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Supplementary Information: Density gradients at hydrogel interfaces for enhanced cell penetration

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Experimental Section

Preparation of the PDMS chambers. Polydimethylsiloxane (PDMS) chambers were fabricated as follows: the silicon elastomer and the curing agent (Sylgard 184, Dow Corning Corporation, USA) were mixed (10:1 in mass) at 2000 rpm for 3 min in an ARE-250 mixer (Thinky Corporation, Japan). The mixture was subsequently poured into poly(methyl methacrylate) (PMMA) molds, where a 500 μm diameter stainless steel wire was positioned to create the holes for the future counter electrode. The mixture was subsequently degassed for 30 min in a vacuum chamber and baked for 4 h at 60 °C. The stainless steel wire and the PDMS form were removed from the PMMA molds and subsequently rinsed with isopropanol (IPA) and MilliQ water.

Preparation of PEG Hydrogels: Metalloprotease (MMP)-sensitive TG-PEG hydrogels were prepared as described previously [1]. In brief, 40 kDa eight-arm PEG precursors containing the pending FXIIIa substrate peptides glutamine acceptor (n-PEG-Gln) or lysine donor with an additional MMP-sensitive linker (n-PEG-$MMP_{sensitive}$-Lys) were mixed 1:1 stoichiometrically (final dry mass content 1.7%, unless otherwise specified) in Tris-Buffer (50 mM, pH 7.6) containing 50 mM calcium chloride. Lys-FITC (1.6 μM), Gln-Alexa 561 (1.6 μM), Gln-RGD (50 μM) were added to the precursor solution prior initiation of cross-linking by 10 U/mL thrombin-activated FXIII (FXIIIa) and vigorous mixing. Lys-FITC was added to gel precursor for the evaluation of the density gradient profiles. For cell penetration studies, Gln-RGD was added to all gel formulations. Additionally, to study cell penetration across the gel-gel interface, Lys-FITC was added to the bottom gel and Gln-Alexa 561 was added to the top gel.

Electrochemical control of PEG polymerization. The precursor mixture was immediately poured into the PDMS chamber (4 mm length, 5.5 mm width and 2 mm thickness) accommodating a platinum wire (0.5 mm in diameter, Alfa Aesar, Ward Hill, USA) used as auxiliary electrode. Cobalt-chromium disks (15 mm in diameter and 0.8 mm in thickness) evaporated with 10 nm chromium and 200 nm gold were used as working electrode to be placed on top of the PDMS chamber. The polymerization of the PEG was allowed to progress during 8 minutes in presence of a direct anodic current applied in galvanostatic mode. The current density was +0.1 $\mu A/mm^2$ or +1 $\mu A/mm^2$.

Confocal laser scanning microscopy (CLSM) of hydrogels. FITC-labelled hydrogels were cut perpendicularly to the hydrogel surface with a razor blade and kept in Tris-Buffer (50 mM, pH 7.6). The sections were imaged using an LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany). The FITC was detected upon excitation at 490 nm (0.7% laser line attenuator transmission) with an emission band-pass filter of 505-550 nm. The pinhole section was set to 30 μm and the focal plane was selected to maximize the intensity over the field of view. The intensity profiles were obtained by setting the minimum intensity as the intensity above the gel surface and by normalizing the values by the intensity measured at 800 μm from the surface.

Colloidal probe force spectroscopy: Force-distance measurements were performed in Tris-Buffer using a commercial FluidFM system (Cytosurge, Switzerland) [2]. For long-range force spectroscopy, the system includes a motorized microscope stage. The setup was mounted on an Observer.Z1 optical microscope (Carl Zeiss, Germany). All devices were controlled by the CYui FluidFM software.

Tipless FluidFM cantilevers with 8 μm apertures (Cytosurge, Switzerland) were used after oxygen plasma cleaning (2 min, Harrick PDC-32G 18 W). Prior to the force measurements, the cantilever spring constant was calculated from the thermal spectrum (in the range of 1.56-

2 N/m) [3]. After filling the cantilever with buffer solution, the optical lever sensitivity in liquid was determined from a deflection-distance curve on a rigid glass surface.

A droplet of approximately 60 µL polystyrene colloids (50 µm in diameter, 1.8×10^5 particles/mL, Micromod, Germany, cat. no. 01-00-504) was deposited on the gel surface to be probed. The colloids were left to sediment for 10 min and the gel was immersed in Tris Buffer (50 mM, pH 7.6). A bead of interest was selected and a first approach with a setpoint of approximately 5 nN was performed on the bead, while applying an underpressure of -700 mbar. When the probe was approached, the colloid was immobilized on the aperture due to the underpressure, avoiding lateral movement of the colloid during the indentation. Force-distance measurements were performed at an indentation speed of 100 nm/s until either the maximal deflection of 9.5 V or the maximal piezo range of 50 µm was reached. Curves acquired at higher indentation speeds (500 nm/s and 1 µm/s) did not significantly vary. After each indentation, the bead was released by applying an overpressure of 1 bar and the next bead was selected. For each sample, 3 force-distance curves were recorded and 3 samples per condition were probed.

Estimation of the elastic modulus: Elastic moduli were estimated by fitting the force-distance curves over a 5 µm indentation using the Hertz model:

$$F = \frac{4}{3}\frac{E}{1-\nu^2}\sqrt{R\delta^3}$$

where F is the applied force, ν is the Poisson's ration (equal to 0.5), E is the elastic modulus, R is the radius of the colloidal probe (equal to 25 µm) and δ is the sample indentation. The estimation of the elastic modulus was performed only on force-distance curves measured on hydrogel sections cut perpendicularly to the surface because the Hertz model is not appropriate to describe the indentation of surface density gradients.

Cell culture: Human-derived bone marrow MSCs, isolated as described elsewhere [4], were cultured in minimal essential medium alpha (MEMalpha, Gibco Life Technologies, cat. no.

22571-020) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco Life Technologies, cat. no. 10500), 1% (v/v) penicillin/streptomycin solution (Gibco Life Technologies, cat. no. 15140-122), 5 ng/mL FGF-2 (Peprotech, cat. no. 100-18B) and 50 nM PDGF (Peprotech, cat. no. 100-14B).

Gel penetration: MSCs were seeded on the hydrogel surface by depositing a 50 µL droplet of medium containing 200'000 cells/mL. The cells were left to sediment on the gel surface for 30 min. Subsequently, the gel was completely covered by medium. MSCs were kept in culture for 1 or 3 days. At each time point, samples were fixed with 4 % paraformaldehyde, rinsed three times and kept in PBS until staining.

Penetration across the gel-gel interface: Cell-containing gel precursors were poured on top of hydrogels produced with an engineered surface and left to polymerize during 8 min. The assembled constructs were subsequently placed in culture for 1 or 3 days. At each time point, samples were fixed with 4 % paraformaldehyde, rinsed three times and kept in PBS until staining.

CLSM of cells in hydrogels: Permeabilization was performed for 30 min at room temperature with 0.1 % Triton X-100 in PBS followed by 2 washing steps with PBS. For f-actin staining, samples were incubated overnight at 4 °C with Alexa 633-labeled phalloidin (Molecular Probes, cat. no. A22284). Afterwards, samples were washed 3 times with PBS before analysis with CLSM. The PEG hydrogels and cells were imaged using an SP5 confocal laser scanning microscope (Leica, Germany). At least 3 samples per condition were analyzed and 3 regions per sample were acquired.

Infiltration quantification: Stacks (125 × 2 µm) acquired by CLSM were reconstructed in 3D, and a side projection was performed. The FITC channel was used to determine the gel surface and the Alexa-633 channel was used to determine the position of cells in the gel cross-section. A threshold was applied to the Alexa-633 channel images, which were subsequently cleaned (noise removal) and segmented into 25 µm thick regions starting from the gel surface. The

amount of positive pixels was quantified in each region as a representation of the cell number. The ratio of cells in each section was calculated as a percentage of the overall amount of cells in the sample. The values represent mean values ± standard deviation of at least 3 gels per time point, in which at least 3 regions were analyzed. T-test and two-way ANOVA (time and treatment) followed by post-hoc multicomparison analysis (Tukey-Cramer range) were performed on data representing the cell infiltration at the hydrogel surface (first 25 µm). Significant difference ($p<0.05$) is indicated with an asterisk.

Supplementary Figures

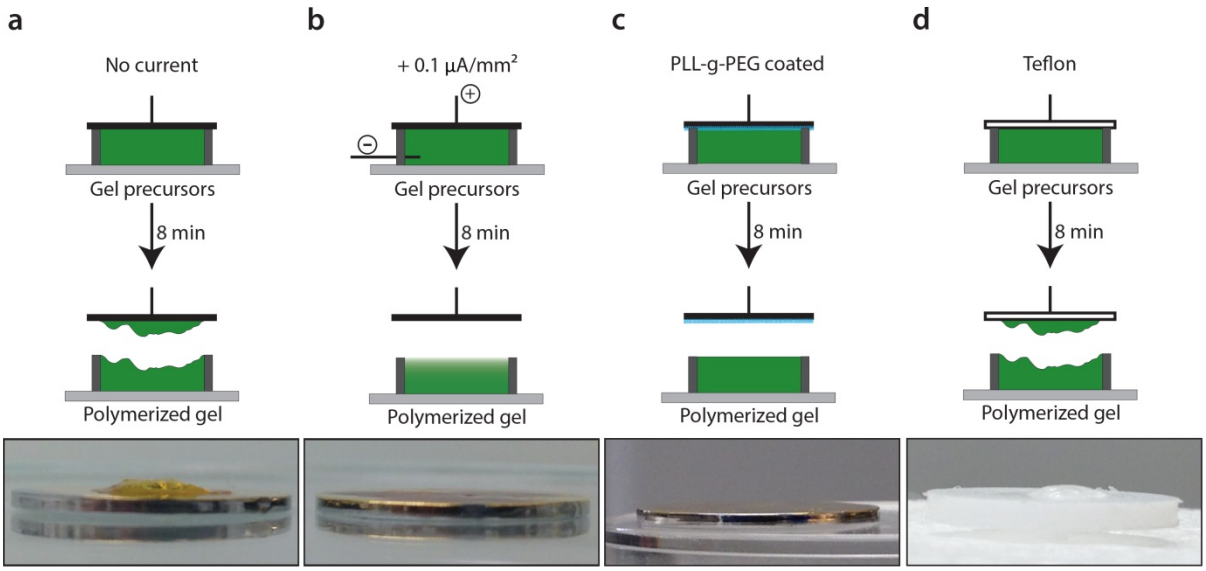
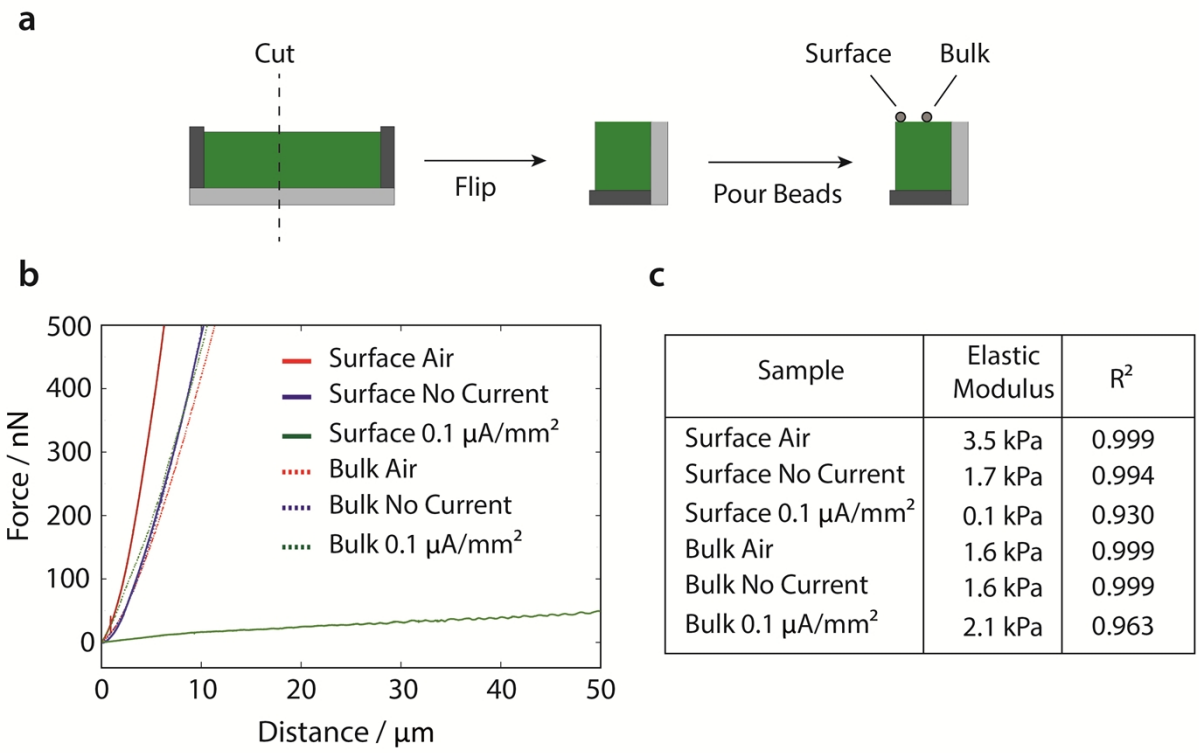


Fig. S1: Removal of the covering surface and effect on the hydrogel integrity. The removal of the non-polarized covering gold electrode and of a covering Teflon disk resulted in the disruption of the hydrogel because of the too strong adhesion between the gel and the cover (a and d). The application of an anodic current (0.1 µA/mm^2) or the application of an anti-adhesive PLL-g-PEG coating to the covering gold surface enabled the removal without hydrogel disruption (b and c). The use of anodic current to decrease the adhesion of PEG hydrogels to metal surfaces was previously reported [5].



Sample	Elastic Modulus	R^2
Surface Air	3.5 kPa	0.999
Surface No Current	1.7 kPa	0.994
Surface 0.1 µA/mm²	0.1 kPa	0.930
Bulk Air	1.6 kPa	0.999
Bulk No Current	1.6 kPa	0.999
Bulk 0.1 µA/mm²	2.1 kPa	0.963

Fig. S2: Mechanical characterization of the hydrogel cross-sections. a) Schematic representation of the experimental setup: Hydrogels were cut perpendicularly to the surface and flipped (cut showing upwards). 50 µm beads were poured on the sections and used to probe the mechanical properties of the hydrogel at different positions: within the 50 µm vicinity of the surface (surface) and at least 500 µm from the surface (bulk). b) Representative force-displacement curves confirmed that all hydrogels had similar bulk rigidities with estimated elastic moduli ranging from 1.6 to 2.1 kPa. By comparing bulk and surface force-displacement curves, we confirmed that the *No current-hydrogel* had a homogenous rigidity (1.7 kPa), whereas the *Air-hydrogel* had a more rigid surface compared to the bulk (3.5 kPa), and the *0.1 µA/mm²-hydrogel* a softer surface compared to the bulk (0.1 kPa). For the thickness of the gel used here, the large extent of the gradient obtained at 1 µA/mm² made the hydrogel difficult to section without disrupting it. For this reason, we do not present this condition in this figure.

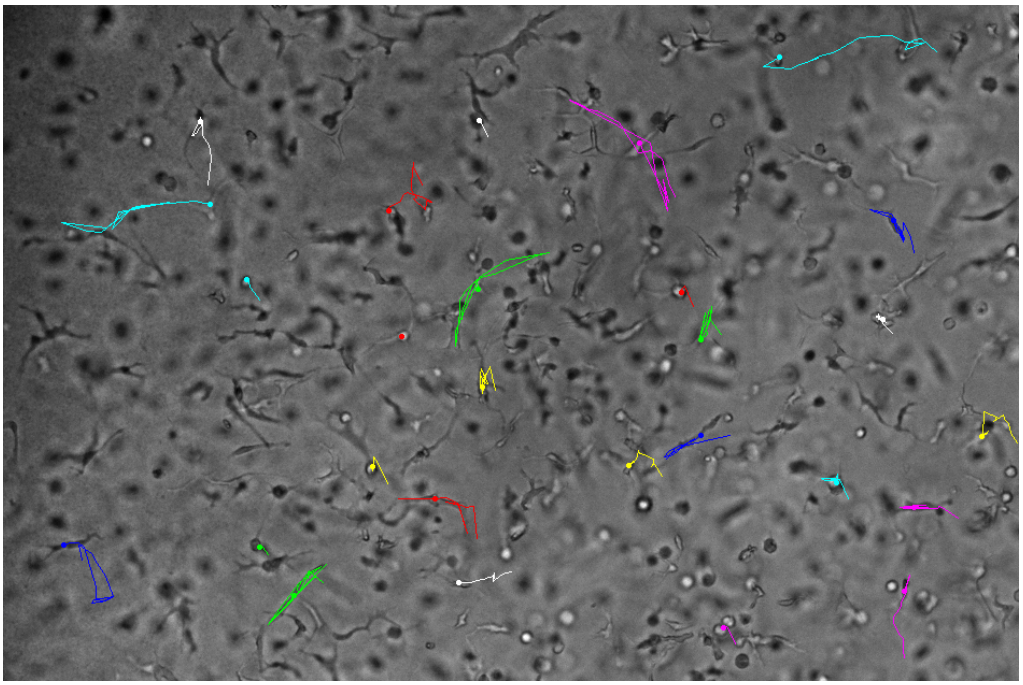


Fig. S3: Cell migration within the hydrogel. MSCs were embedded in PEG hydrogels (final concentration: 0.5 x 10^6 mL^{-1}) and imaged every 20 min for 24 hours (Leica DMI6000 B). Cell migration was followed for 24 hours using the manual tracking plugin in ImageJ. 30 tracked cells had an average migration of 226 ± 44 µm in 24h.

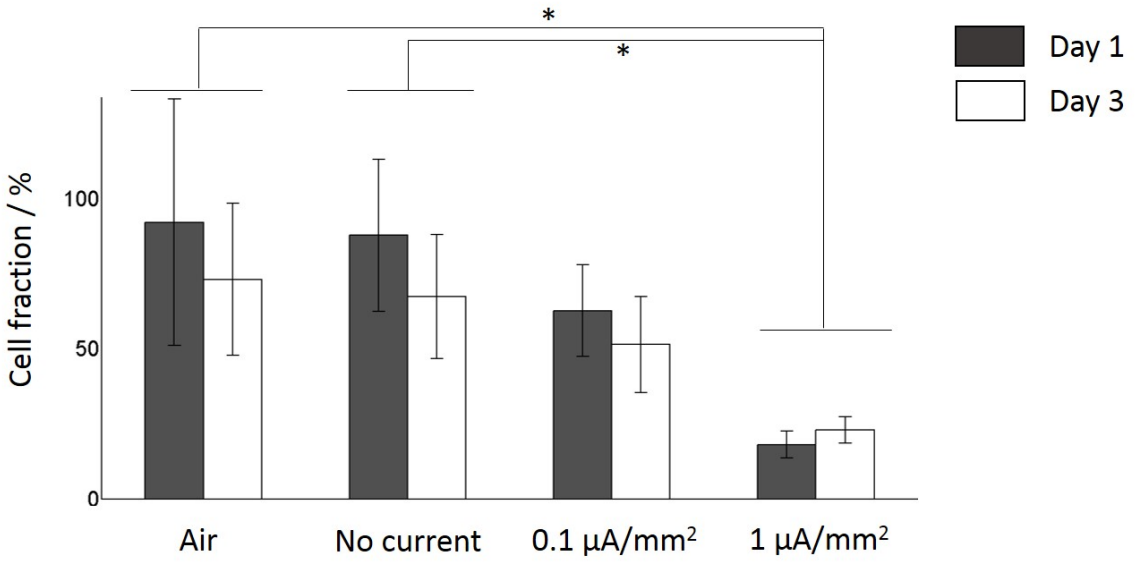


Fig. S4: Two-way ANOVA followed by post-hoc multicomparison test (Tukey-Cramer critical range) of the average cell fraction present at the surface of the hydrogels (first 25 µm). The asterisk indicates significant difference between the groups ($p<0.05$). The entire cell distribution across the hydrogel is presented in Fig. 3.

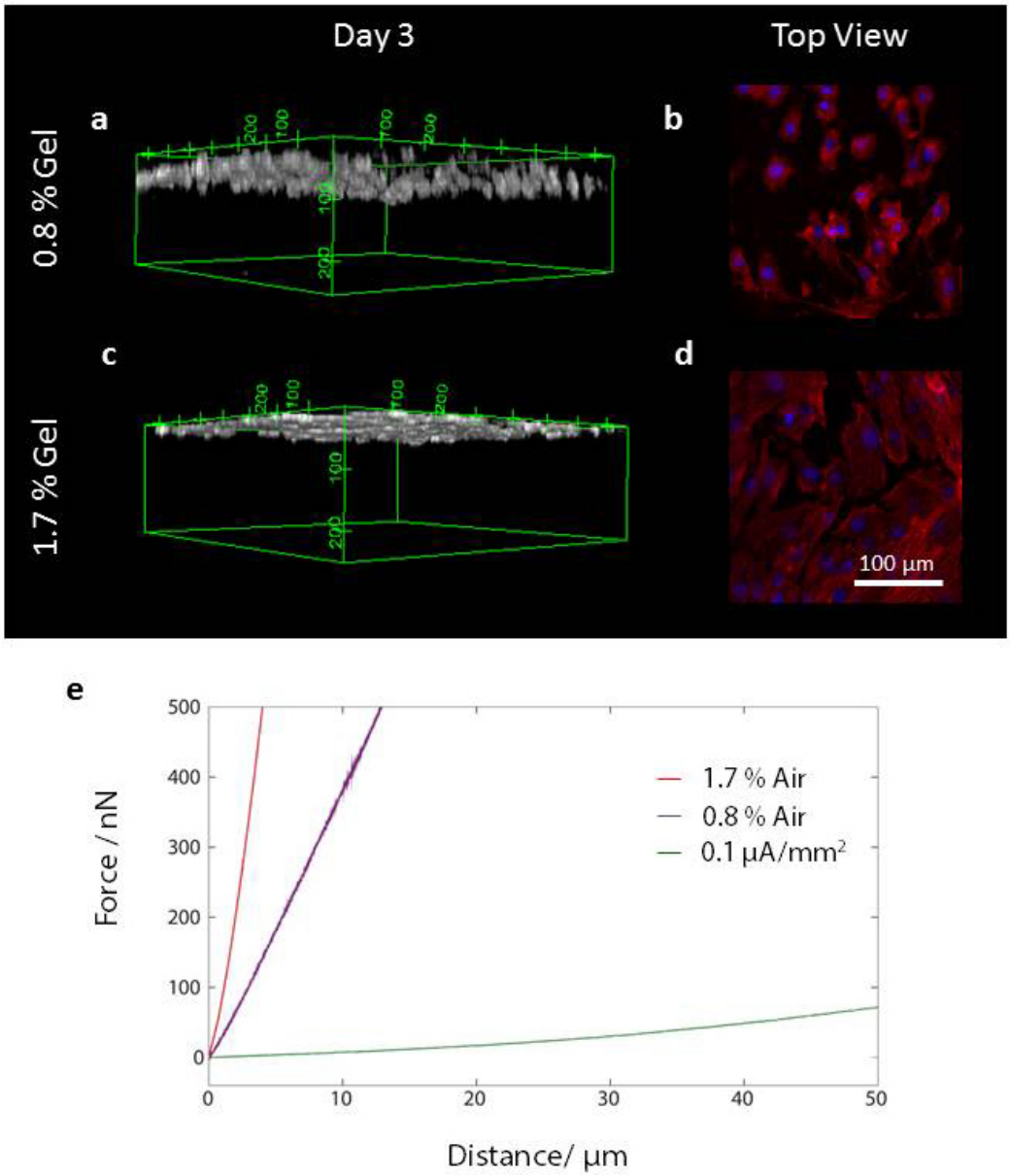


Fig. S5: Human derived bone marrow MSCs seeded on *Air-hydrogels* with lower PEG densities (0.8%). a), c), are 3D reconstruction of 250 µm thick stacks (consisting of 50 images acquired every 5 µm for the 0.8% condition and of 125 images every 2 µm for the 1.7% condition) acquired by LSCM 3 days after seeding: cells formed a 2D sheet on the gel regardless of the rigidity. Cell morphology (top view, right column) show that cells responded to the gel rigidity by spreading more on more rigid gels, as shown in previous studies [6]. e) Force-distance curves measured by indenting 50 µm polystyrene beads deposited on the surface of the hydrogels show that the gels produced with lower PEG density were indeed softer than the 1.7% gel commonly used. These gel surfaces were nonetheless still considerably more rigid than the electrochemically prepared hydrogel surfaces.

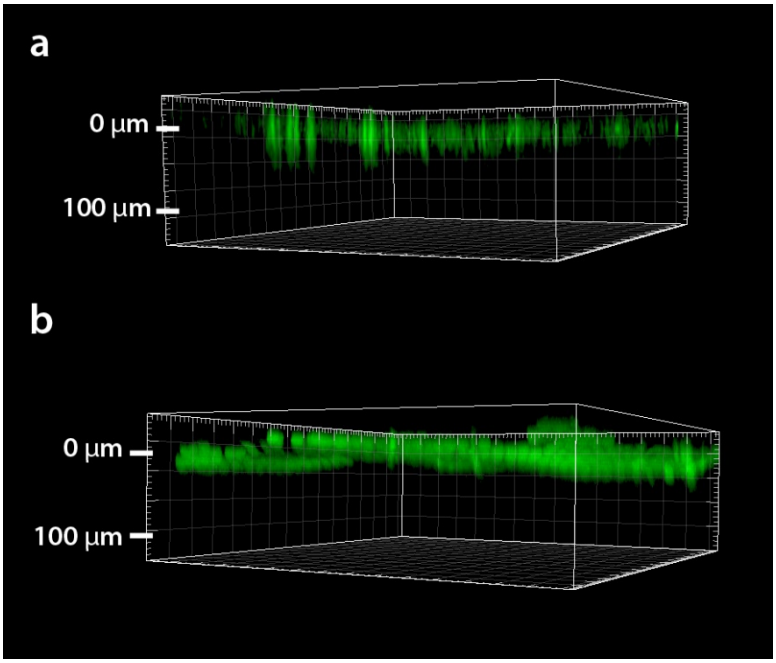


Fig. S6: Fluorescent microbeads on *1 µA/mm²-hydrogel* surface. 3 µm (a) and 20 µm diameter (b) fluorescent beads (Fluoresbrite Plain YG, Polyscience Inc.) were deposited on the hydrogel surface and left to sediment for 1 hour. Their distribution was assessed by acquiring 150 µm thick Z-stacks of the hydrogel surface using a SP5 confocal laser scanning microscope (Leica, Germany).

References for Supplementary Information

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