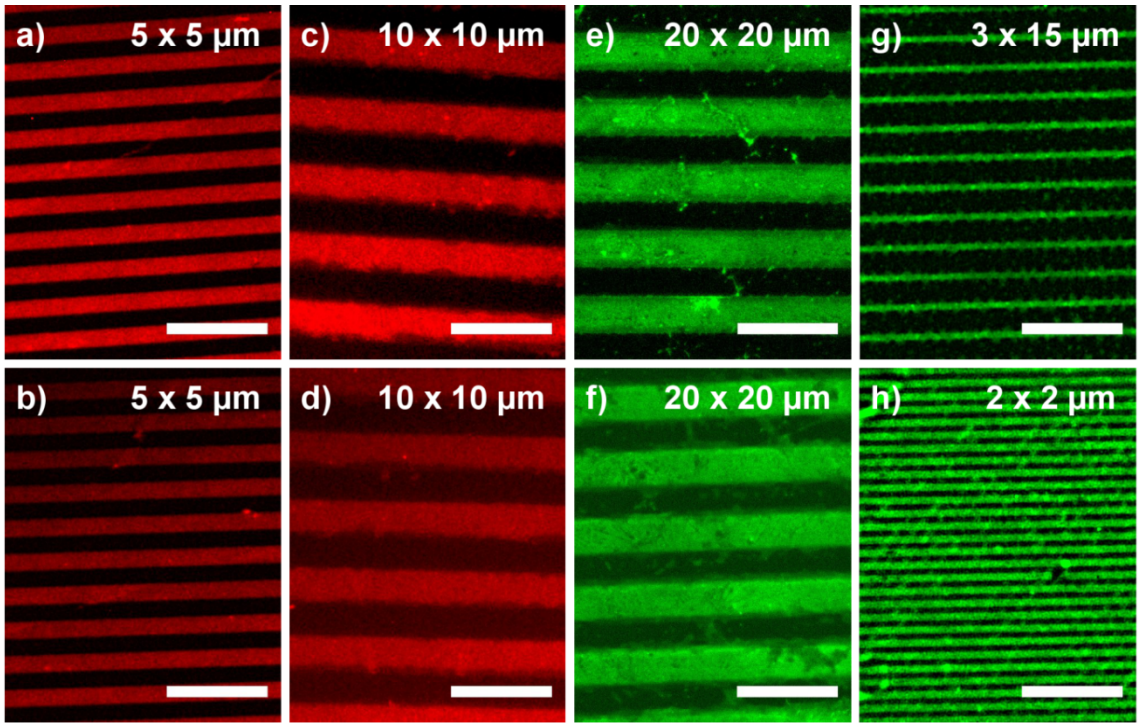

Fig. S2 Fluorescence microscopy images demonstrating the versatility of the extended microcontact printing technique with respect to the size of the pattern, indicated in top right. (a-d) Lines of streptavidin Texas Red® conjugate printed on gelatin. (e, f,) Lines of streptavidin Texas Red® conjugate printed on Matrigel™. (g) Lines of fibronectin printed on Matrigel™. (h) Lines of laminina printed on Matrigel™. Scale bar: 30 µm (a-d, h), 60 µm (e-g)

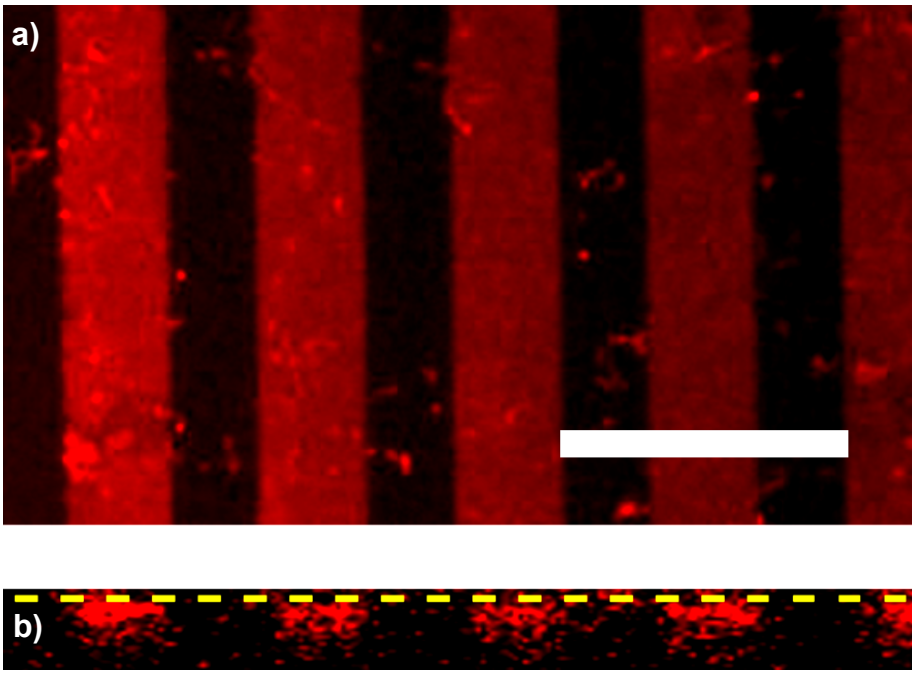

Fig. S3 Fluorescence microscopy and confocal microscopy images showing the depth distribution of streptavidin Texas Red® conjugate 3 h after reconstitution. (a) Lines of streptavidin Texas Red® conjugate printed on gelatin. (b) Cross section showing the protein on the surface and its diffusion profile into the hydrogel bulk (protein diffuses within a region of about 8 µm thick). The dash line represents hydrogel surface. Scale bar (for both a) and b)): 60 µm.

2. Cell culture and immunostaining:

NIH-3T3 mouse embryonic fibroblast cells were resuspended at a density 2 × 10^4 cell mL^{-1} and were seeded onto 2.56 cm^2 area of ProPlate® 4-well microarray system, **at a density of 10^4 cell cm^{-2}, using 0.5 mL of cell suspension. Afterwards, cells were incubated at 37°C and 5% CO_2 for 4 h under serum starvation conditions using cell culture medium supplemented with 1% fetal serum bovine (FBS). Substrates were washed with PBS and cells were fixed in formalin solution (Sigma HT5011, Spain) for 20 min, followed by neutralization by $ClNH_4$ (Merck**

Millipore, Spain) 50 mM in Milli-Q water for 20 min. Then, cells were permeabilized in a 0.1% w/v saponin solution in 1% BSA in 1X PBS for 10 min. Fibronectin micropatterns were stained by using as a primary antibody rabbit anti-fibronectin (1:300) (Sigma, Spain), and as the secondary antibody Alexa Fluor® 568 goat anti-rabbit IgG (1:1000). Phalloidin-FITC (1:500) (Sigma, Spain) was used to stain F-actin and nuclei were stained with Hoechst 33342 (1:1000) (Invitrogen, Spain). All of them were incubated at RT for 1 h. Fluorescence microscopy pictures were acquired by a Nikon Eclipse E1000 upright microscope (Nikon, The Netherlands) equipped with a charge-coupled-device (CCD) camera.

ImageJ free software (http://rsbweb.nih.gov/ij/**) was used to analyze cell shape and alignment with respect to the pattern direction. Cells and nuclei were fitted to an ellipse and the aspect ratio between the long and the short axis w**as measured, as well as the angle described by the long axis of the ellipse with the patterned lines. **All the measurements were obtained from three independent experiments.** Statistical analysis of the variables was performed by **T-**tests. All graphical data are reported as the mean ± standard error (SEM). Significance levels were established at $p < 0.01$.

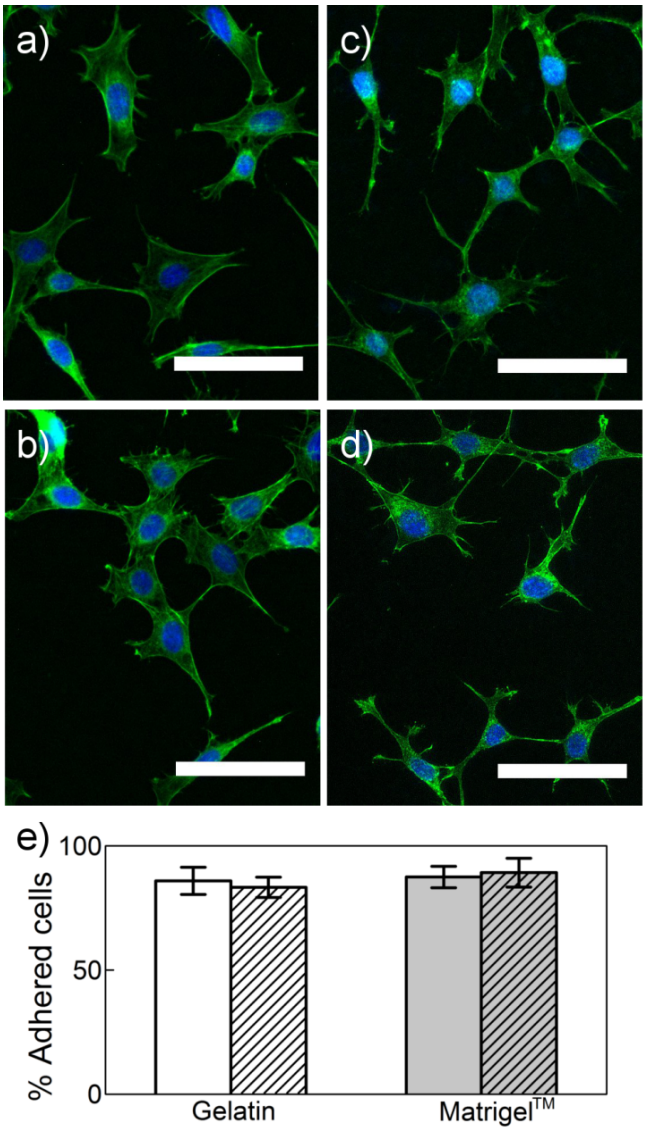

Fig. S4 No morphological differences were found between freeze-dried and non freeze-dried substrates. Representative fluorescent microscopy images of NIH-3T3 cells cultured on freeze-dried gelatin (a) and Matrigel (b) after 4 h of culture. Cell actin cytoskeleton is stained in green and cell nuclei are stained in blue. Scale bar: 75 μm. (e) Graph plotting the percentage of adhered cells on non freeze-dried hydrogels (solid bar) and on freeze-dried hydrogels (stripped bar), presented as the mean with the standard error. No significant differences in cell adhesion were found

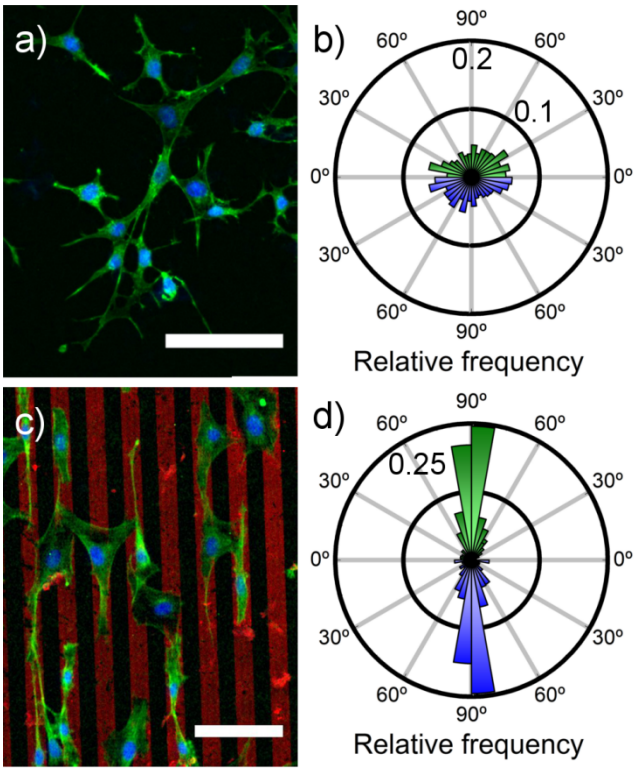

Fig. S5 Cell alignment on micropatterned Matrigel™ and application in cardiac differentiation. Fluorescence microscopy pictures of NIH 3T3 mouse embryonic fibroblasts cultured on (a) non-patterned and (c) patterned (fibronectin lines 20 µm in width) gelatin hydrogels after 4 hours of culture. Cell nuclei are stained in blue, actin cytoskeleton in green and fibronectin in red. Scale bar: 100 µm. Normalized histograms (bin = 10º) depicting the distribution of the angles between cell cytoskeleton fibers (in green) and cell nuclei (in blue) with the pattern direction on (b) non-patterned (d) and patterned gelatin hydrogels

3. Cardiac differentiation studies

Human embryonic stem cells (hESC), provided by the group of Prof. J.C Izpisúa (CMRB-PRBB, Barcelona, Spain), were maintained on a layer of mitotically inactivated mouse embryonic fibroblasts in hESC medium: DMEM/F12 (Invitrogen) supplemented

with 0.1 mM non-essential amino acids (Invitrogen), 1 mM GlutaMAX (Invitrogen), 20% Knockout Serum Replacement (Invitrogen), 55 μM β-mercaptoethanol (Invitrogen) and 10 ng mL^{-1} bFGF (Joint Protein Central). Then, hESCs were cultured on Matrigel (BD Biosciences) with mTeSR medium (Stem Cell Technologies) until high degree of confluence was achieved. Finally, hESC were trypsinized and seeded onto patterned and non-patterned gelatin at density 10^5 cells cm^{-2} with conditioned medium (MEF-CM,) for 24 hours. The following days the media was replaced by KO DMEM (Gibco, Spain) supplemented with 10% fetal bovine serum (Gibco, Spain), 1% non-essentials aminoacids (MEM NEAA 100x Gibco, Spain), 0,1% of 2-Mercaptoethanol 50 nM (Gibco 31350-010), 0,5% of penicillin–streptomycin antibiotic (Gibco 15140-122, Spain), 1% of GlutaMAX 200 mM (Gibco 35050-038, Spain) and ascorbic acid 0.5 mM (Sigma, Spain) to promote cardiac differentiation. Cells were maintained for the following 30 days and the formation of beating foci was monitored. At day 30, cells were fixed with 4% paraformaldehyde for 20 min. Afterwards, samples were rinsed with Tris-buffered saline (TBS) and were incubated 1 hour with block buffer (TBS 1x, 0.5% triton and 6 % donkey serum). The primary antibodies were GATA4 (1.119) rabbit IgG 1:25 and alpha sarcomeric actin (ASA) (1.168) mouse IgM 1:400 in TBS++ (TBS 1x, 0.5% Triton and 6% donkie serum), which were incubated at RT for 2 h. Secondary antibodies were anti-Goat IgG –Cy2 (2.6) and anti-mouse IgM-Cy3 (2.3) in TBS++ 1:200 which were incubated at RT for 2 h. Finally, for nuclei staining cells were incubated with DAPI 1:10000 for 10 min. Bright field and fluorescent images, and videos were acquired by a Leica DMI4000 B microscope equipped with Leica DFC 350 FX digital camera.