Triggering cell detachment from patterned electrode arrays by programmed subcellular release

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Programmed subcellular release is an *in vitro* technique for the quantitative study of cell detachment. The dynamics of cell contraction are measured by releasing cells from surfaces to which they are attached with spatial and temporal control. Release of subcellular regions of cells is achieved by plating cells on an electrode array created by standard microfabrication methods. The electrodes are then biochemically functionalized with an arginine-glycine-aspartic acid (RGD)-terminated thiol. Application of a voltage pulse results in electrochemical desorption of the RGD-terminated thiols, triggering cell detachment. This method allows for the study of the full cascade of events from detachment to subsequent subcellular reorganization. Fabrication of the electrode arrays may take 1–2 d. Preparation for experiments, including surface functionalization and cell plating, can be completed in 10 h. A series of cell release experiments on one device may last several hours.

INTRODUCTION

Programmed subcellular release triggers detachment of distinct parts of a cell from a patterned substrate in a spatially and temporally controlled manner[1]. Subcellular release is accomplished by plating cells on a device with an array of gold electrodes, typically 1–10 μm wide. An adhesion-promoting arginine-glycine-aspartic acid (RGD) peptide sequence[2] is attached to the gold electrodes by a thiol (Au-S-R) linkage (**Fig. 1**). Detachment of specific regions of an adherent cell is triggered by applying a sufficiently negative voltage pulse, resulting in rapid release of the RGD-terminated thiol[3]. The release process is an electrochemical reaction involving reductive desorption of the thiol ($Au\text{-}S\text{-}R + H^+ + e^- \rightarrow Au + HS\text{-}R$). Reductive desorption has been used to release molecules[4–6], fluorescently labeled molecules[3], nanoparticles and proteins[7]. The regions on the glass slide between the electrodes can be modified with polyethylene glycol (PEG) to minimize focal adhesion formation[8,9]. Each stripe is electrically isolated so that the RGD-terminated thiols from a single electrode can be desorbed independently of adjacent electrodes. This design enables the release of a subcellular section of an adherent cell spanning multiple electrodes.

The steps involved in performing subcellular release experiments are as follows. (i) Microfabrication of an array of individually addressable gold electrodes on a glass slide using conventional photolithographic techniques. Electrical contact is made by attaching a wire to a contact pad at the end of each electrode. (ii) Biochemical functionalization of the gold electrode array by immersing the slide into a solution containing RGD-terminated thiol. The glass surface may also be chemically functionalized with PEG. (iii) Plating cells that will span multiple electrodes. (iv) Recording phase-contrast or fluorescence time-lapse movies of cells released under live cell conditions (37 °C, 5% (vol/vol) CO_2, at least 75% humidity). (v) Analyzing cell contraction on phase-contrast images by measuring cell length (or area) and fitting the data to $\Delta L(t)/\Delta L_m = 1 - \exp(-(t-t_0)/\tau)$, where $\Delta L(t)$ is the change in cell length at time t (that is $L_{inital} - L(t)$) divided by the maximum change in cell length ($L_{inital} - L_{final}$). The induction time before retraction of the cell, t_0, and the characteristic contraction time, τ, are two parameters extracted from fitting the data.

Cell types showing a polarized morphology with few cell-cell adhesions are most suitable for programmed subcellular release. Examples of cells released by this method in our laboratory include NIH 3T3 fibroblasts[10], mouse embryonic fibroblasts[11], AG04151 (human skin fibroblasts), HT1080 (human fibrosarcoma cells)[12] and MDA-MB-231 (human breast cancer cells)[13]. Epithelial and endothelial cells tend to form small clusters when plated on surfaces[14,15] and do not release readily from our micropatterned electrodes. Cells with a circular morphology and cells that form multiple nuclei, such as U2OS (osteoblast) cells, do not release well or are not viable after release.

There are very few methods to study cell detachment. Other methods are based on cleavage of actin stress fibers or pharmacological treatment. Laser ablation has been used to cut stress fibers in cells transfected with yellow fluorescent protein–labeled G-actin, allowing quantitative analysis of cell contraction[16]. Chromophore-assisted laser inactivation of fluorescently labeled α-actinin has been used to observe cell contraction after detachment of stress fibers from focal adhesions[17]. These techniques can be used to study the dynamics of cell contraction but do not allow investigation of the full cascade of events from the detachment of integrins from the extracellular matrix to the reorganization of actin filaments and focal adhesion proteins. In a pharmacological approach, nocadozole has been used to induce bulk microtubule disassembly, such that subsequent washout allows visualization of microtubule targeting of focal adhesion disassembly[18]. A drawback of this method is that it can lead to uncontrolled off-target effects.

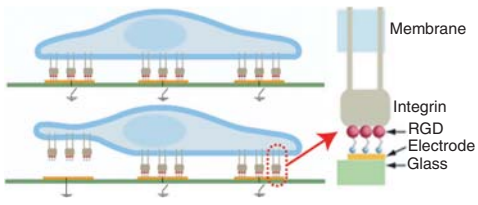

Figure 1 | Schematic illustration of the concept of programmed subcellular release. A cross-section of a cell on an electrode array functionalized with RGD-thiol molecules. The integrins of the cell bind to the RGD, promoting cellular attachments on the gold electrodes. Application of a sufficiently negative voltage pulse causes electrochemical desorption of the thiol molecules, which results in the cleavage of the gold-thiol bond.

Programmed subcellular release overcomes many of the limitations associated with existing methods. Triggering cell detachment by the release of the RGD-terminated thiol is sufficiently upstream to study the full cascade of events involved in cell detachment. Using a fluorescent probe, we have shown that electrochemical desorption of thiols occurs on time scales less than a few milliseconds[3], much faster than processes associated with cell detachment and contraction. Spatial control is achieved, for a cell that spans multiple electrodes, by triggering detachment from one electrode. For example, an NIH 3T3 fibroblast spanning three electrodes can be released by one of the outermost electrodes. Programmed subcellular release has also been shown to mimic the spontaneous cell contraction or 'tail snap' of cells moving on a glass surface[1,19].

Programmed subcellular release can be combined with other techniques, such as immunofluorescence microscopy, pharmacological inhibition studies and real-time live cell imaging of fluorescently labeled proteins to study biochemical and biophysical aspects of cell detachment. Immunofluorescence staining and statistical analysis of ensemble averages can be used to study the effects of cell detachment on focal adhesions[20] and cytoskeletal proteins[21]. Molecular inhibition studies can be performed to deduce the role of a particular molecule or protein in the signaling cascade[22,23]. For example, cells incubated with 50 μM blebbistatin, a drug that inhibits myosin II and is known to influence cell contractility[24,25], result in more than a 100-fold increase in contraction time. Real-time live cell imaging provides a method to probe the kinetics of detachment[26]. For example, we have imaged the dynamics of actin stress fibers during contraction by transfection with GFP-labeled actin[27,28]. Programmed subcellular release can also be used as a versatile tool to study the mechanical properties of normal and diseased cells[11,29] and could be readily combined with complementary cell-mechanics methods, including particle-tracking microrheology[30]. The ability to combine programmed subcellular release with other tools and techniques provides new opportunities to study cell motility, embryonic development, the inflammatory response, wound healing and metastasis of cancerous cells.

Both device fabrication and surface modification are straightforward. Device fabrication involves standard microfabrication methods. The RGD-thiol coupling chemistry and surface functionalization are undemanding and all reagents are commercially available. The live cell chamber on the microscope may need to be modified to accommodate the electrode connections.

MATERIALS

REAGENTS

- Cyclo(Arg-Gly-Asp-D-Phe-Lys) (cyclo RGDfK; Peptides International), 150-μl vials stored in 2 mM aliquots in DMSO at −20 °C (stored and used for up to 2 weeks)
- Dithiobis(succinimidyl undecanoate) (NHS-thiol) was purchased from Dojindo Molecular Technologies and 150 μl was stored in 2 mM aliquots in DMSO at −20 °C (stored and used for up to 3 months)
- Dimethyl sulfoxide (DMSO; Mallinckrodt Baker, ACS reagent grade, <0.1% water, wt/wt)
- Triethylamine (Fisher Scientific) **! CAUTION** Irritant to eye and respiratory tract. Wear gloves, goggles and use a properly ventilated chemical hood when handling.
- 2-[Methoxypoly(ethyleneoxy)propyl]trimethoxysilane (Gelest) (shelf life of about 6 months) **! CAUTION** Irritant to eye, skin and respiratory tract. Wear gloves, goggles and use a properly ventilated chemical hood when handling.
- Toluene **! CAUTION** Flammable. Irritant to eye, skin and respiratory tract. Wear gloves, goggles and use a properly ventilated chemical hood when handling.
- Hydrochloric acid **! CAUTION** Poison! Corrosive. Liquid and mist cause severe burns to all body tissue. May be fatal if swallowed or inhaled. Inhalation may cause lung damage. Wear gloves, goggles, laboratory coat and use a properly ventilated chemical hood when handling.
- Ethanol
- Acetone **! CAUTION** Irritant and flammable. Wear gloves and goggles when handling.
- Isopropanol
- PBS (Dulbecco's phosphate-buffered saline; Gibco)
- Cell line, such as NIH 3T3 mouse fibroblast cells (ATCC)
- Cell culture medium (Dulbecco's modified medium; ATCC) supplemented with 10% (vol/vol) bovine calf serum (ATCC) and 1% (vol/vol) penicillin/streptomycin (Sigma)
- Trypsin (Sigma)
- Opti-MEM (Invitogen)
- Lipofectamine 2000 (Invitrogen)
- FuGENE (Roche Applied Science) **! CAUTION** Highly flammable. Tightly seal and avoid ignition sources.
- 37% (vol/vol) formaldehyde (Fisher Scientific) **! CAUTION** Irritant to eye and skin. Wear goggles and gloves when handling.
- 0.1% (wt/vol) Triton X-100 in PBS (Alfa Aesar)
- Wash buffer (2.5% (vol/vol) fetal calf serum; ATCC) in PBS
- Blocking buffer (10% (vol/vol) fetal calf serum in PBS)
- Monoclonal fluorescein isothiocyanate(FITC)–anti-vinculin antibody produced in mouse (Sigma-Aldrich)
- Alexa Fluor 568 phalloidin (Invitrogen)
- DAPI (Invitrogen)
- ProLong Antifade (Invitrogen)
- DNA plasmids encoding green fluorescent protein (GFP)-tagged paxillin
- Lifeact-GFP
- Silver epoxy (SPI Supplies) **! CAUTION** Irritant to skin; wear gloves when handling.
- CD-26 photolithography developer (Shipley) **! CAUTION** Alkaline liquid and vapor. Irritant to skin, eye and respiratory tract. Onset of symptoms may be delayed. Prolonged, repeated contact, inhalation, ingestion or absorption through the skin may cause toxic effects to internal organ systems. Use in a properly ventilated chemical hood.
- Shipley S1813 photoresist (Shipley) **! CAUTION** Irritant to eye, skin and respiratory tract. Combustible. Prolonged exposure may damage internal organs. Wear goggles, gloves and laboratory coat when handling. Handle only in a properly ventilated chemical hood.

EQUIPMENT

- Photolithography facilities (evaporator, UV lamp, mask containing electrode design, glass slides)
- Gold and chromium sources for evaporation
- Potentiostat (for example, VersaStat 3; Princeton Applied Research)
- V-3 Studio software (Applied Research)
- Cell chamber with 5% CO_2, 50–75% humidity and at 37 °C (LiveCell; Pathology Devices)
- Phase-contrast inverted microscope, ×10 and ×60 objectives
- Digital CCD camera capable of acquiring time-lapse movies (for example, Hamamatsu Orca-4E)
- Image analysis software (for example, Nikon, NIS-Elements)
- Cell release device chamber components (see **Fig. 2**)
- Counter electrode (for example, platinum wire; Alfa Aesar)
- Chloridized silver wire reference electrode (A&M Systems)
- Cell incubator
- Glass slides (Fisherbrand 45 × 50 No. 1 slides)
- Eppendorf tubes

REAGENT SETUP

The volume of reagent required must be sufficient to submerge the electrode array in the well used for subcellular release. The volumes noted in this protocol are based on a well with an o-ring diameter of 19 mm (see **Fig. 2**). About 150 μl of 2 mM cyclo RGDfK and 150 μl of 2 mM NHS-thiol solutions should be prepared in DMSO under argon. Aliquots are stored in 0.5-ml Eppendorf tubes at −20 °C as reaction of the succinimide group to the side chain amine of the lysine is moisture sensitive.

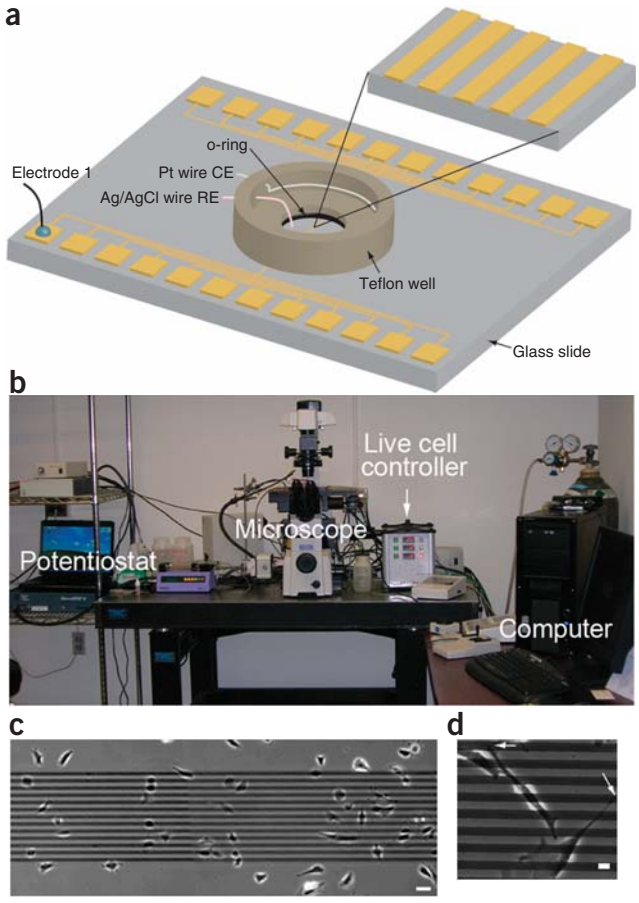

Figure 2 | Experimental setup for programmed subcellular release. (**a**) Schematic illustration of a subcellular release device comprising the electrode array and Teflon well. (**b**) Microscope, live cell chamber, potentiostat and computer. (**c**) Phase microscopy images of NIH 3T3 fibroblast cells on an electrode array. Bar, 50 μm. (**d**) Higher magnification phase microscopy images of NIH 3T3 fibroblast cells. The white arrows show ideal electrodes for release in which cells terminate on the electrode. Bar, 10 μm.

PROCEDURE

RGD-thiol preparation ● TIMING >4 h

1| Add 1.25 μl triethylamine to a vial of 150 μl of 2 mM cyclo RGD peptide and mix by vortexing for a few minutes. Add 150 μl NHS-thiol and vortex for at least 4 h.

▲ **CRITICAL STEP** Reproducible results are achieved when the RGD solution (stored at −20 °C) is used within 2 weeks and the NHS-thiol solution (stored at −20 °C) is used within 2 months. The DMSO should have a low water content to minimize hydrolyzation of the succinimide group (side reaction). Note that smaller preweighed aliquots of NHS-thiol can be obtained from ProChimia Surfaces (Poland). Preconjugated linear RGD-PEG-thiol (ProChimia Surfaces) may be stored in powder form and dissolved in DMSO before use. The remaining powder should then be flushed with nitrogen gas, sealed tightly and stored at −20 °C in an oxygen-free environment.

Note: control studies can be conducted by substituting cyclo RADfK (Arg-Ala-Asp-D-Phe-Lys) for cyclo RGDfK here and in the reagent preparation. RAD is a negative control for RGD[1,31].

Device preparation ● TIMING half a day depending on equipment and availability

2| The gold electrode arrays for subcellular release are fabricated using common photolithography techniques. The key requirements for the electrode array are (i) the electrodes are individually addressable, (ii) the gold film is transparent (we suggest a 7-nm chromium adhesion layer and a 15–35-nm gold layer) and (iii) the cells span multiple electrodes. The wide distribution in cell size among different cell lines can be accommodated by adjusting the width and spacing of the individual electrodes. An array of parallel lines is optimum for most applications; however, the electrode geometry can also be varied if necessary. Fabrication using the liftoff method is recommended as it is difficult to ensure that residual photoresist is completely removed from the gold film when using the etch-back method. Residual photoresist or other contamination of the gold electrodes can result in poor quality monolayers and difficulty with release.

▲ **CRITICAL STEP** Inspect electrode array and contact pads for defects under a microscope.

Optional functionalization of glass with polyethylene glycol ● TIMING 1–2 d (if desired)

3| Clean electrode array by rinsing in acetone and isopropanol to remove any dust or organics that may have accumulated since fabrication. If pegylation of the glass is necessary, add 73 μl of 2-[methoxypoly(ethyleneoxy)propyl]trimethoxysilane to 50 ml toluene and 40 μl concentrated hydrochloric acid.

4| Immerse electrode array in solution and cover for 24–48 h. Rinse and sonicate in ethanol for about 30 s.

5| Clean slides by rinsing in acetone and isopropanol to remove any dust or organics that may have accumulated since fabrication. Make electrical connections to the contact pads (as shown in **Fig. 2b**) using a drop of premixed conductive epoxy. Tape can be used to hold the wires in place temporarily. Remove tape after epoxy has dried, approximately 5 h ● TIMING **5 h**

6| Assemble the device by attaching the well to the glass slide containing the electrode array using an o-ring to ensure a good seal (see **Fig. 2a**). We suggest machining a well from Teflon. The well serves as an electrochemical cell and a cell

culture dish. The o-ring can be located over the electrode array before attaching and securing the well. The well contains the cell culture medium along with the counter electrode (for example, a platinum wire) and reference electrode (for example, a chloridized silver wire). One of the gold electrodes serves as the working electrode in a three-electrode electrochemical cell arrangement. The platinum wire counter electrode should have a submerged area that is at least 10 times larger than the area of one of the electrodes. The tip of the chloridized silver wire reference electrode should be located about a few millimeters above the glass slide. The reference electrode and counter electrode should not be in contact with each other or with any of the electrodes in the electrode array ● TIMING **5 min**

▲ CRITICAL STEP The device components (electrode array and Teflon well) should be cleaned by rinsing extensively in ethanol before and after each experiment. Periodically, the Teflon well should be cleaned by soaking in a solution of sodium hypochlorite diluted with water for a few hours and then rinsed extensively in DI water and ethanol. This cleaning is performed to prevent bacterial contamination and also to remove residual proteins and organics from previous experiments.

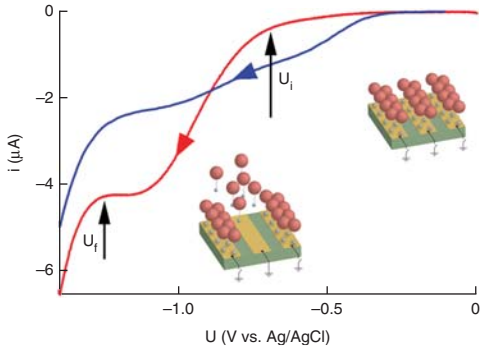

Figure 3 | Cyclic voltammograms of electrodes. RGD-terminated thiol on a single electrode (PBS, 7.4, 100 mV s^{-1}) (red). U_i indicates the potential at which the thiol desorption begins and U_f indicates the end of the thiol desorption process. The thiol monolayer blocks current flow from 0 V to approximately −0.7 V. A characteristic desorption peak (or shoulder) is seen at about −1.2 V. A cyclic voltammogram for a bare gold electrode is shown for comparison (blue).

7| Immerse the electrode array in the well in 300 μl of 1 mM RGD-thiol and incubate for 1–2 h. Ensure that the RGD-thiol solution does not contact the chloridized silver wire reference and platinum counter electrode wires as thiol molecules bind to silver and platinum ● TIMING **1–2 h**

8| After incubation in the RGD-thiol solution, disassemble the device and rinse the electrode array extensively with DMSO and ethanol, and then dry with nitrogen. Rinse the Teflon well extensively with ethanol to remove residual DMSO and dry with nitrogen gas. Reassemble the electrode array and Teflon well using a new viton o-ring rinsed with ethanol (for best results as cells are very sensitive to organic contamination) and add enough PBS to submerge the reference and counter electrodes ● TIMING **5 min**

9| Test the surface functionalization with the RGD-terminated thiol by recording a current-voltage curve (also known as a cyclic voltammogram) for one of the gold electrodes using a suggested potential range of 0 to −1.4 V at a scan rate of 100 mV s^{-1} while measuring the current response. The current–voltage curve should be similar to that shown in **Figure 3** for an electrode functionalized with the RGD-terminated thiol. After recording the current-voltage curve, rinse the electrode array with the device assembled, once with PBS ● TIMING **10 min**

▲ CRITICAL STEP The voltammogram for an RGD-thiol monolayer on an electrode should show (i) a current onset at about −0.7 V, several hundred mV negative for a bare gold electrode and (ii) a well-defined peak or shoulder at about −1.2 V (versus the Ag/AgCl reference). These features are shown in **Figure 3**. One electrode in each device can be used to verify monolayer formation before plating cells. To perform real-time, live cell imaging of fluorescent cellular proteins during cell detachment experiments, it is recommended to see **Box 1**.

? TROUBLESHOOTING

Plating cells on device ● TIMING 20 min

10| Remove cells from T75 cell culture flask by rinsing with 10 ml PBS and adding 1–2 ml trypsin. (A PBS wash is required to remove residual serum from the cell surface so that the trypsin hydrolysis reaction will be most effective.) After cells have detached from the flask, add medium containing serum to quench trypsin reaction. If subcellular release experiments are conducted in serum-free conditions, a centrifugation step may be used to wash the cells (suggested parameters: 2,000 r.p.m. for 5 min).

? TROUBLESHOOTING

11| Add 600 μl medium containing suspended cells to the device. Incubate cells at 37 °C, 5% (vol/vol) CO_2 and high humidity ● TIMING **4–18 h**

▲ CRITICAL STEP Cell release has been achieved for cells incubated for 4–18 h. The plating time may influence the global cell contraction kinetics.

BOX 1 | REAL-TIME, LIVE CELL IMAGING OF FLUORESCENT CELLULAR PROTEINS ● TIMING 2D

Live cell imaging can be performed by transiently transfecting cells with DNA plasmids that encode a GFP-labeled protein of interest and replating cells on release device 12–36 h after transfection. Cells can be released as described by this protocol with the consideration that many GFP-labeled proteins (with the exception of Lifeact-GFP actin) photobleach within seconds. Therefore, the exposure time and frequency of image acquisition must be adjusted accordingly. Insert the following steps between Steps 9 and 10 in the standard protocol.

1. Remove cells from the surface of a T-75 cell culture flask by adding 1.5 ml trypsin. After cells have detached from the flask, add 8.5 ml cell medium containing serum to quench trypsin reaction. Add 1.5 ml of these cells to a 25-mm cell culture dish. Add an additional 2.5 ml medium and incubate at 37 °C, 5% CO_2 and high humidity overnight.

Transfect cells according to transfection procedure. Two examples of typical transfection procedures for paxillin and actin are given:

2. GFP-Paxillin: after cells are fully spread, add 250 µl of Opti-MEM to two Eppendorf tubes. Add 10 µl lipofectamine to one tube and 4 µg DNA plasmids encoding green fluorescent protein (GFP)-tagged paxillin to the other tube. Gently mix by pipetting. Incubate 5 min. Add the tube containing lipofectamine dropwise to the tube containing the DNA, mix gently and incubate for 20 min. Gently add DNA solution to the cells.

Lifeact-GFP: add 100 µl of Opti-MEM to an Eppendorf tube. Gently add 2 µg DNA plasmids encoding Lifeact-GFP and mix. Add 8 µl FuGENE to the DNA by inserting the pipette tip containing the FuGENE directly into the solution, minimizing contact with the plastic vial. Chemical residues from the plastic vial can reduce the biological activity of the FuGENE. Mix gently by vortexing.

3. Remove medium containing transfection reagents after 6 h. Rinse cells in PBS and add fresh medium. The best time to view GFP-transfected cells is 24–48 h after transfection (minimum time is 16 h).

12| Perform Option B (below) if you would like to do inhibition studies, otherwise follow the steps in Option A.

(A) Without inhibition studies ● TIMING 5 min

(i) After cells have been incubated for the desired time, remove medium, rinse gently with PBS, and add enough fresh medium to cover the electrode array and the counter and reference electrodes (about 3 ml for the cell release well shown in **Fig. 2a**).

(ii) Arrange the reference and counter electrodes as described in Step 2.

(iii) Transfer the cell release device to the microscope stage in a live cell chamber (see **Fig. 2b**) at 37 °C and high humidity with 5% CO_2.

(B) Inhibition studies ● TIMING 30 min–4 h

(i) After cells have been incubated for the desired amount of time, remove medium, rinse gently with PBS and add fresh medium containing the desired concentration of molecular inhibitor to the well. Ensure that the electrode array, counter electrode and reference electrode are submerged.

(ii) Incubate cells for the appropriate amount of time before continuing with the cell release experiment procedure (Steps 11–14). Note that the experimental conditions, such as the frame rate and imaging time, may need to be adjusted as the molecular inhibitor may change the release dynamics (induction time and contraction time).

Releasing cells using time-lapse microscopy ● TIMING 10 min

13| Using phase contrast mode, focus the microscope at the desired magnification (typically ×60 objective), and select a cell for release (release is most readily accomplished on electrodes on which a cell terminates; see white arrows in **Fig. 2d**). Inspection of the electrode array before release (Step 2) is recommended to ensure the electrode is not broken. Connect the wire for this electrode to the working electrode of the potentiostat. The correct wire can be identified by counting the electrodes from one side under the microscope. Connect the reference and counter electrode wires to the potentiostat.

▲ **CRITICAL STEP** Cells must appear healthy before release. Healthy 3T3 fibroblasts typically spread well axially and show a smooth, regular membrane.

14| Using image acquisition software, such as Nikon NIS-Elements, you should program the camera to acquire phase contrast, time-lapse images at 1 frame per second.

▲ **CRITICAL STEP** To establish a baseline, you should capture images for several minutes before release. View images before release to verify that the cell is not contracting before the voltage has been applied.

15| Program the potentiostat to apply a potential of −1.3 V (versus the Ag/AgCl wire reference electrode) for up to 50 s. The electrode should be held at open circuit potential before and after the voltage pulse. The open circuit potential should be stable before release of the cell. Note that the potential of the reference electrode is dependent on the chloride ion concentration of the medium and will shift by 60 mV for an order of magnitude change in chloride ion concentration. This should not be a problem in most cases. If necessary use a double-junction Ag/AgCl electrode containing a reference solution (for example, BAS MF-2052) to avoid changes in medium composition. However, even small double-junction reference electrodes may be difficult to incorporate into the well that must fit in the live cell chamber (see **Fig. 2a**).

BOX 2 | IMMUNOFLUORESCENCE STAINING STUDIES ● TIMING 3.5–4H

These may be conducted in conjunction with cell release experiments by fixing and staining released cells after Step 16 as follows:

1. Following cell release, fix cells by adding 80 µl of 30% paraformaldehyde directly to cell medium on the microscope stage.
2. After 1 h, gently rinse cells with PBS. To stain for cytoplasmic proteins, permeabilize cell membrane by incubating cells in 0.1% Triton X-100 for 10 min.
3. Rinse cells with wash buffer three times.
4. Block cells with blocking buffer for at least 20 min (cells can be left overnight at this step).
5. Dilute primary antibody 1:40 (for example, monoclonal FITC–anti-vinculin antibody) in blocking buffer and add to cells for 1 h.
6. Wash cells three times in wash buffer.
7. Dilute any secondary antibodies, Alexa Fluor 568 phalloidin (1:40) and DAPI (1:100) in blocking buffer add to the cell and incubate for 1 h.
8. Wash cells three times in wash buffer.
9. Add a drop of ProLong Antifade solution to the cells and cover with a glass coverslip. Let solution dry before imaging cells with fluorescence microscopy.

16| Begin image acquisition and then initiate the potential pulse on the potentiostat. Record the time difference between the image acquisition and the potentiostat. Immunofluorescence staining studies may be conducted at this point (see **Box 2**) ● TIMING **3.5–4 h**
? TROUBLESHOOTING

Image analysis of cell release videos ● TIMING 1 h

17| Images may be analyzed by measuring the length of the cell along the contraction axis (see **Fig. 4a**) as a function of time using image analysis software (for example NIS-Elements). Experiments can be compared by plotting normalized cell contraction versus time, as shown in **Figure 4b**. The cell perimeter may also be traced and the normalized area may be plotted against time. In general, contraction dynamics are the same for cell length and cell area. The choice of cell length or area may depend on cell shape. For example, highly polarized 3T3 cells are most easily analyzed by measuring cell length along the contraction axis over time. In contrast, more circular, less polarized cells may be better analyzed by measuring the change in cell area over time.
? TROUBLESHOOTING

● TIMING
Microfabrication: 1–2 d depending on the number of devices and level of experience
Step 1, RGD-thiol: >4 h
Step 2, Electrode array fabrication: half a day depending on equipment and availability
Steps 3 and 4, Surface pegylation: 1–2 d (if desired)
Step 5, Electrode array preparation—wire contacts: 5 h (can be performed in parallel with Step 1)
Step 6, Assembled device preparation—assembly of electrode array and Teflon well: 5 min
Step 7, Assembled device preparation—surface functionalization: 1–2 h
Step 8, Assembled device preparation—rinsing: 5 min
Step 9, Assembled device preparation—test surface functionalization: 10 min
Box 1, Live cell imaging: at least 2 d
Step 10, Plating cells on assembled device—trypsinization of cells: 20 min
Step 11, Plating cells on assembled device—cell incubation: 4–18 h
Step 12A, Plating cells on assembled device—rinse: 5 min

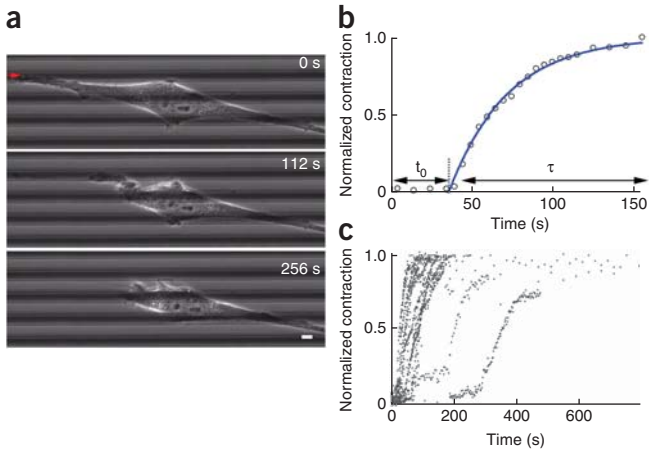

Figure 4 | Programmed subcellular release. (**a**) Typical release experiment of an NIH 3T3 fibroblast cell monitored under phase-contrast microscopy. A voltage was applied to an electrode (red arrow) at t = 0 s. After release of the cell from the uppermost gold line, there was an induction time before the cell began to contract. Magnification, ×60. Bar, 5 µm. (**b**) Plot of normalized cell contraction verse time for the cell shown in **a**. The induction time (t_0) and contraction time (τ) are indicated by arrows. The solid line is a fit to the equation $\Delta L(t)/\Delta L_m = 1 - \exp(-(t-t_0)/\tau)$, with t_0 = 37.1 s and τ = 34.2 s. (**c**) Plot of the normalized cell contraction curves for 22 cells.

Step 12B, Incubation of cells with molecular inhibitors: typically 30 min to 4 h (depends on inhibitor)
Steps 13–15, Cell release—identification of a cell to release: 10 min
Step 16, Cell release—release and contraction of cells: 15 min (as needed)
Box 2, Immunofluorescence staining: 3.5–4 h
Step 17, Image analysis: 1 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible solution
9	Cyclic voltammogram does not match that of **Figure 3**	The cyclic voltammogram should show (i) a current onset at about −0.7 V, several hundred mV positive of the current onset for a bare gold electrode, and (ii) a well-defined peak or shoulder at about −1.2 V (see **Fig. 3**). For small electrodes, reductive desorption peak is not as well defined as for a large electrode. Failure to observe these two features indicates improper formation of the thiol monolayer due to contamination or degradation of the chemicals. Contamination can be overcome by careful cleaning of the electrode array. Degradation of the RGD-thiol can be overcome by using fresh chemicals
10	Cells appear unhealthy or do not adhere to device	This suggests that the device may be contaminated. Every part of the device exposed to the RGD-thiol solution during the surface functionalization step must be rinsed extensively with ethanol to remove residual DMSO and excess thiol. Replacing the o-ring between the device and the chamber before culturing cells may be necessary
16	Cells do not contract after release of the RGD-terminated thiol	Cell release is most readily accomplished when a highly polarized cell terminates on the release electrode
	Bubbles form on the electrode during release	The applied potential is too large. At potentials negative to −1.5 V (Ag/AgCl), hydrogen evolution can occur resulting in bubble formation. Regular calibration of reference electrode wires is recommended; this can be accomplished by measuring the potential about a double-junction reference electrode and in a known solution
17	The plot of normalized cell contraction verse time does not fit the exponential model shown in **Figure 4**	Cell contraction curves for NIH 3T3 fibroblast cells may show multiple exponential contractions. This effect is usually associated with cells that span at least three electrodes where contraction slows down as the contracting cell crosses another electrode

ANTICIPATED RESULTS

An example of release of an NIH 3T3 fibroblast cell is shown in **Figure 4a**. The red arrow indicates the release electrode on which the thiol molecules have been electrochemically desorbed at t = 0 s. After a brief induction time, t_0 = 37.1 s, the cell begins to contract with a time constant τ = 34.2 s. The end-to-end distance of the cell along the contraction axis was measured and plotted as normalized contraction versus time as in **Figure 4b**. **Figure 4c** shows the normalized contraction for 22 cells with an average induction time of 57 ± 14 s and average contraction time of 39 ± 7 s.

Programmed subcellular release can be combined with other methods, such as immunofluorescence staining, molecular inhibition studies and real-time live cell imaging of fluorescently labeled proteins. An example of a cell that was released and stained for paxillin, actin and DAPI is given in **Figure 5**. **Figure 6** reveals that releasing cells in the presence of myosin II inhibitor, blebbistatin significantly increases both the induction and contraction times. Real-time live cell imaging of fluorescently labeled proteins

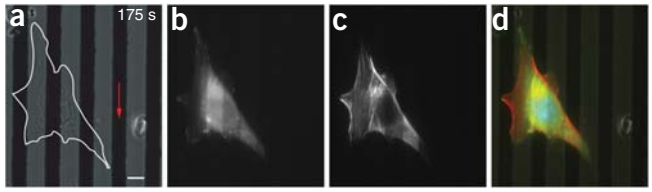

Figure 5 | Immunofluorescence staining of a cell during cell release. (**a**) Phase-contrast image of an NIH 3T3 fibroblast cell 175 s after release from electrode labeled with red arrow. (**b**) Fluorescence microscopy image of the cell in panel **a** stained with FITC–anti-vinculin. (**c**) Fluorescence microscopy image of the cell in panel **a** stained with phalloidin. (**d**) Overlaid fluorescence images of a fixed NIH 3T3 fibroblast spanning multiple electrodes (dark diagonal lines) stained for actin fibers (Alexa Fluor 568 phalloidin, red), vinculin (FITC–anti-vinculin, green) and cell nucleus (DAPI, blue).

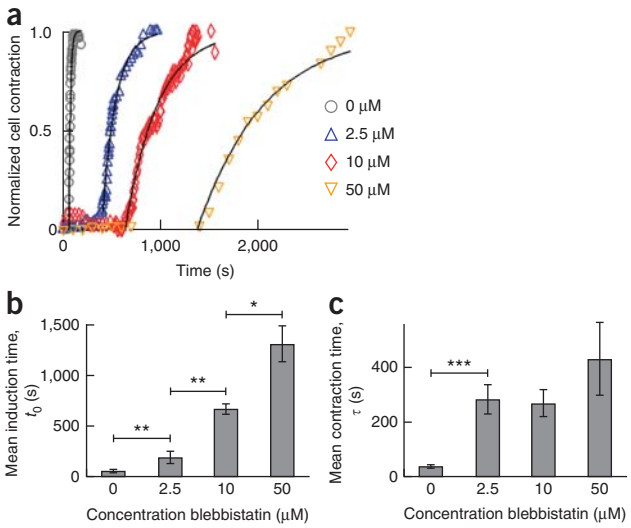

Figure 6 | Effect of blebbistatin concentration on cell contraction. Normalized cell contraction curves and mean induction and contraction times with 0 μM (n = 18), 2.5 μM (n = 4), 10 μM (n = 3) and 50 μM (n = 3) blebbistatin.

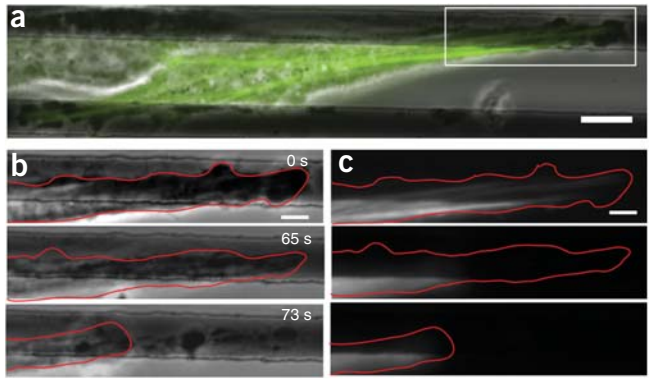

Figure 7 | Live cell imaging of Lifeact-GFP actin during release. (**a**) Overlay of phase-contrast and fluorescent Lifeact-GFP images of an NIH 3T3 fibroblast spanning two electrodes. (**b**) Phase-contrast and (**c**) fluorescent images during release, of the cell in panel **a** as indicated by the white box.

can be used to study the effects of cell release on cytoplasmic proteins such as actin and paxillin. **Figure 7** depicts a cell release experiment conducted on a cell transiently transfected with Lifeact-GFP actin.

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