

High-Frequency Mechanostimulation of Cell Adhesion

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Abstract: Cell adhesion is regulated by molecularly defined protein interactions and by mechanical forces, which can activate a dynamic restructuring of adhesion sites. Previous attempts to explore the response of cell adhesion to forces have been limited to applying mechanical stimuli that involve the cytoskeleton. In contrast, we here apply a new, oscillatory type of stimulus through push–pull azobenzenes. Push–pull azobenzenes perform a high-frequency, molecular oscillation upon irradiation with visible light that has frequently been applied in polymer surface relief grating. We here use these oscillations to address single adhesion receptors. The effect of molecular oscillatory forces on cell adhesion has been analyzed using single-cell force spectroscopy and gene expression studies. Our experiments demonstrate a reinforcement of cell adhesion as well as upregulated expression levels of adhesion-associated genes as a result of the nanoscale “tickling” of integrins. This novel type of mechanical stimulus provides a previously unprecedented molecular control of cellular mechanosensing.

Cell adhesion is a prominent biological activity that is regulated by protein interactions as well as by mechanical stimuli, which are regarded as cues to prompt cell functions and stem cell differentiation.^[1,2] A devised adhesion environment allowing the dynamic control of such interactions via protein modulation will have a significant impact on a number of life-essential processes such as embryogenesis and cancer development.^[3,4] However, the application of mechanical stimuli has been limited to large scale forces that are often exerted by microneedles or through substrate stretching.^[5,6] This results in an indirect transmission of forces to adhesion sites that might lead to undesired activation of signal transduction pathways. Such macroscale forces neither provide the precision of mechanical stimuli at the molecular scale nor the reversibility required to resemble the dynamics of cell adhesion. In this work, we investigate a dynamic interface composed of photoactive push–pull azobenzene molecules

that operate as nanomanipulators to individual adhesion sites. These molecules are capable of oscillating continuously between two isomeric states when exposed to visible light. This fully reversible process is associated with a change in azobenzene length of about 3.5 Å, which can be transferred to adhesion sites as mechanical stimuli at high frequencies in the millisecond regime. Our study is the first to report the use of push–pull azobenzenes in biological applications, particularly in the field of mechanosensing.

“Conventional” azobenzenes switch between two well-defined isomeric states using two wavelengths and have been used to modulate cell adhesion via ligand availability. One of these wavelengths is usually in the UV regime, which can be harmful for cells when exposed for extended irradiation times. The bistability of regular azobenzenes, which function by either exposing or hiding adhesion ligands via kinking the azobenzene units,^[7] also impairs efficient force transduction to adhesion sites.

Therefore, in this work we exploited push–pull-substituted azobenzene molecules with integrin ligand c(RGDfK) headgroups in a novel method of modulating cell adhesion via nanoscale oscillations. Push–pull azobenzenes carry an electron withdrawing substituent in one ring and an electron-donating group in the other ring, both usually in *para* or *ortho* positions with respect to the azo group. This dipolar substitution pattern induces a red-shift of the π – π^* absorption into the visible region,^[8–10] an increase of absorbance, and a very fast thermal back-reaction from the photochemically generated bent *cis* state to the thermodynamically more stable stretched *trans* state. Hence, upon irradiation with visible light of a single wavelength, these molecules perform a very rapid movement or mechanical oscillation of the azobenzene headgroup ($\approx 10^2$ – 10^5 Hz, depending on the substitution, light intensity and environment). Once irradiation is terminated, the molecules return to the *trans* configuration.^[8,11] Push–pull azobenzenes embedded in polymers exert large forces onto the surrounding polymer matrix. This process has been frequently used to generate surface relief grating in polymer surfaces.^[12,13] In our work, we employed the light-induced mechanical movement of push–pull azobenzenes to apply forces to integrins and to serve as nanomechanical molecularly defined stimulators.

Push–pull azobenzenes were first coupled to glass surfaces along with a biologically passive polyethylene glycol (PEG) layer, then the molecules were functionalized with the integrin ligand c(RGDfK) (Figure 1a and Supporting Information).^[14] The molar ratio of c(RGDfK)–azobenzene and PEG2000 was set to 1:99, which provides suitable adhesion conditions for fibroblast cells.^[7,15] The c(RGDfK)–azobenzene packing density on the substrates was 1.07 ± 0.33 molecules nm^{-2} , as determined with UV-vis spectroscopy. Fig-

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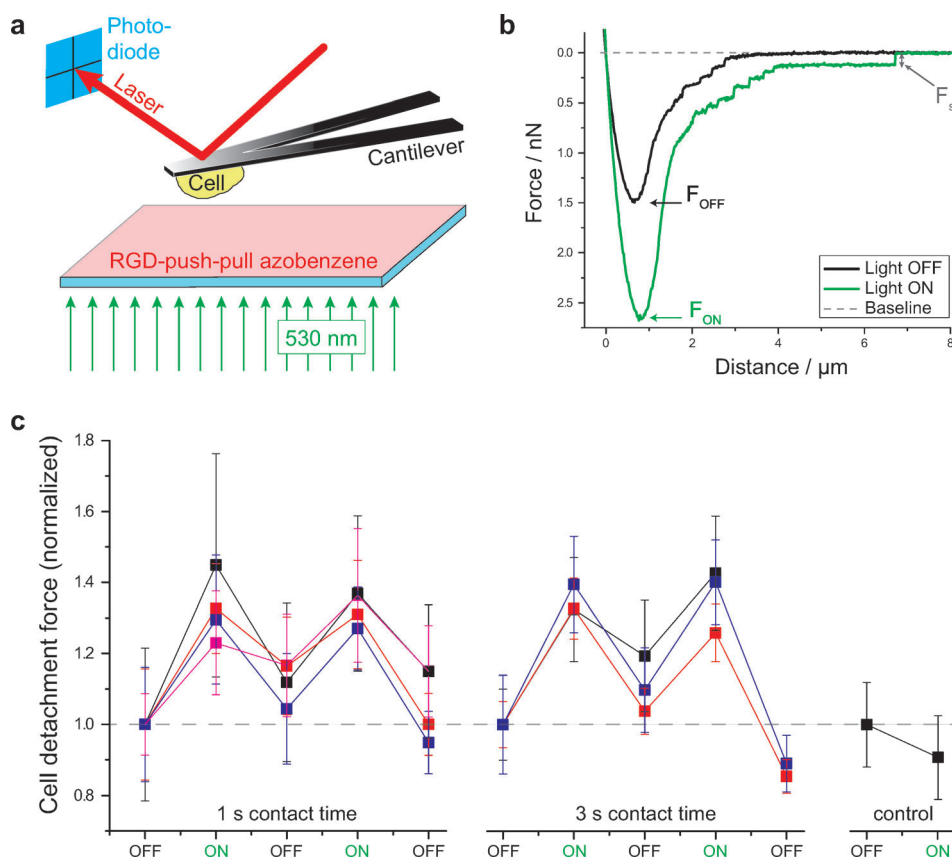


Figure 2. Cell adhesion as a function of RGD push-pull azobenzene oscillation. a) A cell is immobilized on a tipless cantilever and an atomic force microscope is used to measure the deflection of the cantilever during approach and retraction of the cell. b) Representative force–distance curves in the dark and under continuous irradiation with light (530 nm). F_{OFF} and F_{ON} are the forces needed to detach cells from the surface when the light is switched off and on, respectively. F_{S} is the force associated with the last rupture event. c) Normalized cell detachment force in subsequent irradiation cycles for both 1 s and 3 s contact times. The increase in detachment force due to azobenzene oscillation is statistically significant (Student's *t*-test, $p < 0.001$). Data from a control experiment (1 s contact time on bare glass) are also shown. Each square represents the mean value of ≥ 40 force curves; error bars denote standard deviation. Each color symbolizes an independent experiment. Cell detachment forces increase considerably if the molecules oscillate.

response to large-scale external forces, which occurs at timescales of a few minutes.^[5] Furthermore, the observed reinforcement in our experiments is fully reversible.

To investigate the effect of RGD push-pull azobenzene oscillations on single rupture events, we analyzed the force associated with the final rupture event in the force–distance curves. This parameter contains information on the rupture properties of single molecules and tiny adhesion clusters.^[24] Figure 3 demonstrates that the force associated with the last rupture is indeed increased if the RGD push-pull azobenzenes oscillate, both for 1 s and 3 s cell-surface contact time. The maxima of the Gaussian fits for the last rupture force distributions are shifted by approximately 6 pN (1 s) and 8 pN (3 s). These shifts are significant at a level of $p < 0.001$ (Student's *t*-test) for both cell-surface contact times. A significant increase was also observed for other parameters in conjunction with irradiation, that is, for the position of the last rupture and the length of the last tether (Supporting Information). The observed impact of RGD push-pull

azobenzene oscillation on single rupture force, detachment length and tether length is the first sign that a molecularly defined mechanical stimulation influences integrin binding.

After the initial integrin contact to an adhesive surface, integrins are connected to actin filaments through a number of linker proteins, such as vinculin, paxillin, zyxin and talin in so-called focal adhesion clusters. These proteins are known to transfer extracellular signals to the cytosol, as well as to stabilize the focal adhesion clusters by regulating the relative expression of genes through a feedback system. To investigate larger scale effects of the RGD push-pull azobenzene-induced mechanical stimulation on cell adhesion and to explore the effect at longer timescales, we examined the gene expression level of these linker proteins. Fibroblast cells were seeded on RGD push-pull azobenzene-coated surfaces and pre-incubated for 30 min. The samples then underwent a consecutive series of light exposure and incubation cycles, each with a defined time period and number of repetitions (Figure 4a). The results show a clear upregulation of vinculin, paxillin, zyxin

and talin in response to irradiation of cells on an RGD push-pull azobenzene surface in comparison to cells on uncoated glass surfaces (Figure 4b). Talin is upregulated even for the shortest stimulation protocols, which is a significant result as talin is a protein that binds directly to the cytosolic tail of integrins. Whereas focal adhesion clusters are known to react to external forces, for example, with maturation and growth, previously large-scale forces at the cell level were necessary to induce changes in focal adhesion clusters.^[5] In contrast, our RGD push-pull azobenzene surface coating exerts only tiny but oscillatory and continuous mechanical stimulation on the integrins in the cell membrane. Still, this molecularly defined “tickling” has a significant and large-scale effect on the cells.

Our results show that cell adhesion is significantly enhanced at different timescales and at different levels (i.e. adhesion, gene expression) by the tiny and continuous oscillations of RGD push-pull azobenzenes. SCFS experiments have demonstrated reinforcement of cell adhesion at the level of single molecule ruptures and the level of cells;

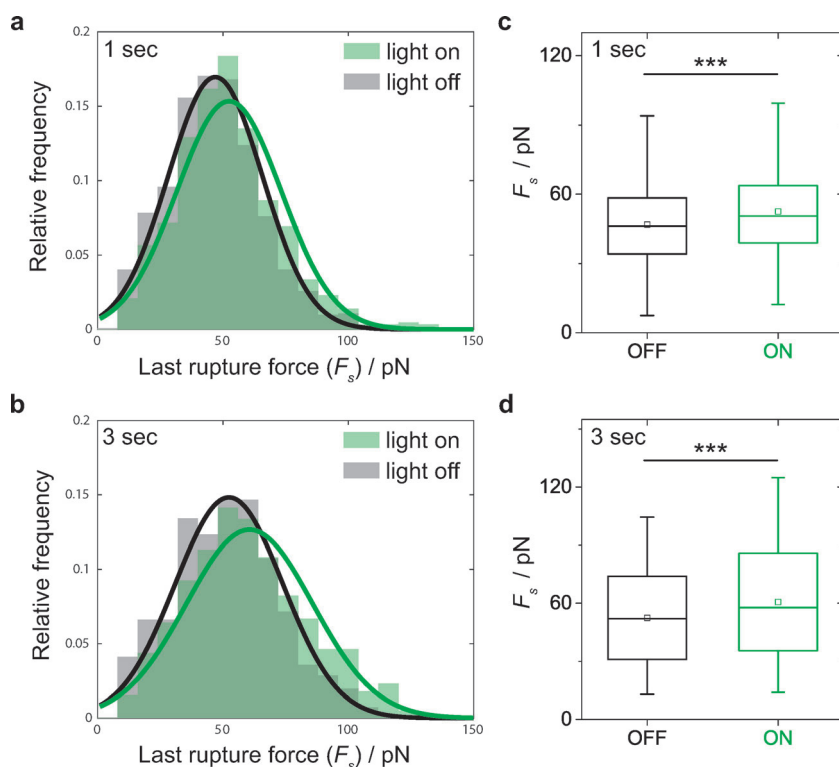


Figure 3. Relative frequencies of the force (F_s) associated with the last rupture event in force-distance curves. a,b) Force distributions for 1 s and 3 s cell-surface contact times. c,d) Boxplots of the force distributions (interquartile range; line in each box: median; dot: mean) for 1 s and 3 s contact times. The force shifts due to RGD push-pull azobenzene oscillations are statistically significant (Student's t-test, $p < 0.001$, number of analyzed rupture events: 868 (ON) and 1318 (OFF) for 1 s, 392 (ON) and 559 (OFF) for 3 s).

likewise, gene expression data revealed a significant upregulation in the expression of essential focal adhesion cluster proteins that are involved in mechanosensing.^[5] As a potential light-induced heating of the environment by the oscillation of azobenzenes can be neglected and as a heating would rather decrease detachment forces,^[25,26] we attribute the reinforcement of adhesion to mechanosensory processes either at the level of integrins or at the level of tiny adhesion clusters. Mechanical stimulation could be induced via integrins that act as catch bonds^[23] or via other components of focal adhesion clusters that have been proposed as mechanosensors, such as talin^[27] and vinculin.^[28] It is well-known that cells react to very small changes in their environment,^[2] particularly on tiny mechanical changes, for example, changes in ligand tethering.^[29] In contrast, the exertion of light-controlled oscillatory “tickling” forces to single adhesion proteins provides a completely novel method for cell stimulation. Our molecular oscillation approach is useful to elucidate mechanisms of cell adhesion due to its spatio-temporal resolution and because the stimulus can be dynamically tuned by

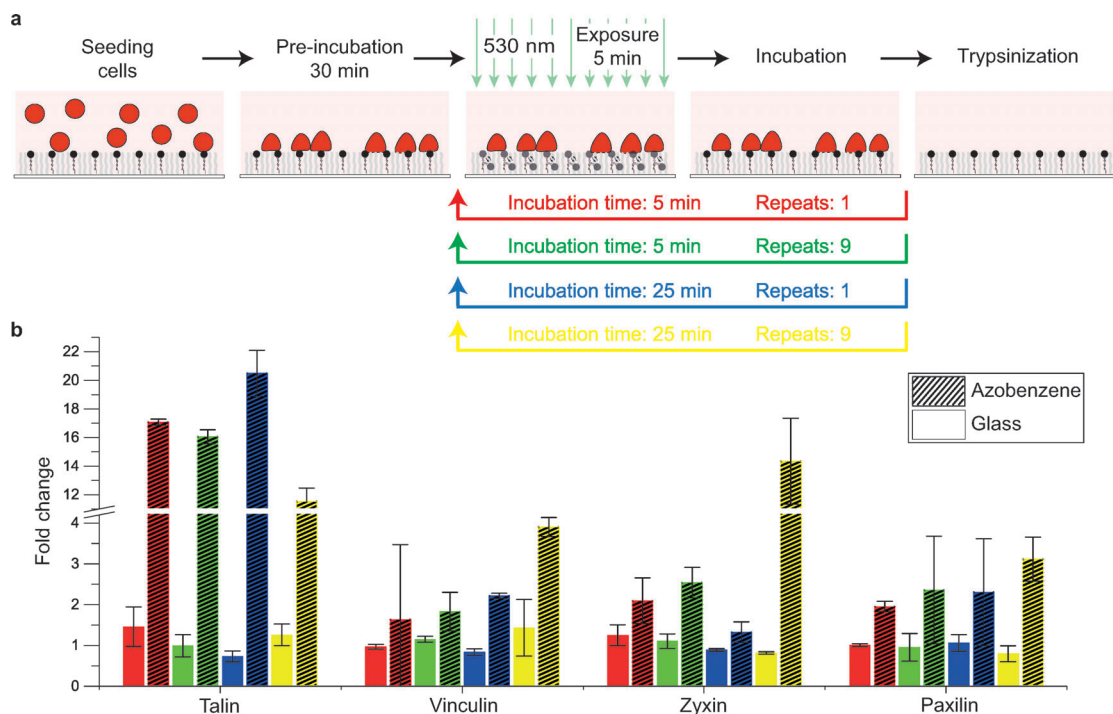


Figure 4. Gene expression as a function of RGD push-pull azobenzene oscillation. a) Experimental steps showing four different types of experiments with different light stimulation protocols (color codes). b) Average fold changes in gene expression in response to different light stimulation protocols on RGD push-pull azobenzene surfaces. Three samples were analyzed with at least two technical replicates for each experiment setting. Error bars denote standard deviation.

controlling the light intensity and the duration of illumination.

The strategy of employing biointerfaces that apply an externally controlled oscillatory nanoscale stimulus on molecules in the cell membrane opens prospects for numerous applications, given the central role of dynamic extracellular environments in biological systems. As a large variety of different push-pull azobenzene chemistries are available,^[8,10,11] different wavelengths and oscillation frequencies can be used to tailor molecularly defined photo-oscillatory properties of surfaces. This will enable elucidating nanoscale mechanisms of mechanosensing at the molecular level in a large variety of biomolecular binding systems and biological applications.

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Keywords: azobenzene · cell adhesion · integrins · mechanosensing · single-cell force spectroscopy

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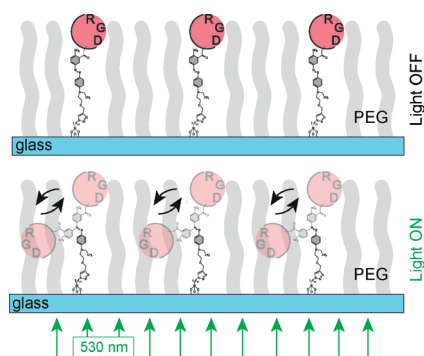
Communications



Cellular Mechanosensing

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High-Frequency Mechanostimulation of
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Tickling cells: Biofunctionalized push–pull azobenzene molecules were synthesized and coupled to glass substrates in order to exert molecularly defined, oscillatory forces onto cells. The applied forces activate a mechanosensory mechanism in which cells respond by strengthening of their adhesion to the substrate as well as by upregulating the expression level of adhesion-related genes.