Maleimide Photolithography for Single-Molecule Protein–Protein Interaction Analysis in Micropatterns

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Spatial organization of proteins into microscopic structures has important applications in fundamental and applied research. Preserving the function of proteins in such microstructures requires generic methods for site-specific capturing through affinity handles. Here, we present a versatile bottom-up surface micropatterning approach based on surface functionalization with maleimides, which selectively react with organic thiols. Upon UV irradiation through a photomask, the functionality of illuminated maleimide groups was efficiently destroyed. Remaining maleimides in nonilluminated regions were further reacted with different thiol-functionalized groups for site-specific protein immobilization under physiological conditions. Highly selective immobilization of Histagged proteins into tris(nitrilotriacetic acid) functionalized microstructures with very high contrast was possible even by direct capturing of proteins from crude cell lysates. Moreover, we employed phosphopantetheinyl transfer from surface-immobilized coenzyme A to ybbR-tagged proteins in order to implement site-specific, covalent protein immobilization into microstructures. The functional integrity of the immobilized protein was confirmed by monitoring protein–protein interactions in real time. Moreover, we demonstrate quantitative single-molecule analysis of protein–protein interactions with proteins selectively captured into these high-contrast micropatterns.

Lateral organization of proteins into functional microstructures has a major impact not only in fundamental research but also in analytical and biomedical applications.1–3 Numerous powerful approaches for photolithographic and microcontact printing-based patterning of proteins into structures with a spatial resolution around the diffraction limit of light have been reported.4–11 Biophysical and biotechnological applications, however, require protein micropatterning under native conditions in order to maintain the functional integrity of the immobilized protein. This is, in particular, important for probing protein function on the single-molecule level, which requires homogeneous functionality and minimum background binding. Moreover, simple and generic surface fabrication techniques are required for broad application. For this purpose, photolithographic surface patterning is ideally suited because it is based on relatively simple equipment and procedures. Protein capturing to surfaces rendered biocompatible by use of a thin, protein-repellent polymer coating has proven to be very suitable for functional protein micropatterning. For site-specific capturing of target proteins, spatially resolved functionalization of these layers with suitable capturing groups is required.9 In the case of recombinant proteins, suitable affinity tags such as the oligohistidine tag12 or tags for site-specific biotinylation13,14 have proven useful for ensuring site-specific protein immobilization.15–18 Ideally, this strategy enables for direct capturing of proteins from crude cell lysates into micropatterns,19 thus avoiding demanding purification of the target proteins.

Here, we aimed to meet these requirements by implementing a highly generic approach for functional surface patterning, which is amenable with different methods for protein capturing. To this end, we devised a bottom-up surface patterning approach based on a dense poly(ethylene glycol) (PEG) polymer brush, which was previously shown to very efficiently protect surfaces against

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nonspecific binding of proteins.\textsuperscript{20} Moreover, highly functional immobilization of fragile proteins such as receptor domains and molecular motor proteins has been demonstrated on such surfaces.\textsuperscript{18,21} For surface patterning, the terminal amine groups of the PEG polymer brush were reacted with a heterobifunctional cross-linker to obtain maleimide functionalities. This offers versatile means for site-specific protein capturing either directly through exposed cysteine residues within proteins or through thiol-functionalized ligands for protein capturing. Here, we explored photodestruction of surface maleimides upon UV irradiation through a photomask as a patterning strategy (Scheme 1). Thus, subsequent surface modification with thiol-functionalized molecules is possible only in nonirradiated areas. In addition to surface functionalization with biotin, we have adapted this method for generic, site-specific immobilization of recombinant proteins: (i) covalent immobilization by enzymatic phosphopantetheinyl transfer (PPT) based on coenzyme A (CoA)-functionalized surfaces\textsuperscript{22–27} and (ii) affinity-based immobilization utilizing the high-affinity interaction of the oligohistidine tag of recombinant proteins with tris(nitrilotriacetic acid) (tris-NTA) functionalized surfaces.

**EXPERIMENTAL SECTION**

**Materials.** Homofunctional diaminopoly(ethylene glycol) (DAPEG) with an average molecular mass of 2000 g/mol was purchased from Rapp Polymere, Tübingen, Germany. Biotin-OEG\textsubscript{3}-undecylthiol (BT-thiol) was purchased from ProChimia, Sopot, Poland. Streptavidin (SAv) was purchased from Serva Electrophoresis GmbH, Heidelberg, Germany. Thiol-functionalized tris-NTA was synthesized as described previously.\textsuperscript{28} Manganese(II) chloride tetrahydrate, imidazole, ethylenediaminetetraacetic acid (EDTA), N-(2-hydroxyethyl)piperazine-N'\textprime-2-ethanesulfonic acid (HEPES), sodium chloride, and asbo-


lute ethanol were purchased from Carl Roth, Karlsruhe, Germany. Streptavidin labeled with ATTO 655 (ATTO-SAv) and ATTO 655 maleimide were purchased from ATTO-TEC GmbH, Siegen, Germany. Microstructured masks for photopatterning (chrome on quartz) were obtained from NB Technologies, Bremen, Germany. Phosphopantetheinyl transferase Sfp and CoA conjugated with Dy-547 (CoA547) were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany). CoA conjugates with ATTO 655 and Alexa Fluor 568 (Invitrogen) were synthesized and purified according to published protocols. All other chemicals were purchased from Sigma Aldrich.

**Protein Production and Labeling.** Enhanced green fluorescent protein carrying an N-terminal hexahistidine tag (H6-EGFP) cloned in the plasmid pET21a was expressed in Escherichia coli and purified by immobilized metal ion chromatography and size-exclusion chromatography by using standard protocols. For binding experiments with crude cell lysates, cells were sonicated and purified by immobilized metal ion chromatography and size-exclusion chromatography by using standard protocols. For binding experiments with crude cell lysates, cells were sonicated and purified by immobilized metal ion chromatography and size-exclusion chromatography by using standard protocols. For binding experiments with crude cell lysates, cells were sonicated and purified by immobilized metal ion chromatography and size-exclusion chromatography by using standard protocols.

**Surface Chemistry and Patterning.** Surface chemistry was carried out on transducer slides for reflectance interference spectroscopy (RIfS) detection (a thin silica layer on a glass substrate) as well as standard glass cover slides for fluorescence microscopy. Surface coating with a thin PEG polymer brush and further functionalization with maleimide groups was carried out as described in detail previously. After surface cleaning in fresh Piranha solution (one part 30% H2O2 and two parts concentrated H2SO4; caution, highly corrosive), the surface was activated by reaction with pure (3-glycidyloxypropyl)trimethoxysilane for 1 h at 75 °C. Subsequently, the surface was reacted with molten DAPEG for 4 h at 75 °C, followed by an incubation of 1 M mercaptoethanol in N,N-dimethylformamide (DMF) for 10 min at room temperature in order to block thiol-reactive sites. For functionalization with maleimide groups, the amine-functionalized surfaces were incubated under a saturated solution of 3-(maleimido)propionic acid N-hydroxysuccinimide ester (MPSA-NHS) in dry DMF for 30 min at room temperature. If not stated otherwise, photolithographic patterning was performed by irradiation for 5 min through a photomask with a 75 W xenon lamp equipped with a 280–400 nm dichroic mirror (Newport Spectra-Physics). Thereafter, the chemically modified slides were washed with absolute ethanol. Further functionalization was performed by incubation with 500 µM BT-thiol or tris-NTA-thiol in HBS for 30 min at room temperature. Functionalization with CoA was carried out in situ by injecting 1 mM CoA in HBS under flow through conditions for 2–5 min.

**Binding Assays by Solid-Phase Detection.** Protein binding to surfaces functionalized before and after photodesorption of maleimides was monitored in real time by RIfS. Label-free detection by RIfS is based on probing changes in optical thickness of a thin silica layer by white light interference. Changes in the surface loading upon protein binding and dissociation are detected in real time as a shift of the interference spectrum on the wavelength axis. A change in surface loading by 1 pg/mm² leads to a shift of the interference minimum (1.5th order) by 1.2 pm as determined by calibration experiments with radioactively labeled proteins. The measurements were performed under continuous flow through conditions in a home-built setup as described in detail earlier. Photoirradiated maleimide-functionalized RIfS transducer slides (prepared as described above) were equilibrated in HBS containing 0.01% Triton X-100 and then reacted with 1 µM BT-thiol in HBS. The presence of biotin groups was confirmed by a subsequent injection of 100 nM SAv.

**Fluorescence Imaging of Protein Micropatterns.** Ensemble fluorescence imaging of protein micropatterns was performed in a confocal laser-scanning microscope (CLSM, FluoView 1000, Olympus) equipped with an argon ion laser (488 nm line for excitation of EGFP), a 559 nm diode laser (excitation of AlexaFluor 568), and a 635 nm diode laser (excitation of ATTO-655). Proteins were diluted in HBS containing 1% (w/v) bovine serum albumin (BSA). All binding assays were carried out in a flow through format. Protein samples were injected through a 500 µl sample loop via an injection valve and incubated at continuous flow for 2–5 min. Before imaging of immobilized ATTO-565-SAv and H6-EGFP, the flow cell was thoroughly rinsed with HBS. Binding of IFNα2 to immobilized IFNAR2-H10 and ybbR-IFNAR2 was probed by injecting 50–100 nM fluorescence-labeled IFNα2, and images were acquired without rinsing. The dissociation kinetics of fluorescence-labeled IFNα2 were probed by subsequent injection of unlabelled 1 µM IFNα2 and time-lapse imaging in the presence of unlabeled IFNα2. All images were acquired without flow.

**Single-Molecule Imaging of Protein–Protein Interactions.** Single-molecule fluorescence imaging was carried out with an inverted microscope (Olympus IX71) equipped with a single-line total internal reflection (TIR) illumination condenser (Olympus) and a back-illuminated electron-multiplied (EM) charge-coupled device (CCD) camera (Xon DU897D, 512 × 512 pixel from Andor Technology). An argon krypton laser (C70 Spectra, Coherent)
was coupled into the microscope through a polarization-maintaining monomode fiber (KineFlex, Pointsource). The 647 nm laser line was selected by means of an acousto-optical tunable filter (AOTF, AAoptics). A 60× objective with a numerical aperture of 1.45 (PLAPON 60×/1.45 TIRFM, Olympus) was used for TIR excitation. The excitation beam was reflected into the objective by a quadruple-line dichroic beamsplitter (Di R405/488/561/647, Semrock), and the fluorescence was detected through a quadruple bandpass filter (FF01 446/523/600/677-25, Semrock). Fluorescence imaging was performed by excitation at 647 nm with a typical power output of 5 mW at the objective. The camera was operated at −80 °C with a typical EM gain of 300 and a frame rate of 1–10 Hz, depending on the kinetics of the interaction. The laser was synchronized to the camera readout by use of the AOTF. All binding experiments were carried out at room temperature in HBS containing 0.01% Triton X-100 and 50 µM EDTA supplemented with oxygen scavengers [0.5 mg/mL glucose oxidase (Sigma), 40 mg/mL catalase (Roche AppliedScience), 5% (w/v) glucose, and 1 mM Trolox] to minimize photobleaching.

Localization and residence times of individual IFN\(\text{R}_2\) molecules were determined from trajectories obtained by the multiple target tracker. Trajectories of less than three frames were not considered in the evaluation of residence times. Histograms of the frequency of different residence times were fitted by a monoexponential decay function.

### RESULTS AND DISCUSSION

The functionalization of glass-type surfaces with maleimide groups was carried out with a dense PEG polymer brush, which was covalently coupled to silanized surfaces. We have previously demonstrated the high stability of these PEG layers and the efficient functionalization of terminal amine groups of the PEG. An efficient surface functionalization with maleimide groups was confirmed by monitoring the reaction with CoA by RIfS (Figure S-1, Supporting Information), yielding a final surface concentration of 0.4 pmol/mm\(^2\) (0.24 molecules/nm\(^2\)). Moreover, rapid kinetics of the reaction was observed with a rate constant of 80 M\(^{-1}\)·s\(^{-1}\). Thus, saturated binding can be obtained within a few minutes of incubation of a thiol-functionalized compound at millimolar concentration.

In the first step, optimum conditions for maleimide-based protein micropatterning were established by use of the immobilization of BT-thiol for a functional readout (Figure 1a). After reaction of maleimide-functionalized surfaces with BT-thiol, strong, highly specific binding of streptavidin was detected by RIfS (Figure 1b). Upon UV irradiation of maleimide-functionalized surfaces prior to the reaction with BT-thiol, substantially reduced binding of streptavidin was detected. Rapid photodestruction of surface maleimides with a half-life of 13 s was observed (Figure 1c). This photodestruction can occur due to photoinduced radical polymerization of maleimide groups on the surface. This effect was readily employed for micropatterning of biotin on surfaces, as confirmed by fluorescence imaging using streptavidin labeled with ATTO655 (AT655SAv, Figure 1d). Strong binding of AT655SAv to nonilluminated surface areas was observed, while no significant fluorescence was detectable in areas where the maleimide groups were destroyed by UV irradiation. Besides demonstrating the proof-of-principle of maleimide-based protein

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micropatterning, such architectures can be readily employed for capturing biotinylated proteins.

Efficient and selective protein capturing into micropatterns even from complex sample matrices is an important prerequisite for biological applications, where small amounts of proteins are available and purification results in loss of function and material. We therefore aimed to establish selective protein capturing into micropatterns from crude cell lysates. To this end, we employed stable yet reversible immobilization of His-tagged proteins by surface functionalization with tris-NTA.18 For micropatterning of tris-NTA through maleimide photolithography, we employed a thiol-functionalized tris-NTA derivative (cf. Scheme 1), which was generated in situ by reduction of the corresponding disulfide.28 This compound was incubated on surfaces subjected to maleimide patterning by UV illumination through a photomask (Figure 2a). After the NTA moieties were loaded with Ni(II) ions, selective binding of H6-EGFP to tris-NTA-functionalized regions was observed with a contrast of \( >50:1 \) compared to nonfunctionalized areas (Figure 2b). While very stable binding of H6-EGFP during rinsing for extended time periods was observed, rapid quantitative elution was observed upon injection of imidazole, which competes with the His tag (Figure 2c). After being washed with imidazole, the surface could be repeatedly reloading with H6-EGFP without requiring incubation of Ni(II) ions (Figure 2c). However, after removal of complexed Ni(II) ions from the surface, binding of H6-EGFP was completely abolished (Figure 2c), confirming specific protein capturing at the surface.

In order to explore the selectivity of protein capturing into micropatterns, we incubated a crude cell lysate, which was prepared from E. coli cells expressing H6-EGFP, on micropatterned tris-NTA surfaces. The lysate was diluted into HBS to a final concentration of 1 \( \mu \)M H6-EGFP as determined by the absorption at 480 nm. Strikingly, selective and very efficient targeting of H6-EGFP into micropatterns was achieved (Figure 2d), even in this very complex sample matrix containing all soluble E. coli proteins. Again, specific binding of H6-EGFP was confirmed by elution with imidazole, and repetitive loading was possible.

For site-specific covalent immobilization of recombinant proteins, we employed enzymatic PPT transfer from CoA coupled to maleimide-functionalized surfaces through its thiol group (cf. Scheme 1). Micropatterned CoA-functionalized surfaces were obtained by coupling CoA after photodestruction of surface maleimides through a photomask. As a target amino acid sequence for enzymatic PPT, we chose the peptide tag ybbR, which is a suitable substrate for the PPTase Sfp.23 This tag was fused to the N-terminus of the ectodomain of the type I interferon receptor subunit IFNAR2 (ybbR-IFNAR2) as a model protein for probing protein–protein interactions. We have recently performed a detailed characterization of the immobilization of ybbR-IFNAR2 onto CoA-functionalized surfaces by PPT.27 Highly efficient and functional immobilization of ybbR-IFNAR2 was achieved by this method, which was confirmed by monitoring the interaction with its ligand IFNa2 by real-time label-free detection. In contrast, quantitative denaturation was observed upon physisorption of IFNAR2 on glass-type surfaces.35 Here, functional immobilization of ybbR-IFNAR2 into micropatterns was detected by probing the interaction with fluorescence-labeled IFNa2 (Figure 3a). Upon incubation of 50 nM IFNa2 labeled with Dy-547 (Dy547IFNa2), selective binding to nonilluminated surface areas was detected (Figure 3b). In a similar experiment carried out with IFNa2 labeled with AlexaFluor 568 (AF568IFNa2), we quantitatively
assessed the contrast between functionalized and nonfunctionalized regions. For this purpose, part of the structure was photobleached by the confocal laser beam (Figure 3c), and fluorescence intensity in the photobleached areas was compared with fluorescence intensity in the nonbleached areas. A very low difference between the background fluorescence signals in the photobleached area compared to the nonbleached area was observed (Figure 3d). From these signals, a contrast of >60:1 was estimated, confirming the very efficient photodestruction of surface maleimides and the highly selective coupling of thiol-modified compounds to nonilluminated areas.

In order to probe reversible interaction between IFNa2 labeled with ATTO 655 (AT655IFNa2) and immobilized ybbR-IFNAR2, we monitored the fluorescence intensity within functionalized microstructures upon chasing with 1 µM unlabeled IFNa2 (Figure 4). Nearly quantitative elution of AT655IFNa2 was observed (Figure 4a,b). The dissociation curve obtained from the fluorescence intensity within the microstructures was well-fitted by a monoexponential decay function (Figure 4c). A dissociation rate constant of 0.02 s⁻¹ was obtained from the fit, which is in perfect agreement with the dissociation rate constant previously obtained for this interaction. These reversible protein–protein interactions in micropatterns. (a) Schematic of the assay: site-specific immobilization of ybbR-IFNAR2 to micropatterned CoA in the presence of the PPTase Sfp, followed by binding of AF568IFNa2. (b) Patterning of ybbR-IFNAR2 as detected by its interaction with Dy547IFNa2. The intensity profile across the indicated line is shown on the right. (c) Determination of contrast: after binding of AF568IFNa2 to micropatterned ybbR-IFNAR2, a selected area (indicated by the white rectangle) was photobleached. Subsequently, the fluorescence in functionalized and nonfunctionalized areas was determined. (d) Comparison of the fluorescence intensities in bleached and nonbleached areas (as indicated in panel c).
interaction assays confirmed functional immobilization of a fragile protein into micropatterns. The measurements demonstrated the possibility to quantitatively probe protein–protein interactions in these micropatterns by ensemble measurements.

Quantitative interaction studies on the single-molecule level are even more challenging with respect to binding specificity and selectivity and the functionality of the immobilized protein. In order to evaluate the suitability of our micropatterning technique for single-molecule analysis methods, we aimed to monitor the interactions of individual $^{\text{AT655}}$IFNα2 molecules with micropatterned IFNAR2. For this purpose, IFNAR2-H10 was immobilized on micropatterned tris-NTA. Specific and reversible binding of $^{\text{AT655}}$IFNα2 was confirmed by ensemble measurements (Figure 5a,b). For single-molecule experiments, we employed the IFNα2 mutant M148A, which binds to IFNAR2 with an approximately 30-fold decreased binding affinity, based on a 30 times higher dissociation rate constant ($\sim 0.6 \text{ s}^{-1}$). IFNα2-M148A labeled with AT655 ($^{\text{AT655}}$IFNα2-M148A) was incubated on tris-NTA micropatterns loaded with IFNAR2-H10, and binding was monitored under steady-state conditions at a frame rate of 10 Hz. Transient binding of $^{\text{AT655}}$IFNα2-M148A was observed preferentially to surface areas covered with IFNAR2-H10 (Figure 5c). A maximum intensity overlay of consecutive 1000 frames confirmed the high contrast observed for ensemble experiments. From localizing individual binding events in each frame, a contrast of >100:1 was determined for $^{\text{AT655}}$IFNα2-M148A (Figure 5d), which is in good agreement with the ensemble measurements. In the presence of unlabeled IFNα2, as well as in the absence of immobilized IFNAR2-H10, only very few binding events were detected in the microstructures (Figure 5e), confirming specific binding of $^{\text{AT655}}$IFNα2-M148A to immobilized IFNAR2-H10. A histogram of the residence times of individual $^{\text{AT655}}$IFNα2-M148A binding to micropatterns loaded with maltose binding protein carrying a decahistidine tag (MBP-H10) instead of IFNAR2-H10 is shown. (f) Histogram of residence times for individual $^{\text{AT655}}$IFNα2-M148A binding to micropatterns loaded with IFNAR2-H10 (red) and MBP-H10 (blue) measured over 1000 frames. Black lines are the best fits by an exponential decay function.

Figure 5. Single-molecule detection of transient protein–protein interactions in micropatterns. (a) Binding of $^{\text{AT655}}$IFNα2 to IFNAR2-H10 immobilized on micropatterned tris-NTA. (b) (Top) $^{\text{AT655}}$IFNα2 bound to IFNAR2-H10 micropattern before (left) and after (right) chasing with unlabeled IFNα2, and (bottom) intensity profile across the indicated line (red, left image; black, right image). (c) Image of individual $^{\text{AT655}}$IFNα2-M148A molecules binding to IFNAR2-H10 immobilized into tris-NTA functionalized micropatterns (image dimensions 30 $\times$ 30 µm$^2$). (d) Maximum intensity overlay image of 1000 consecutive frames acquired during incubation of 1 nM $^{\text{AT655}}$IFNα2-M148A in the absence (left) and presence (right) of 5 µM unlabeled IFNα2. White squares indicate the patterned tris-NTA regions. (e) Average number of binding events (molecules/µm$^2$) within 1000 frames detected inside (dark gray) and outside (light gray) the tris-NTA functionalized squares upon incubation of 1 nM $^{\text{AT655}}$IFNα2-M148A in the absence (−IFNα2) and presence (+IFNα2) of 5 µM unlabeled IFNα2. As another negative control, binding of 1 nM $^{\text{AT655}}$IFNα2-M148A on the same tris-NTA micropattern loaded with maltose binding protein carrying a decahistidine tag (MBP-H10) instead of IFNAR2-H10 is shown. (f) Histogram of residence times for individual $^{\text{AT655}}$IFNα2-M148A binding to micropatterns loaded with IFNAR2-H10 (red) and MBP-H10 (blue) measured over 1000 frames. Black lines are the best fits by an exponential decay function.


CONCLUSIONS

Maleimide chemistry has frequently been employed for selective immobilization of oligonucleotides, peptides, proteins, and other chemical compounds because of its highly selective reaction with thiol groups under physiological conditions. Owing to these ideal properties of the thiol–maleimide coupling reaction, it is considered as metal-free “click chemistry”. Here, we have exploited efficient photodestruction of maleimides covalently immobilized on a protein-repellent PEG polymer brush by UV irradiation. Previous work on the photochemistry of maleimides has demonstrated the polymerization of maleimide and its N-substituted derivatives in the absence of photoinitiators, which explains the UV-induced loss of reactivity. We have employed maleimide photodestruction for micropatterned surface functionalization with a very high contrast, which in our hands was substantially higher than those observed for other photopatterning techniques such as uncaging of functional groups or photodestruction of NTA moieties. While uncaging offers the possibility for binary functional patterning, photodestruction of maleimides is ideal for generating microstructures with a single functionality. On the basis of this approach, we have implemented two complementary methods for capturing proteins into micropatterns under physiological conditions. Very efficient noncovalent targeting of His-tagged proteins into microstructures functionalized with tris-NTA-thiol was achieved, yielding micropatterns with very high contrast. We demonstrate micropatterning of His-tagged proteins directly from crude cell extracts. Thus, small quantities of highly fragile proteins and protein complexes in complex sample matrices can be assembled into functional microstructures. Proteins immobilized into tris-NTA micropatterns were efficiently eluted with imidazole, and repetitive loading into the microstructures was possible. Thus, patterned substrates can be used for sequentially targeting different His-tagged proteins or for binding experiments with different protein densities. Since most recombinant proteins are fused to a His tag for affinity purification by immobilized metal ions, this generic approach is highly applicable for in situ protein micropatterning. As an alternative approach, we implemented site-specific covalent protein immobilization by PPT into microstructures functionalized with CoA. For this approach as well, functional protein micropatterns with a very high contrast were obtained. This method is advantageous for applications that require very robust, irreversible protein immobilization. Maleimide photopatterning is simple and compatible with many substrates and surface architectures, and it can be used with many other thiol-functionalized compounds, for example, for direct coupling of peptides or proteins with free cysteine residues into micropatterns or for micropatterning of thiol-functionalized oligonucleotides.

In addition to the versatile application of maleimide-based immobilization, excellent functionality of micropatterned proteins was demonstrated. Owing to the high patterning contrast and the excellent protein-repelling properties of the surface, transient protein–protein interactions could be detected and quantitatively analyzed on the single-molecule level. While micropatterned surfaces have previously been applied for probing protein interactions on the cell surface, as well as for real-time protein–protein interaction analysis, quantitative analysis of interactions between isolated proteins in micropatterns on the single-molecule level has not yet been reported to our knowledge. The key challenge in such experiments is the presence of labeled protein in the bulk, so that even weak nonspecific binding to the surface will result in substantial background signals. The very good signal-to-background ratio observed in our study suggests that interactions of protein complexes with dissociation constants of up to 10 \( \mu \)M can be readily measured. Thus, the kinetics of protein–protein interactions with such low binding affinities can be reliably quantified on the single-molecule level.

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SUPPORTING INFORMATION AVAILABLE

Three figures showing binding curve for coupling of CoA, single-molecule dissociation kinetics of wild-type IFNα2, and photobleaching kinetics for single-molecule interaction studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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