Programmed sub-cellular release for studying the dynamics of cell detachment

Bridget Wildt, Denis Wirtz & Peter C. Searson

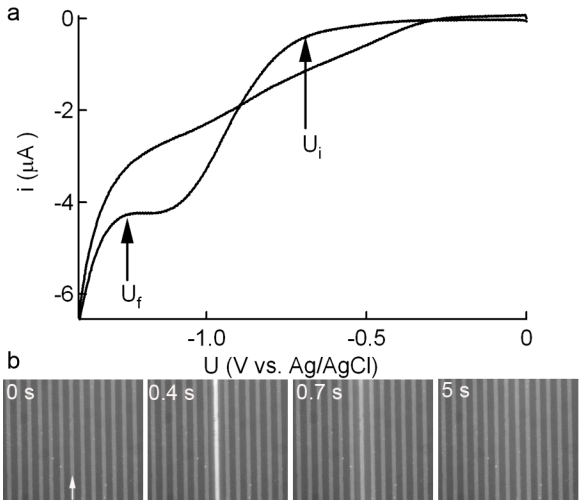
Supplementary Methods

Supplementary Movies

Supplementary Movie 1. Time lapse movie of sub-cellular release (see Fig. 1f). Magnification, 10x.

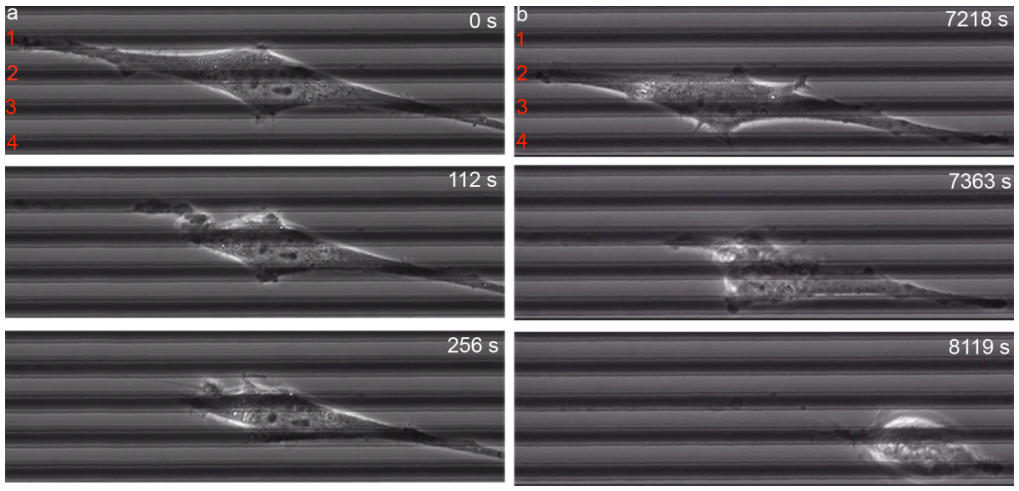
Supplementary Movie 2. Time lapse movie of sub-cellular release (see Supplementary Fig. 2a). Magnification, 10x.

Supplementary Movie 3. Time lapse movie of sub-cellular release (see Supplementary Fig. 2b). Magnification, 60x.



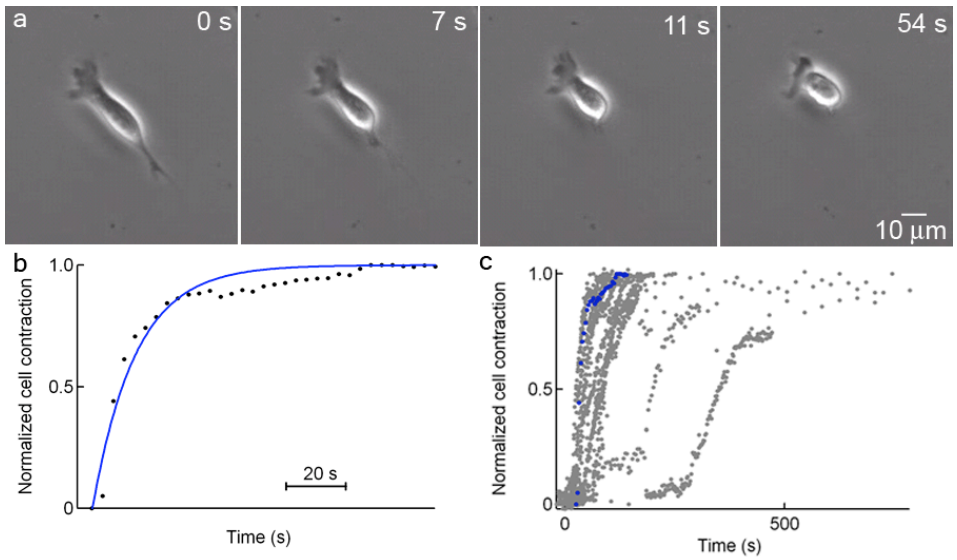
Supplementary Figure 1. Programmed thiol release. (a) Cyclic voltamogram for RGD-terminated thiol on a single electrode in PBS (pH 7.4) at a scan rate of 100 mV s^{-1}. (b) Sequence of fluorescence images of release of a fluorescently-labeled thiol from one electrode (see arrow) of an electrode array.

The cyclic voltamogram in Supplementary Fig.1 shows the current response of a single gold electrode functionalized with a fluorophore-terminated thiol in PBS. The device was functionalized with an amine-terminated thiol (HSC11-EG6NH2, Prochimia, Poland) tethered to a fluorescent probe, Alexa fluor 568 (Invitrogen, USA), via an N-hydroxysuccinimde linkage. At positive potentials, from 0 V to -0.8 V, the current is negligible as the self-assembled monolayer acts as a molecular resist. The peak at -1.1 V corresponds to reductive desorption of the RGD-terminated thiol from the gold surface according to R-S-Au + H^+ + e^- → R-SH + Au.[1-3] The onset and completion of desorption are indicated by U_i and U_f, respectively. Supplementary Fig. 1b shows a sequence of time lapse images of a single electrode (see arrow) after applying -1.3 V (Ag/AgCl). The intensity is initially very weak due to fluorescence quenching, however, 400 ms after release, the desorbed fluorophore-terminated thiol has diffused far enough away from the surface that the fluorescence is no longer quenched by the gold and the electrode appears bright. At longer times, the fluorescence becomes diffuse as the fluorophore-terminated thiol diffuses away from the electrode into the bulk solution. These results demonstrate that thiol release is very fast in comparison to the induction time in the cell release experiments.



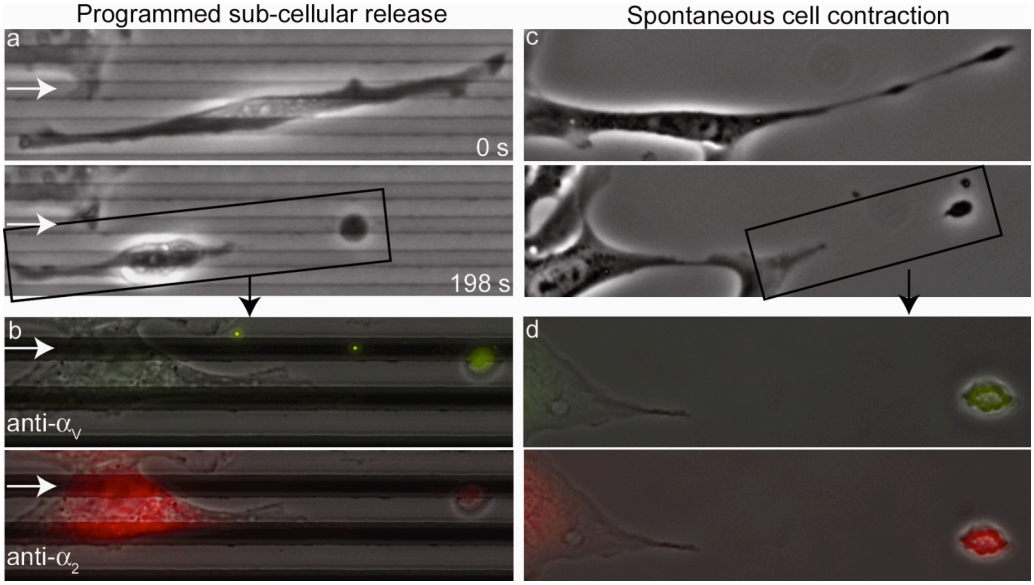
Supplementary Figure 2. Sequential sub-cellular release of a 3T3 fibroblast cell plated on a device containing 5 μm wide lines with 10 μm spacing in which the glass surface has been modified with polyethylene glycol. (a) The cell was released from electrode #1 and from the contraction dynamics we determine t_0 = 37 s and τ = 34 s. (b) After two hours, the cell had spread along electrode #2, and was subsequently released. The cell contracted to electrode #3 (t_0 = 31 s and a τ = 157 s) after which the cell remained viable.

Supplementary Fig. 2 shows a sequential release experiment, verifying that cells are completely viable after release and in fact, most start to spread immediately after contraction.



Supplementary Figure 3. Spontaneous release (tail snap) of a 3T3 fibroblast cell on glass. (a) Phase contrast images of a fibroblast cell during contraction. (b) Normalized contraction of the cell in (a). (c) Normalized contraction curve for this cell (blue) plotted with results from sub-cellular release experiments (gray). Time-lapse phase contrast microscope images were obtained at a rate of 16.7 frames per minute and the change in cell length was measured with respect to the initial position and normalized to the maximum change in length. The release curves were fit to the equation: $\Delta L(t)/\Delta L_m = 1 - \exp[-(t-t_0)/\tau]$.

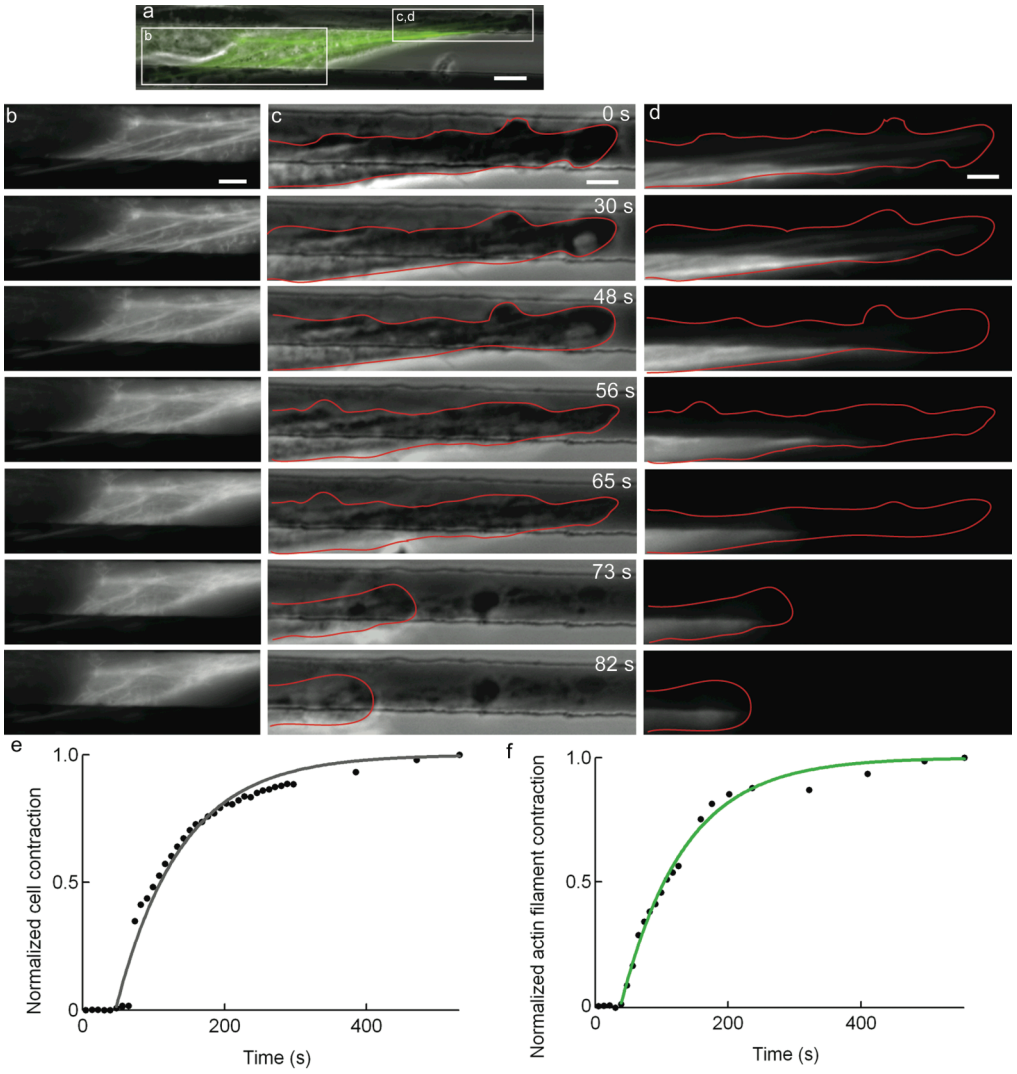
Cell contraction during tail snap in 3T3 fibroblast cells follows identical dynamics to our programmed sub-cellular release experiments. A typical example is shown in Supplementary Fig. 3. The cell contraction is exponential with a characteristic contraction time τ = 14.4 s, within the range obtained from our programmed sub-cellular release experiments (9.9 – 69.6 s). Figure 3c shows the spontaneous contraction of this cell (blue) with cells that have released by programmed sub-cellular release (gray). This result demonstrates that the global contraction dynamics in our method are identical to spontaneous cell contraction. Furthermore, the tail snap experiments highlight the problem of establishing a reference point in time when studying cell contraction, a problem that is overcome with our device.



Supplementary Figure 4. Integrin staining of cellular debris left behind after contraction. (a) Phase contrast image of a 3T3 fibroblast cell on a device before and after programmed release and subsequent cell contraction (magnification, 15x). The release electrode is indicated by the white arrows. During contraction, cellular material is left behind along the contraction trajectory. (b) Immunofluorescence images of α_V (Alexa fluor 488, green) and α_2 (Alexa fluor 568, red) integrins reveal their presence in the debris (magnification, 60x). Images were obtained approximately 30 minutes after programmed sub-cellular release (cell began to re-spread) of the cell in (a) using Alexa fluor 488 conjugated antibodies to the integrin sub-units. (c) Phase contrast image of a 3T3 fibroblast cell before and after spontaneous cell contraction on glass (magnification, 15 x). (d) Immunofluorescence image of α_V (Alexa fluor 488, green) and α_2 (Alexa fluor 568, red) integrins show their presence in the debris (magnification, 60x).

Cellular material, or debris, is left behind on a substrate during normal cell locomotion. This material often contains integrins[4,5]. Debris is also observed along the contraction trajectory after

programmed sub-cellular release and after spontaneous cell contraction on glass (Supplementary Figs. 4a and 4c). In both cases the debris was found to contain the integrin sub-units α_v and α_2 (see Supplementary Methods). The integrin sub-unit α_v commonly binds to RGD receptors and the α_2 integrin sub-unit is associated with collagen receptors[6]. The presence of both α_v and α_2 integrins in the debris suggests that attachments are not limited to RGD-integrin interactions.



Supplementary Figure 5. Live cell imaging of lifeact-GFP actin during release. (a) Overlay of phase contrast and fluorescent lifeact-GFP (green) images of a 3T3 fibroblast cell spanning two electrodes. (b) Fluorescent lifeact-GFP images of the region of the cell in (a) indicated by the white box labeled "b". This panel verifies that actin filaments do not photobleach in the 82 seconds following cell release and contraction. (c) Phase contrast and (d) fluorescence images of the region of the cell in (a) indicated by the white boxes labeled "c" and "d". This panel reveals that the actin filaments contract prior to global cell contraction. All bars, 5 μm. (e) Normalized cell contraction curve with t_0 = 65 s and τ = 91 s. (f) Normalized actin filament contraction curve with t_0 = 38 s and τ = 95 s.

Supplementary Methods

Programmed Sub-Cellular Release: Device Fabrication

The devices for sub-cellular release were fabricated on 50 x 75 mm glass slides. E-beam evaporation was used to deposit a 25 nm thick gold film with a 10 nm chromium adhesion layer onto the glass slides. For high resolution imaging, No. 1 glass coverslips were used and the thickness of the film was decreased to 15 nm gold and 5 nm chromium to increase the transparency. Due to the small currents associated with desorption, the IR drop is negligible. Optical lithography was used to pattern 10 μm and 5μm wide gold lines with 20 μm, 15 μm and 10μm spacing.Each line terminated in a contact pad. Copper wires were bonded (silver epoxy, SPI supplies, West Chester, PA) to the contact pads terminating each line, allowing each line to be individually addressable. For release experiments the device was assembled into an electrochemical cell with a platinum counter electrode and a chloridized silver wire reference electrode (A&M Systems, Carlsborg, WA).

RGD-thiol Synthesis

The gold lines on the device were functionalized with an RGD-terminated thiol synthesized by reacting a cyclo-RGD with a succinimide-terminated thiol. Cyclo(Arg-Gly-Asp-D-Phe-Lys), RGD, was purchased from Peptides Int'l (Louisville, KY), stored in 2mM aliquots in DMSO at -20˚C. The peptide was used within two weeks. Dithiobis(succinimidylundecanoate), NHS-thiol, was purchased from Dojindo (Gaithersburg, MD) and stored in 2 mM aliquots in DMSO at -20˚C. Both solutions were prepared and aliquoted in a glove box as the subsequent reactions are moisture sensitive. Prior to functionalizing the gold lines, 1.5 μL concentrated triethylamine (Fischer, Scientific Fairlawn, NJ) was added to a 150 μL aliquot of the RGD peptide. The sample was mixed for a few minutes to ensure that all the primary amines on the lysine amino acid were unprotonated. The RGD peptide was then added to 150 μL NHS-thiol and vortexed for 4 - 6 h.

Polyethylene Glycol Functionalization of Glass

In some experiments it is useful to modify the glass with polyethylene glycol to control cell spreading and reduce focal adhesions on the glass. Polyethylene glycol-modification of the glass can be accomplished by immersing glass slides containing gold electrodes in 73 μl 2-

[Methoxypoly(ethyleneoxy)propyl]trimethoxysilane (Gelest, Tullytown, PA) in 50 ml toluene and 40 μl conc. hydrochloric acid. After at least 24 hours at room temperature the devices were carefully rinsed in toluene and ethanol. The gold on these devices was then functionalized with RGD-thiol as described below.

RGD Functionalization of Gold

The devices were incubated with the RGD-terminated thiol for 1 - 2 h and then rinsed and sonicated for 5 min in DMSO (J.T. Baker, Phillipsburg, NJ), and then sequentially rinsed in ethanol (Warner Graham Co., Cockeysville, MD) and PBS (Dubecco's phosphate buffered saline, Gibco, Grand Island, NY) to remove all non-specifically adsorbed RGD-thiol. Thiols are chemisorbed to gold but do not have a strong affinity for glass, hence the functionalization step results in RGD-thiols chemically adsorbed to the gold while the glass is unfunctionalized. 2 mL PBS was added to the device such that the reference and counter electrodes were submerged. As a negative control, cyclo(Arg-Ala-Asp-D-Phe-Lys), RAD, was purchased from Peptides Int'l and used in the exact same manner as the RGD. Cells plated on RAD-thiol functionalized devices, did not appear healthy and the majority of the cells did not release.

Before each cell release experiment, a cyclic voltammogram was obtained from one of the electrodes to verify the functionalization procedure was successful (see Supplementary Figure 1a). Cyclic votammetry was performed using a potentiostat (PAR 263A, Princeton Applied Research, Oak Ridge, TN) by scanning the potential between 0 V and -1.4 V (Ag/AgCl), at 100mV s^{-1}. The device is then rinsed with PBS and sterilized for use in cell culture by spraying lightly with ethanol.

Cell Culture

NIH-3T3 mouse fibroblast cells (ATCC, Manassas, VA) were cultured in modified media (ATCC, Dubecco) supplemented with 10% bovine calf serum (ATCC) and 1% penicillin/streptomycin (Sigma, St. Louis, MO). Cells were passed by washing in PBS and trypsinizing (Sigma). Experiments were conducted with cells between passages 3 and 20. Cells were plated on a device 12-18 h prior to release experiments. Release experiments were conducted in a chamber (Live Cell, Pathology Devices, Westminster, MD) with 5% CO_2, 50 - 75% humidity, and at 37˚C, mounted on an inverted microscope (TE2000-E, Nikon). Cell

release from a specific electrode was achieved by applying -1.3 V with respect to the Ag/AgCl reference electrode.

Cell Transfection

3T3 fibroblast cells were trypsinized and plated in a 6 well dish to about 80% confluency. Cells were incubated with 10 μl cationic-lipid based transfection agent Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 4 μg GFP-paxillin plasmid (Promega midiprep kit, Madison, WI) in Optimem (Invitrogen) for about 16 h. The transfection media was then changed and 1 - 4 days later the cells were trypsinized and re-plated on EPSR devices. A similar procedure was followed for the lifeact-GFP actin with the exception that 8 μl Fugene (Roche, Basel Switzerland) was added to 2 μg lifeact-GFP plasmid in Optimem. To enhance the clarity of the focal adhesions in the GFP-paxillin experiments, the images in Figure 3 panel (b) were modified using Nikon software with the intensity transformation set to multiply by 2.

Programmed Sub-Cellular Release: Image Acquisition and Analysis

Time lapse images were acquired using NIS-Elements software (Nikon) at 1 s intervals for the duration of the release using 10-15X magnification. High resolution images were obtained using a 1.40 NA objective with 60X magnification. Images were analyzed using the same software and the change in cell length was measured with respect to the initial position and normalized to the maximum change in length. The area of the cell was determined by tracing the outline of the cell in different time frames. The release curves were fit to the equation: $\Delta L(t)/\Delta L_m = 1-\exp\left[-\left(t-t_0\right)/\tau\right]$ from which values for the induction time and contraction times were extracted using IGOR PRO software. The induction and contraction times from different experiments were analyzed by one-way ANOVA statistics, with a significance level of 0.05 using Origin software.

Molecular Inhibition Studies

Cell release studies under drug treatment were conducted in the same manner as the control release experiments with the exception that prior to the experiment, drugs (all from Sigma) were added to the media and incubated for a certain amount of time. The table below gives the relevant information for the drug studies:

Inhibitor	Supplier	Incubation time prior to experiment
2.5 μM-50 μM Blebbistatin	Sigma	45 min
0.3 μM Latrunculin B	Sigma	30 min
20 μM ML-7	Sigma	45 min

Immunofluoresence microscopy

Immunofluoresence microscopy was performed by fixing cells with 3.7% formadehyde (Fischer Scientific) in PBS for 1 h, rinsing with wash buffer (2.5% fetal calf serum (ATCC) in PBS with 10 mM glycine (Sigma)), permeablizing the cell membrane by adding 0.1% Triton X-100 (Alfa Aesar, Ward Hill, MA) for 10 min, rinsing with wash buffer and blocking with blocking buffer (10% fetal calf serum in PBS) for at least 20 min. 40:1 blocking buffer to monoclonal anti-vinculin-FITC antibody produced in mouse (Sigma) was added for 1 h and cells were then rinsed with wash buffer. A solution of 40:1 blocking buffer, phalliodin 568 (Invitrogen, Carlsbad, CA), and goat anti mouse Alexafluor 488 (Invitrogen) and 300 nM DAPI (Invitrogen) were added to the cell and incubated for 1 hour. Cells were rinsed with wash buffer, preserved with Prolong, (Invitrogen), a glass coverslip and nail polish. Immunofluorescence images were acquired on the inverted microscope using NIS-elements software. Fluorescent images were acquired with exposure times of 30 ms (Cy3), 100 ms (DAPI), and 1000 ms (FITC) with a 1.40 NA objective with 60x magnification. Focal adhesion images were analyzed by zooming in and then tracing the outlines of cell and focal adhesions and using NIS-elements software to determine the area.

Immunofluorescence microscopy (integrin staining) of cellular debris

Programmed sub-cellular release experiments were conducted as described above. If the cell left behind a substantial amount of debris that appeared, the device was fixed with 3.7% formaldehyde (Fischer Scientific) in PBS for 1 h, rinsing with wash buffer (2.5% fetal calf serum (ATCC) in PBS with 10 mM glycine (Sigma)), permeablizing the cell membrane by adding 0.1% Triton X-100 (Alfa Aesar, Ward Hill, MA) for 10 min, rinsing with wash buffer and blocking with blocking buffer (10% fetal calf serum in PBS) for at least 20 min. 50:1 blocking buffer to polyclonal goat anti-α_V integrin (sc-6616, Santa Cruz Biotech., Santa Cruz, CA) and polyclonal rabbit anti-α_2 (sc-9089, Santa Cruz Biotech.) was added for 1 h and cells were then rinsed with

wash buffer. A solution of 100:1 blocking buffer to secondary antibodies for goat (Alexafluor 488 donkey anti-goat, Invitrogen) and rabbit (Alexa fluor 568 donkey anti-rabbit, Invitrogen) were added to the cell and incubated for 1 hour. Cells were rinsed with wash buffer, preserved with Prolong (Invitrogen), a glass coverslip and nail polish. Immunofluorescence images were acquired on the inverted microscope using NIS-elements software. Fluorescence images were acquired with an exposure time of 100 ms (Cy3 and FITC) with a 1.40 NA objective and 60x magnification.

Spontaneous cell contraction of cells plated on glass (see *Cell Culture*), were obtained by using the multipoint feature of the Nikon software to capture different groups of cells moving on the glass. Images were acquired at 5 minute intervals for approximately 2 - 3 hours. During this time some of the cells contracted and left behind cellular material. At the end of the experiment the cells were fixed and stained for the integrin antibodies described above.

Supplementary Movies. These phase contrast movies were acquired with an inverted microscope such that the cells are viewed from underneath the device. At t = 0 a voltage pulse is applied to the indicated electrode resulting in release of the RGD-terminated thiols. The speed of each movie is increased ten times.

References

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