

# Molecular dynamics imaging in micropatterned living cells

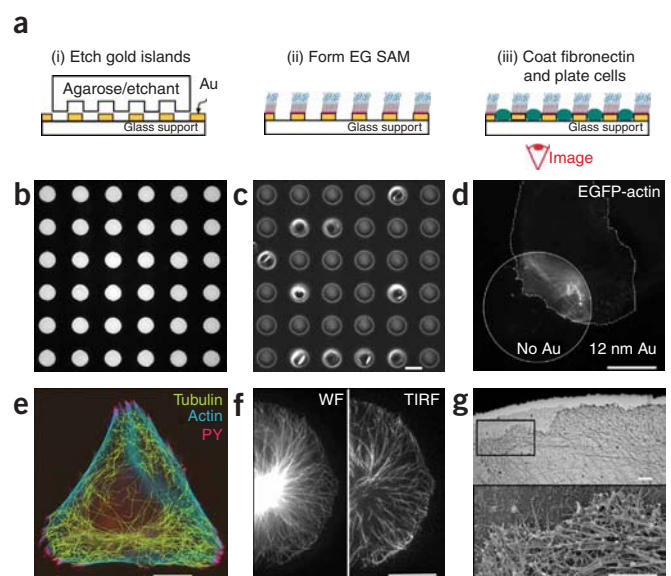
Kristiana Kandere-Grzybowska<sup>1</sup>,  
Christopher Campbell<sup>2</sup>, Yulia Komarova<sup>1</sup>,  
Bartosz A Grzybowski<sup>2</sup> & Gary G Borisy<sup>1</sup>

**Micropatterning approaches using self-assembled monolayers of alkyl thiols on gold are not optimal for important imaging modalities in cell biology because of absorption of light and scattering of electrons by the gold layer. We report here an anisotropic solid microetching (ASOMIC) procedure that overcomes these limitations. The method allows molecular dynamics imaging by wide-field and total internal reflection fluorescence (TIRF) microscopy of living mammalian cells and correlative platinum replica electron microscopy.**

In the most common approach for cell micropatterning—microcontact printing of self-assembling monolayers (SAMs)<sup>1,2</sup>—an elastomeric stamp made of poly(dimethyl siloxane) (PDMS) is used to pattern islands of methyl-terminated alkyl thiols onto gold. Remaining regions of the gold substrate are covered by a SAM of oligo(ethylene glycols) (EG SAM) that is resistant to cellular adsorption<sup>1,2</sup>. Cells adhere to the patterned islands and assume their shapes. This and related approaches have been used to

investigate the relationship between cell shape, extracellular matrix, growth and directional motility<sup>3–8</sup>. A major drawback of microcontact printing, however, has been its unsuitability for fluorescence imaging of molecular dynamics in living cells and for correlative electron microscopy. This is because the adhesive islands on which the cells reside are supported by a layer of gold, which attenuates the fluorescence signal and scatters electrons. Here we demonstrate how this limitation can be overcome by patterning cells on substrates with transparent adhesive islands (that is, bare glass) surrounded by gold derivatized with an anti-adhesive EG SAM. Such substrates are fabricated by ASOMIC<sup>9</sup> that uses reaction diffusion processes<sup>10,11</sup> to chemically remove gold from an initially uniform layer into a micropatterned hydrogel stamp. We demonstrate that ASOMIC allows fluorescent imaging of living, geometrically defined cells as well as TIRF and electron microscopy. In particular, we demonstrate monitoring of microtubule dynamics using yellow fluorescent protein fusion with microtubule plus-end cytoplasmic linker protein-170 (YFP-CLIP170) as a marker<sup>12</sup>. Initial results with triangular cells suggest a pathway for guidance of microtubule growth.

We performed ASOMIC microetching (Fig. 1a) using agarose stamps patterned in bas relief with arrays of raised microfeatures—either circles or equilateral triangles. We prepared agarose stamps by casting hot degassed high-gel-strength agarose against oxidized PDMS templates (Supplementary Methods online). For etching, we placed stamps feature-side-down unto gold-coated glass



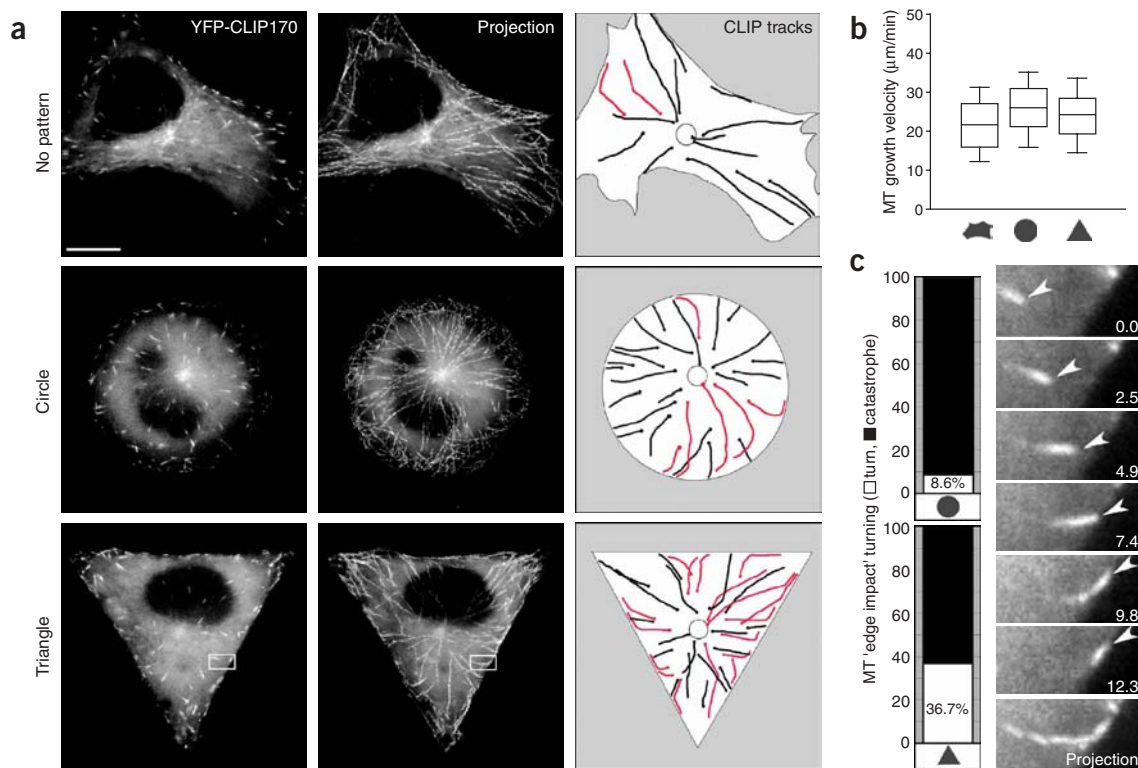
**Figure 1** | ASOMIC method allows for cell micropatterning compatible with fluorescence imaging and electron microscopy. **(a)** Cell patterning using ASOMIC. **(b)** Bright-field image shows etched circles (diameter 40  $\mu\text{m}$ ; spacing 80  $\mu\text{m}$ ) in white (no gold) and remaining gold in black. **(c)** Corresponding phase-contrast image shows that cells assume the shapes of etched islands. **(d–g)** ASOMIC substrata are compatible with wide-field **(d,e)**, TIRF imaging **(f)** and platinum replica electron microscopy **(g)**. The attenuation of fluorescence signal by gold demonstrated by plating EGFP-actin expressing B16F1 cells on etched substrate (circle delimits etched island, dotted line shows cell boundary **(d)**). Immunofluorescence staining for phosphotyrosine (PY), microtubules (tubulin) and phalloidin-Alexa488 staining for actin in B16F1 cell on a triangular microetched island **(e)**; see also Supplementary Fig. 1. Wide-field (WF) and TIRF observation of tubulin immunostaining in circular cells **(f)**. Actin structure in cells on etched substrate visualized by platinum replica electron microscopy; note difference in optical contrast between etched and nonetched regions in upper image; boxed inset is contrast-inverted and enlarged to visualize actin filaments **(g)**. Bars: **c**, 40  $\mu\text{m}$ ; **d–f**, 10  $\mu\text{m}$ ; **g**, 0.5  $\mu\text{m}$ .

<sup>1</sup>Department of Cell and Molecular Biology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611-3008, USA. <sup>2</sup>Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3120. Correspondence should be addressed to G.G.B. (g-borisy@northwestern.edu) or B.A.G. (grzybor@northwestern.edu).

coverslips. When the stamp's features come into conformal contact with the substrate, etching occurs at the interface between the two materials (**Fig. 1a**). This reaction decreases the concentration of the etchant and increases that of the reaction products near the surface; diffusion from and toward the bulk of the stamp resupplies the former and clears away the latter. A key feature is that the stamp etches into the solid anisotropically—that is, without lateral spreading of the etchant over the solid's surface. The result is an array of transparent islands of shapes identical to those of the stamp's features (**Fig. 1b**). Notably, the unetched gold regions were not protected and could be subsequently covered with an anti-adhesive EG SAM (**Fig. 1a**). In contrast, in conventional microcontact printing, gold has to be protected first and only then etched<sup>13</sup>; EG thiols, however, cannot be used as a protective layer because they lose their antifouling properties under harsh etching conditions. We incubated ASOMIC etched coverslips in a 5 mM solution of EG-terminated alkyl thiol SHC<sub>11</sub>EG<sub>3</sub> (ProChimia) in ethanol for 2 h, washed them with ethanol and dried them in a nitrogen stream. We then affixed the dry patterned coverslips to the bottoms of plastic observation chambers, coated them with fibronectin (50 µg/ml) and plated cells at a density of 10,000–20,000 cells/cm<sup>2</sup> in culture medium containing serum (**Fig. 1a**).

ASOMIC produced large arrays (~2 cm × 2 cm) with perfectly etched features, and, unlike with microcontact printing patterns, the quality of the array could be evaluated readily by low-power bright-field microscopy—that is, the islands (light) could be easily distinguished from the gold background (dark; **Fig. 1b**). Corresponding phase-contrast image illustrates that cells attached to the etched islands, but not to the substratum covered with the EG SAM (**Fig. 1c**).

Molecular dynamics imaging has three major requirements: (i) high spatial resolution (~250 nm), (ii) high temporal resolution (few seconds) and (iii) extended image sequences (>100 frames). With the advent of green fluorescent protein (GFP) and its derivatives in cell biology, the importance of obtaining extended fluorescence sequences to analyze dynamic processes in living cells cannot be overestimated. Others have attempted to increase the transparency of micropatterned substrata by reducing the thickness of the gold layer<sup>14</sup>. Such efforts have allowed immunofluorescence imaging in fixed cells<sup>6–8,14</sup>, but the residual gold still diminished imaging efficiency. To determine signal attenuation in our system, we allowed B16F1 cells expressing enhanced GFP fused to actin (EGFP-actin) to spread onto both the islands and the gold regions (**Fig. 1d**). To allow adhesion onto the latter, we omitted the



**Figure 2** | Microtubule edge impact turning depends on cell shape. **(a)** Patterned substrata with circular and triangular adhesive islands were prepared by ASOMIC. Left column shows one frame in time-lapse series (3-s interval; **Supplementary Videos 1–3**) of *Yfp-CLIP170*-transfected B16F1 cells cultured on unpatterned substrate or islands of defined geometry coated with fibronectin. Projection (middle column) shows microtubule growth paths obtained by superimposing 16 frames. Representative CLIP tracks (right column) show paths of microtubule growth (dots, origin; circle, centrosomal area; red, turning microtubules, black, microtubules that do not turn). Bar, 10 µm. **(b)** Restriction of cells to the islands did not inhibit microtubule growth velocity. Instantaneous velocities were obtained from displacement of CLIP spots over time (100–110 microtubules, 5 cells, 2,000–3,000 s of total observation for each pattern; mean ± s.d. for velocities displayed as box plot). **(c)** CLIP spots within 1 µm from the edge were scored as either disappearing (catastrophe) or changing direction ('edge impact' turning). Microtubules that impact straight edges of triangular cells turn more frequently than those encountering convex edges of circular cells (left). Montage (right) of seven frames of enlarged boxed inset shown in **a**. Time, s; arrowhead, CLIP spot at tip of growing microtubules. Projection shows microtubule turning upon impact with cell edge.

incubation of microetched substrate in EG alkyl thiol and instead coated it uniformly with fibronectin. We compared fluorescence intensities in  $40 \times 40$ -pixel regions from both etched and unetched regions within the same cell. Gold layers of 50-nm thickness were almost opaque; even thin gold layers (12 nm) attenuated the fluorescence signal by  $\sim 75\%$  ( $74.0 \pm 10.4\%$ ;  $n = 10$  cells), a level incompatible with molecular dynamics imaging.

Attenuation by the gold layer also precludes other important imaging modalities such as TIRF and electron microscopy. Replica electron microscopy<sup>15</sup> generally uses thin layers ( $< 3$  nm) of platinum. Because gold will bond to the platinum in the preparation of the replica, even thin layers of gold ( $< 12$  nm) will degrade contrast to an extent that renders the replicas useless. Our ASOMIC approach improves wide-field fluorescence imaging (Fig. 1d,e and Supplementary Fig. 1 online) demonstrates that, by removing the gold, both TIRF (Fig. 1f) and electron microscopic (Fig. 1g) modalities are now compatible with micropatterning.

Avoiding fluorescence attenuation allowed unprecedented ability to monitor molecular dynamics in live, geometrically-defined cells. We transfected B16F1 cells with YFP-CLIP170, a protein that marks growing microtubule plus ends<sup>12</sup>. We imaged cells plated onto ASOMIC patterned glass with a Nikon Diaphot 300 inverted microscope using  $100\times$  objective (Plan, 1.25 NA) as previously described<sup>12</sup> (Supplementary Videos 1–3 online). Notably, images had brightness similar to that recorded for control cells plated on unpatterned glass, and effective exposure times were the same (500 ms), allowing acquisition of extensive time-lapse sequences (100–200 frames). In contrast, attempts to carry out dynamic imaging on ‘all-gold’ substrates patterned by conventional micro-contact printing were hampered by unacceptable trade-offs. For rapid cellular events such as tracking microtubule elongation with a plus-end binding protein, compensation of attenuation by increased exposure time resulted in unacceptable loss of temporal resolution. Conversely, election of exposure times matched to microtubule dynamics resulted in images that were too dim to quantify essential features, as shown by a direct comparison of microtubule plus ends in a time-lapse sequence of cells partially on and off a 12-nm gold film (Supplementary Fig. 2 online).

The ability to image YFP-CLIP170 molecular dynamics allowed analysis of microtubule growth velocity, growth persistence and growth path. Microtubule growth persistence (CLIP track length; Fig. 2a) and growth velocity (Fig. 2b) did not differ appreciably between unpatterned and patterned substrata. Thus, cell shape had no substantial effect on these basic parameters of microtubule dynamics. We noticed that CLIP spots that reached the edge of the cell had two possible fates—they either disappeared, indicative of microtubule catastrophe, or they turned and continued to grow along the edge of the cell. Which of the fates predominated was dependent on the shape of the cell edge—CLIP spots encountering the straight edges of triangular cells turned more often (36.7%, 188 microtubules, 4 cells) than those encountering the concave perimeter of circular cells (8.6%, 175 microtubules, 4 cells; Fig. 2a,c). Whether this ‘edge impact’ turning is due to a molecular or mechanical interaction with edge structures, possibly actin

bundles (Fig. 1e and Supplementary Fig. 1), remains to be determined. Microtubule growth guided along cell edges may provide a mechanism to concentrate microtubule plus-end activity where it is needed most—in the tail and retracting edges of the cell—ensuring efficient destabilization of adhesions, thus facilitating cell movement.

We have developed a versatile and robust method for preparation of microetched substrata that allows the use of new imaging strategies with geometrically-defined cells. The method optimizes molecular dynamics imaging of fluorescently labeled structures in living cells with controlled geometries and is compatible with other important imaging modalities such as TIRF and electron microscopy. It may be used with confocal or two-photon microscopy. In general, it may prove useful in micropatterning applications in which signal attenuation by gold needs to be minimized—such as biosensing and photomanipulation—and we expect it will be of broad applicability in cell biology. The method opens new vistas for studying cytoskeletal dynamics and already has demonstrated the potential to reveal new cellular behaviors. Finally, the ability to microetch large areas, the sharp optical contrast between adhesive regions and nonadhesive barriers and the stability of etched gold–SAM plates make them potential platforms for high-throughput, high-content cell-based assays.

Note: Supplementary information is available on the Nature Methods website.

#### ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health grant GM 25062 (G.G.B.), Camille and Henry Dreyfus New Faculty Award (B.A.G.), Baxter Health Care Corporation (G.G.B. and B.A.G.) and Department of Defense Breast Cancer Research Program postdoctoral fellowship (K.K.G.). We thank O. Chaga for help with electron microscopy.

#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at <http://www.nature.com/naturemethods/>  
Reprints and permissions information is available online at  
<http://npg.nature.com/reprintsandpermissions/>

- Whitesides, G.M., Ostuni, E., Takayama, S., Jiang, X. & Ingber, D.E. *Annu. Rev. Biomed. Eng.* **3**, 335–373 (2001).
- Mrksich, M. *Curr. Opin. Chem. Biol.* **6**, 794–797 (2002).
- Ireland, G.W., Dopping-Hepenstal, P., Jordan, P. & O'Neill, C. *J. Cell Sci.* **8** (Suppl.), 19–33 (1987).
- Singhvi, R. *et al. Science* **264**, 696–698 (1994).
- Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M. & Ingber, D.E. *Science* **276**, 1425–1428 (1997).
- Jiang, X., Bruzewicz, D.A., Wong, A.P., Piel, M. & Whitesides, G.M. *Proc. Natl. Acad. Sci. USA* **102**, 975–978 (2005).
- Lehnert, D. *et al. J. Cell Sci.* **117**, 41–52 (2004).
- Parker, K.K. *et al. FASEB J.* **16**, 1195–1204 (2002).
- Smoukov, S.K., Bishop, K.J.M., Campbell, C.J. & Grzybowski, B.A. *Adv. Mater.* **17**, 1361–1365 (2005).
- Klajn, R. *et al. Nat. Mater.* **3**, 729–735 (2004).
- Campbell, C.J., Klajn, R., Fialkowski, M. & Grzybowski, B.A. *Langmuir* **21**, 418–423 (2005).
- Komarova, Y.A., Vorobjev, I.A. & Borisy, G.G. *J. Cell Sci.* **115**, 3527–3539 (2002).
- Xia, Y.N., Zhao, X.M., Kim, E. & Whitesides, G.M. *Chem. Mater.* **7**, 2332–2337 (1995).
- Mrksich, M. *et al. Proc. Natl. Acad. Sci. USA* **93**, 10775–10778 (1996).
- Svitkina, T.M. & Borisy, G.G. *Methods Enzymol.* **298**, 570–592 (1998).

## Supplementary Methods

### Reagents

High gel strength agarose (OmniPur, Darmstadt, Germany); NANO SU-8 2010 photoresist (Microchem, Newton, MA); poly (dimethyl siloxane) (PDMS) prepolymer (Sylgard 184 Silicone Elastomer; Dow Chemical Co; Midland, MI); KI/I<sub>2</sub> based TFA type gold etchant (Transene Co. Inc., Danvers, MA); ethylene glycol (EG)-terminated alkyl thiol HS(CH<sub>2</sub>)<sub>11</sub>(OCH<sub>2</sub>CH<sub>2</sub>)<sub>3</sub>OH (ProChimia, Gdansk, Poland).

Fibronectin was from Sigma-Aldrich. We used the following primary antibodies for immunostaining: mouse monoclonal antibody against phosphotyrosine was purchased from Upstate Cell Signaling Solutions (Waltham, MA); rat monoclonal antibody against  $\alpha$ -tubulin from Abcam; phalloidin-Alexa488 from Molecular Probes (Portland, OR).

Secondary antibodies conjugated to Cy5 or rhodamine were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). B16F1 mouse melanoma cell line stably expressing EGFP- $\beta$ -actin<sup>1</sup> and untransfected B16F1 cells were from Dr. C. Ballestrem (Weizmann Institute of Science, Rehovot, Israel).

### Designing patterns

We designed real-size patterns by using layout software CleWin (WieWeb software, Eschede, The Netherlands). Features were printed on transparency sheets to generate photomasks by commercial vendor (CAD/ Art Services, Inc., Poway, CA). We chose feature dimensions to cover the size range of freely spreading B16 mouse melanoma cells (cell area  $1,590 \pm 540 \text{ m}^2$ ,  $n=10$  cells). Circles of diameters 35-45  $\mu\text{m}$  (areas 962-1,590  $\text{m}^2$ , respectively) were optimal for cell spreading over an entire island; triangles used had areas in the same range.

### Preparation of agarose stamps

We prepared agarose stamps by casting hot degassed 8 % w/v solution of high gel strength agarose (OmniPur, Darmstadt, Germany) in deionized water against oxidized poly (dimethyl siloxane) (PDMS) templates. PDMS templates were prepared by standard photolithography and molding approaches<sup>2,3</sup> and had their surfaces patterned with arrays of microscopic depressions (depth  $\sim 10$ -15  $\mu\text{m}$ ) corresponding to the negative of desired patterns in agarose. After gelation at room temperature, we peeled agarose away from the PDMS template and cut it into 1-2 cm x 1-2 cm x 2-5 mm rectangular blocks ("stamps").

### Preparation of microetched substrata and cell micropatterning.

We soaked agarose stamps for  $\sim 5$  minutes in a solution of a gold etchant containing potassium iodide and iodine (KI/I<sub>2</sub>) complex (Gold Etchant TFA, Transene Co. Inc., Danvers, MA) diluted 1:5 in deionized water. We then blotted soaked stamps against filter paper and air-dried them for 5 minutes to remove excess liquid. For etching, we placed stamps for 1-3 minutes feature-side-down onto standard glass coverslips (# 1.5, 22 mm x 22 mm) covered with e-beam evaporated gold films (12, 20, 30 or 50 nm) supported by a 5 nm adhesion layer of titanium. The thicker gold films required longer times of etching. We washed microetched substrata thoroughly with ethanol and then incubated them in 5 mM solution of ethylene glycol (EG)-terminated alkyl thiol HS(CH<sub>2</sub>)<sub>11</sub>(OCH<sub>2</sub>CH<sub>2</sub>)<sub>3</sub>OH (ProChimia, Gdansk, Poland) in ethanol for 2 h. We thoroughly washed substrata with ethanol and dried in a nitrogen stream. We used vacuum grease to affix dry patterned coverslips to the plastic observation chambers. We washed the dishes twice with phosphate buffered saline (PBS) and coated them with fibronectin (50  $\mu\text{g/ml}$ ) for 1 h at room

temperature. We plated cells at a density of 10,000-20,000 cells/cm<sup>2</sup> in DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (gentamycin). At low plating density, each adhesive island was occupied by one or two cells, or was empty. Since most cells assumed the shapes of the islands within two hours, we typically performed imaging experiments 2-8 hours after cell plating.

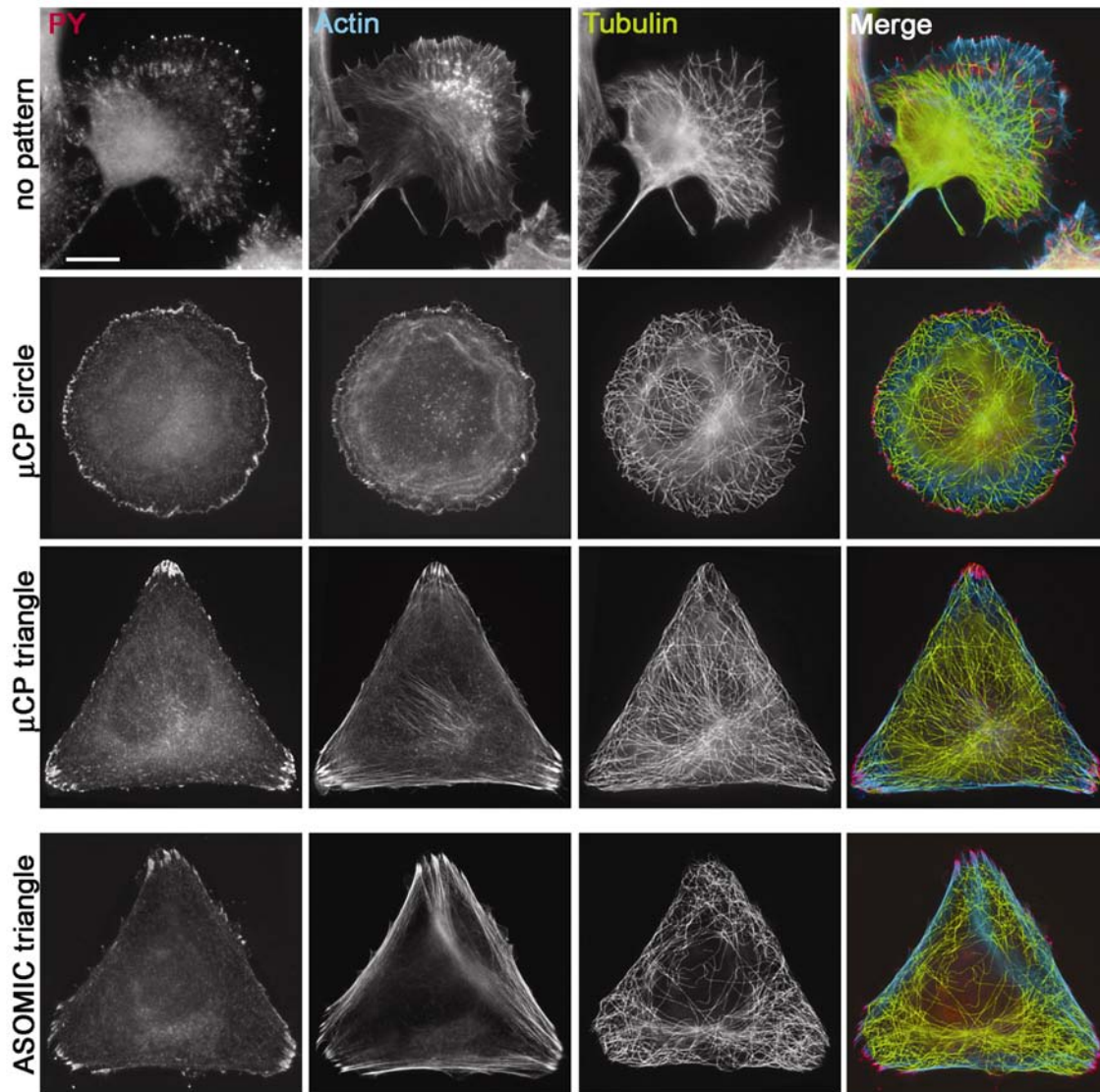
### **Microscopy, imaging and data analysis.**

For light microscopy, we used an inverted microscope (Diaphot 300; Nikon) equipped with a Plan 100X, 1.25 NA objective and slow-scan cooled CCD camera (model CH350; Photometrics, Tucson, AZ) driven by MetaMorph<sup>®</sup> imaging software (Universal Imaging Corp., Worchester, PA.). For total internal reflection (TIRF) microscopy, we used arc lamp-delivered TIRF microscope (Olympus) equipped with 60X 1.45 NA objective and Hamamatsu Electron Multiplier CCD camera (model C9100). We performed immunostaining after extraction-fixation of cells in 0.2% glutaraldehyde (GTA), 0.5% Triton-100X in PBS for 3-5 min followed by post-fixation in 0.2% GTA in PBS for 20 min. Observation of microtubule (MT) dynamics in live cells was done as previously described<sup>4</sup>. Briefly, we transfected B16F1 cells with plasmid encoding MT plus-end marker YFP-CLIP170. We generated YFP-CLIP170 by subcloning a fragment of a rat brain CLIP-170 cDNA (positions 192-4597; sequence accession number AJ237670) into *XhoI* and *Sall* sites of pEYFP-C1 (Clontech). Twenty-four hours after transfection, we plated cells onto microetched glass. During the observation cells were kept on microscope stage in Leibowitz-15 culture medium supplemented with 10% FBS at temperature 36-37° C. We obtained time-lapse series of 100-200 frames ~3 sec intervals. For presentation we generated .MOV format Videos by using MetaMorph<sup>®</sup> and compressed movies with Sorenson3 compression module in Quicktime Pro software. We obtained instantaneous MT growth velocities from frame-to-frame displacement of CLIP spots over time (100-110 MTs, 5 cells, 2000-3000 sec of total observation for each pattern). For turning analysis, we followed CLIP spots found within 1 μm from the cell edge for three subsequent frames and scored them as either disappearing—signifying catastrophe—or changing direction—‘edge impact’ turning (170-190 MTs, 4 cells).

We performed platinum replica electron microscopy as described previously<sup>5</sup>.

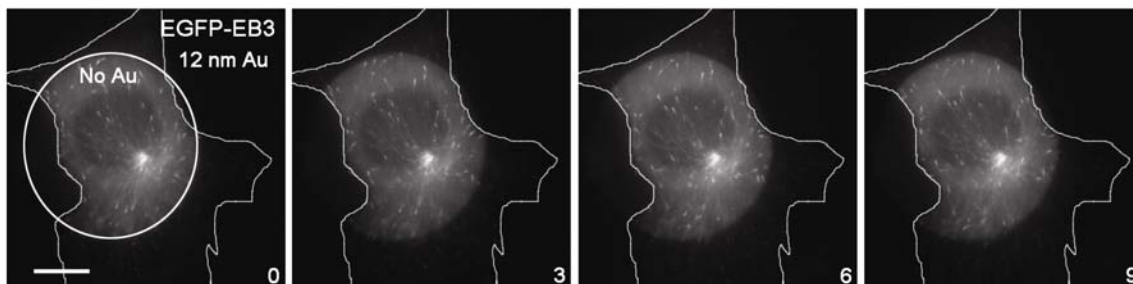
### **References**

1. Ballestrem, C., Wehrle-Haller, B., & Imhof, B.A. *J. Cell Sci.* **111**:1649–1658 (1998).
2. Whitesides, G.M., Ostuni, E., Takayama, S., Jiang, X. & Ingber, D.E. *Annu. Rev. Biomed. Eng.* **3**: 335-373 (2001).
3. LeDuc, P., Ostuni, E., Whitesides, G. & Ingber, D. *Methods Cell Biol.* **69**: 385-401 (2002).
4. Komarova, Y.A., Vorobjev, I.A. & Borisy, G.G. *J. Cell Sci.* **115** (Pt 17): 3527-3539 (2002).
5. Svitkina, T. M. & Borisy, G.G. *Methods Enzymol.* **298**:570-592 (1998).



**Supplementary Figure 1** Distribution of focal adhesions, actin filaments and microtubules in micropatterned cells.

B16F1 cells cultured on substrata without a pattern (upper row) or with fibronectin-coated circle- (40  $\mu\text{m}$  diameter; middle row) or triangle-shape islands (lower two rows). Immunofluorescence staining with antibody to phosphotyrosine (pY; left column), actin revealed by phalloidin staining (second left column) and microtubules revealed by staining with antibody to tubulin (third left column) in fixed cells. Cytoskeletal organization is similar in cells patterned using microcontact printing ( $\mu\text{CP}$ ) (third row) and ASOMIC (lower row) approaches. Scale bar corresponds to 10  $\mu\text{m}$ .



**Supplementary Figure 2** Fluorescence signal attenuation by thin gold layers.

B16F1 cells expressing EGFP-EB3 on etched substrate uniformly coated with fibronectin (circular line delimits etched circle from—No Au—from unetched regions—12 nm Au; line is also drawn around the cell perimeter; time, sec).