

Light-Guided Surface Engineering for Biomedical Applications

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Free radical species generated through fluorescence photobleaching have been reported to effectively couple a water-soluble species to surfaces containing electron-rich sites (1). In this report, we expand upon this strategy to control the patterned attachment of antibodies and peptides to surfaces for biosensing and tissue engineering applications. In the first application, we compare hydrophobic attachment and photobleaching methods to immobilize FITC-labeled anti-M13K07 bacteriophage antibodies to the SiO₂ layer of a differential capacitive biosensor and to the polyester filament of a feedback-controlled filament array. On both surfaces, antibody attachment and function were superior to the previously employed hydrophobic attachment. Furthermore, a laser scanning confocal microscope could be used for automated, software-guided photoattachment chemistry. In a second application, the cell-adhesion peptide RGDS was site-specifically photocoupled to glass coated with fluorescein-conjugated poly(ethylene glycol). RGDS attachment and bioactivity were characterized by a fibroblast adhesion assay. Cell adhesion was limited to sites of RGDS photocoupling. These examples illustrate that fluorophore-based photopatterning can be achieved by both solution-phase fluorophores or surface-adhered fluorophores. The coupling preserves the bioactivity of the patterned species, is amenable to a variety of surfaces, and is readily accessible to laboratories with fluorescence imaging equipment. The flexibility offered by visible light patterning will likely have many useful applications in bioscreening and tissue engineering where the controlled placement of biomolecules and cells is critical, and should be considered as an alternative to chemical coupling methods.

1. INTRODUCTION

Strategies for the directed patterning of biomolecules at specific sites on diverse material surfaces are highly desired for multiplexed, array-based screening paradigms (2), as well as technologies such as tissue engineering, which rely on micro- or nanoscale cell–protein interactions (3). Recently, a fluorophore-based immobilization technique was described for the high-resolution, site-specific patterning of proteins such as enzymes within microfluidic channels (1, 4). This method utilizes photobleaching, a singlet oxygen-dependent immobilization mechanism, to couple dye-labeled proteins to glass and polydimethylsiloxane (PDMS) surfaces. Visible light patterning has two main advantages over other biomolecular patterning strategies. Nondamaging wavelengths, such as those used in aryl azide and benzophenone chemistries (5, 6), are avoided. Second, the reaction can be rapidly carried out in aqueous, neutral buffers preserving protein functionality.

In order to facilitate the implementation of photoattachment chemistry in the development biomolecular and/or cellular arrays, further studies are necessary to expand upon the scope of materials which can be surface engineered using this process, namely, polymer surfaces. Also, efforts to facilitate photopatterning, such as implementation with laser scanning confocal microscopes and software-driven, automated bleach parameters, are relatively unexplored. In addition, a reverse-coupling technique would be desirable. In this case, instead of labeling the soluble protein with a dye, the target surface is conjugated to a fluorophore. This has several advantages. Dye labeling of proteins is not required, and in this scenario, one photoactivable surface could be employed for the patterning of multiple biomolecules.

In this study, we explored the utility of visible light-guided surface engineering for site-specific antibody immobilization on a differential capacitance-based viral biosensor (7) as well as a polyester filament-based fluorescence detection platform (8–10). We then extended this photopatterning technique to couple the cell-adhesion peptide RGDS (11) to a surface layer of poly(ethylene glycol)-fluorescein (PEG-FITC) with the intent of developing a substrate for site-specific biomolecular and cellular patterning. This latter example also features low nonspecific adsorption, a limitation not addressed in previous visible-light photopatterning techniques (4). In these initial studies, we observed that a variety of surfaces are amenable to photopatterning, and that the simplicity of these techniques makes automated surface patterning readily accessible to biological laboratories with access to a laser scanning confocal microscope. This method may have broad applicability in the field of biosensors which rely on an *a priori* pattern of binding partners as well as tissue engineering applications which rely on spatial control of cells in their construction. Photocoupling can also be used to functionalize nanoparticles and other bioconjugates bearing amine or PEG-FITC moieties.

2. DETAILED EXPERIMENTAL PROCEDURES

Antibodies were photocoupled onto silicon dioxide and polyester surfaces for sandwich immunoassays. In the third portion of this report, peptides were photoimmobilized on PEG-FITC-coated capture substrates in order to modulate cell attachment.

2.1. Photopatterning of Capture Antibody on Capacitive M13K07 Sensor. A previously characterized, capacitive sensor for the detection of the M13K07 bacteriophage (7) was prepared for use under dry argon at 25 °C with three rinses of anhydrous acetone (Sigma, St. Louis, MO). The surface was then immersed in a 4% solution of 3-aminopropyltriethoxysilane (United Chemical Technologies, Bristol, PA) in anhydrous

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acetone for 10 min, followed by 5 min immersions in anhydrous acetone and ultrapure water, and stored at 25 °C in a desiccator. Successful silanation of capacitor surfaces was verified by comparing the adsorption of fluorescein-conjugated bovine serum albumin (1 mg/mL in borate pH = 8.5) on treated and untreated chips. Immediately prior to use, the silicon dioxide surface was layered by micropipette with 100 μ L of a 100 μ g/mL FITC-anti-M13K07 monoclonal antibody (mAb), 2.8 mol FITC/mol IgG) in 100 mM sodium bicarbonate buffer, pH = 8.5.

The FITC-labeled mAb (anti-M13K07 Ms IgG₁, GE Healthcare) was immobilized onto the biosensor surface using an upright laser scanning confocal microscope (LSM 510, Carl Zeiss). The timed bleach function provided in the microscope manufacturer's software package, as used for fluorescence recovery after photobleaching (FRAP) studies (12), was used to control the laser intensity of the 488 nm line of a 30 mW Argon laser, as well as the number of scanning iterations. A 4 $\times$, 0.1 NA objective was used to scan the 200 μ m² capacitor surface. Two capacitors were each bleached using different bleach iterations (50 vs 10, using 100% laser intensity). Using the manufacturer's software, the laser was rastered in parallel with the plates of the capacitor, and the bleaching time was less than three minutes. The surface was rinsed three times with PBS, pH = 7.4, for 1 min each. Next, the capacitors were coated with the same amount and concentration of FITC-mouse IgG₁ isotype control mAb (3 mol FITC/mol IgG), and bleached at 100% intensity for 50 iterations.

The functionality of the photoimmobilized surface was ascertained by sandwich immunoassay. The surface was rinsed with PBS, and exposed to a solution of 100 μ L of M13K07 bacteriophage (3.3×10^{11} virions/mL) for 30 min at 25 °C. The surface was rinsed with PBS and incubated with 100 μ L of Cy5-conjugated anti-M13K07 mAb (1.72 mol Cy5/mol IgG, 50 μ g/mL in PBS) for 30 min at 25 °C. The chip was rinsed with PBS and imaged immediately on a Nikon TE2000U inverted fluorescence microscope equipped with a Cy5 fluorescence cube, an Exfo X-Cite 120 metal halide excitation light source, and a C7780 Hamamatsu cooled CCD camera. Mean fluorescence intensities were measured by analyzing ROIs defined along capacitor boundaries using *Image Pro Plus 5.0* (Media Cybernetics), with data plotted using *SigmaPlot 9.0* (SYSTAT).

2.2. Photopatterning of Antibody on Polyester Filament-Based M13K07 Sensing Array. We investigated the utility of photoattachment chemistry in the development of high-throughput biomolecular screening. A previously described polyester monofilament-based system for viral detection based on hydrophobically immobilized capture mAb for multiplexed sandwich immunoassays (9) was adapted for the detection of M13K07 bacteriophage, a test virus, based on *photopatterned* mAb. All procedures were carried out at 25 °C. A 120 μ m polyester monofilament (Sulky Corp.) was wrapped around a PhastGel sample applicator (GE Healthcare) and inlaid within the concave teeth as previously described (9). The filament/comb apparatus was rinsed in successive washes of 70% ethanol, 10% HCl, and Milli-Q water and dried overnight prior to use. A 0.75 μ L solution containing 100 μ g/mL of anti-M13K07 mAb or mouse IgG₁ isotype control mAb in 100 mM borate buffer, pH = 8.5, was pipetted on each loading tooth for either timed passive incubations or photobleaching sessions. The distance between neighboring teeth is 0.5 cm. For photobleaching, the comb was inverted and suspended within a humidified 60 mm Petri dish upon the Nikon TE2000U microscope stage. For photobleaching, the X-Cite 120 metal halide light source was used in conjunction with a FITC cube and 4 $\times$ objective at full lamp intensity (1.4 W from microscope emission port, as

determined by the manufacturer) for specific durations. Bleaching was monitored by measuring filament mean fluorescence using *Image Pro Plus 5.0*.

To test the functionality of the passively adsorbed and photopatterned surfaces, combs were rinsed three times in PBS, immersed in a multichannel pipet basin containing 3.3×10^{11} virions/mL M13K07 in PBS for 30 min, and rinsed three times with PBS. The combs were subsequently immersed in a basin containing 50 μ g/mL Cy5-anti-M13K07 mAb for 30 min and rinsed in PBS.

Filaments were immediately fastened to a standard, pre-cleaned microscope slide with tape, and the fluorescence measured using a Genepix 4000B microarray scanner (Axon Instruments) equipped with a 635 nm laser line as previously described (9). The mean fluorescence intensity of ROIs defined along discrete filament spots was measured by *Image Pro Plus* on grayscale 16-bit images, and plotted using *SigmaPlot*.

2.3. Photopatterning of Poly(D-lysine)-Coated Glass Substrate with PEG-RGDS. Poly(D-lysine)-coated 35 mm glass-bottom dishes with no. 1 borosilicate coverglass (MatTek Corp.) were coated with a heterobifunctional PEG spacer (MW = 3400) containing an *N*-hydroxysuccinimide ester (NHS) amine-reactive group and a fluorescein isothiocyanate moiety (NHS-PEG-FITC, Nektar Therapeutics) by incubating the surface with 0.2 mg of NHS-PEG-FITC in 100 mM borate buffer, pH = 8.5, for 1 h, followed by three rinses in borate buffer. Unreacted amine groups were quenched by 20 min incubation with 1 mg of sulfo-NHS-acetate (Pierce Chemical) in borate buffer. This acetylation reaction rendered the remaining amines unreactive. The glass dish was then incubated with 100 μ g/mL of the fibronectin-derived cell-adhesion peptide RGDS (>95% purity, Genscript Corp.) in borate buffer. Photobleaching of 1 mm rectangular areas delineated by taped foil photomasks was achieved using a 10 $\times$ objective with the aforementioned inverted fluorescence microscope and light source for timed intervals, with mean fluorescence levels monitored to confirm photobleaching. Typically, the time necessary to reduce intensity levels 20% of initial fluorescence was less than 5 min. Photobleaching and RGDS passive adsorptions were followed by three rinses in borate. RGDS presence on the glass substrate was qualitatively assessed using fluorescamine.

Since RGDS has been demonstrated to promote fibroblast adhesion and spreading (11), we evaluated the bioactivity of the coupled RGDS using a fibroblast adhesion assay. 3T3 fibroblasts (ATCC) were cultured to 70% confluence in DMEM containing 10% bovine calf serum under incubation at 37 °C, 5% CO₂. The cells were then seeded in each dish at a concentration of 1000 cells/mL and incubated overnight under the same culture conditions. The next day, dishes were rinsed three times with PBS, and fixed for 20 min at 25 °C in 4% paraformaldehyde in PBS. Dishes were incubated with 0.5% trypan blue prior to the fixation step to ascertain cell viability via dye exclusion. The dishes were then rinsed in PBS containing 100 mM glycine and imaged by phase contrast.

2.4. Comparison of Antibody Chemical Crosslinking and Photoattachment on PEG-FITC and PEG-Maleimide-Coated Glass Substrates. Poly(D-lysine)-coated MatTek dishes were coated with 0.2 mg of NHS-PEG-FITC or thiol-reactive NHS-PEG-maleimide (NHS-PEG-MAL, MW = 3400, Nektar) and acetylated as described in section 2.3. To generate sulfhydryl groups for covalent coupling of mAb to NHS-PEG-MAL, 5 mg of anti-M13K07 mAb in 1 mL PBS-EDTA, pH = 7.4, was reacted with a 10-fold molar excess of 2-iminothiolane (Traut's reagent, Sigma) for 30 min at 25 °C. Excess Traut's reagent was removed by three centrifugation cycles of mAb/Traut's reagent on an Amicon-4 Ultra 100K MWCO spin column filtration device (Millipore) using PBS-EDTA as the rinse and

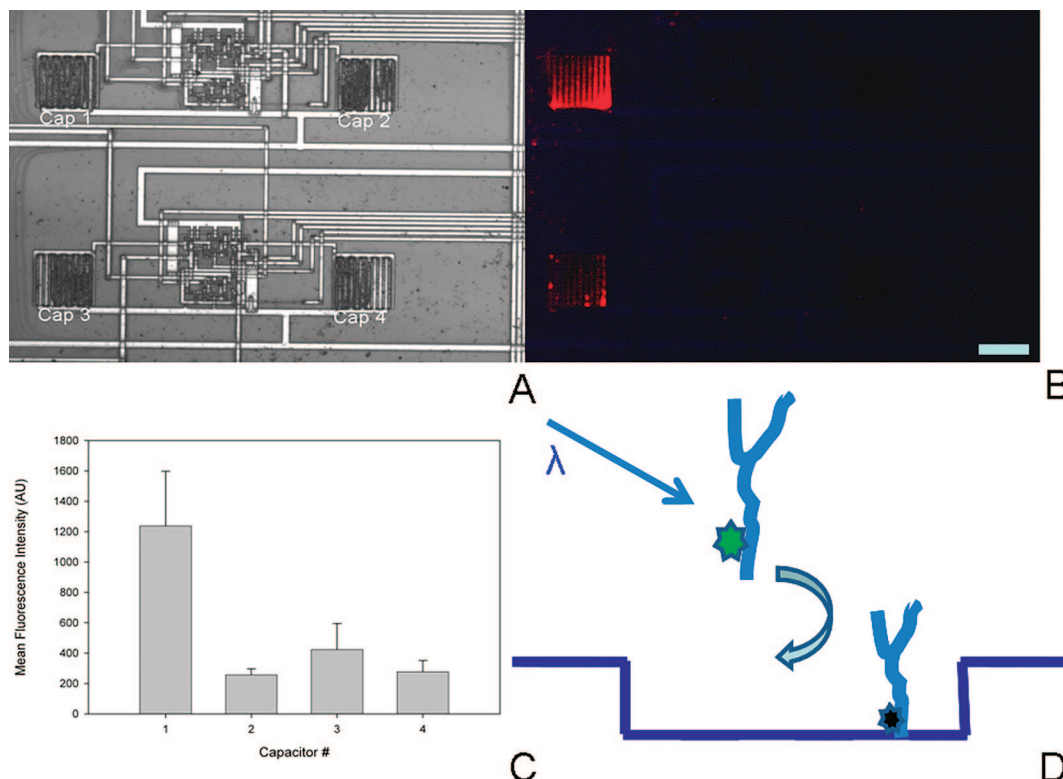


Figure 1. Light (A) and fluorescence (B) micrographs of a multiplexed capacitive biosensor photopatterned with anti-M13K07 monoclonal antibodies and isotype controls, with measured fluorescence intensities shown in (C). Parameters and reagents used for photoattachment using a confocal microscope: Cap 1, 30 mW, 50 bleach iterations, anti-M13K07 photopatterned; Cap 2, 30 mW, 50 bleach iterations, anti-Ms IgG photopatterned; Cap 3, 30 mW, 10 bleach iterations, anti-M13K07 photopatterned; Cap 4, no patterned antibody. (C) Fluorescence intensity measurements of Cy5-anti-M13K07-incubated sensors, reported as mean $\pm$ SD, $n = 3$. Bar = 10 μ m.

resuspension buffer. For chemical cross-linking, 200 μ g of mAb in 200 μ L PBS-EDTA was pipetted onto the PEG-MAL surface and was reacted overnight at 4 $^{\circ}$ C. For photocrosslinking, 200 μ g of purified mAb in a 200 μ L solution of PBS-EDTA was pipetted on the PEG-FITC surface. The microwell of the dish was then photobleached using the X-Cite 120 light source (1.4 W) using a 2 $\times$ objective for 10 min. Following both reactions, the dishes were rinsed 3 $\times$ with PBS, saving the initial aspirate. Aspirate was collected from 20 MatTek dishes bearing either the PEG-FITC or PEG-MAL attachment linker. Protein concentration was determined using the Coomassie Plus assay (Pierce) according to manufacturer instructions.

3. RESULTS AND DISCUSSION

Our studies suggest that photobleaching can be used to pattern proteins and peptides on SiO₂, polyester, and glass surfaces. Photopatterning of anti-M13K07 mAb to capacitor surfaces was site-specific, with an achieved spatial resolution below 10 μ m (Figure 1a,b). No signal was detected due to photopatterned IgG₁ control mAb capture of virus, or nonspecific binding of the virus itself, as indicated by mean fluorescence output (Figure 1c). In addition, no secondary antibody fluorescence was detected beyond the capacitor regions of the chip, indicating that diffusion of the bleached fluorophore did not interfere significantly with site-specific coupling, and that nonspecific adsorption was not significant. Specifically, the average fluorescence from 10 different noncapacitor regions of the chip was 237 ± 67 , which was similar to control capacitor values (Figure 1c).

The fluorescence signal from the sandwich immunoassay generated from filament-coupled anti-M13K07 mAb using photoattachment chemistry was 2- to 3-fold higher than the signal generated by passively adsorbed antibodies (Figure 2).

The enhanced signal could be due to either (a) an initially higher coupling efficiency using the photobleaching technique, and/or (b) greater preservation of bioactivity of photocoupled mAb. Although further studies would be required to differentiate between these two possibilities, we did observe that photocoupled antibodies appeared to be more tightly coupled, since successive washes of filament were associated with loss of passively adsorbed antibodies but with low loss of photocoupled antibody (data not shown).

Our studies also demonstrated that cell-adhesion peptides can be site-specifically coupled on tissue culture substratum (Figure 3). Irradiated PEG-FITC regions (Figure 3a) promoted fibroblast attachment similar in density and morphology observed on the native poly (D-lysine)-coated MatTek surface (Figure 3e). Nonirradiated PEG-FITC regions on the same were resistant to cell adhesion (Figure 3b). The lack of cell adhesion in nonirradiated regions was most likely due to the PEG coating on the surface which blocks adsorption of RGDS (Figure 3c), as well as the fibroblasts themselves (Figure 3f). The PEGylation of the surface was found to be important for controlling cell attachment, as acetylation of the surface alone was not sufficient (Figure 3d). Complete photobleaching of the entire PEG-FITC surface (from 1 to 3 h of continuous lamp illumination) did not result in cell attachment (Figure 3g,h). In a separate experiment, we demonstrated that attachment of 3T3 cells to PEG-RGDS was most likely due to specific peptide–integrin interactions, since competitive inhibition experiments (addition of 2 mM soluble RGDS peptide) reduced cell attachment by 82% (Supporting Information).

The mAb coupling efficiency of photoattachment and chemical crosslinking was compared and found to be similar. The efficiency of photocoupling was measured to be 3.3 pmol/cm². Thiol-maleimide crosslinking, a technique which has long been

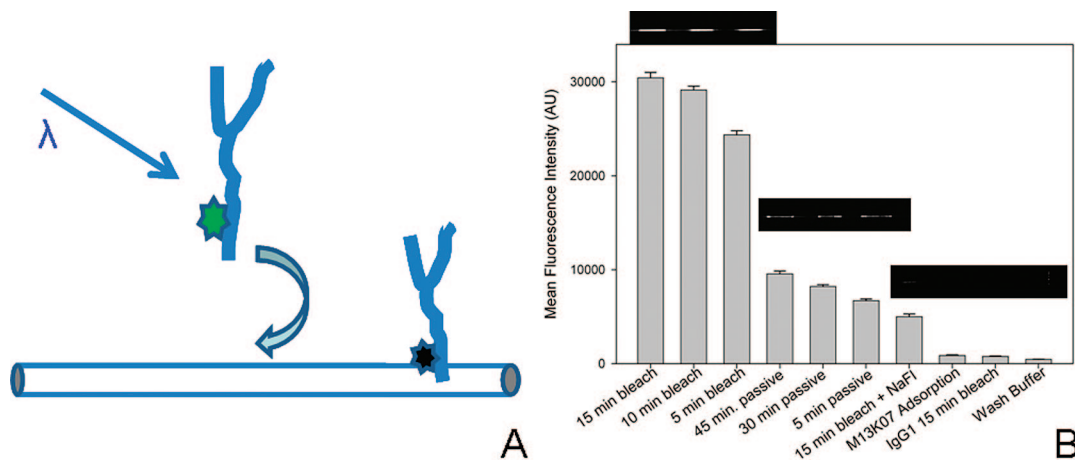


Figure 2. Schematic of a strategy for photocoupling of antibody to polyester filament (A) and resulting sandwich immunoassay Cy5-anti-M13K07 intensities (B). A representative filament is overlaid on the graph in (B).

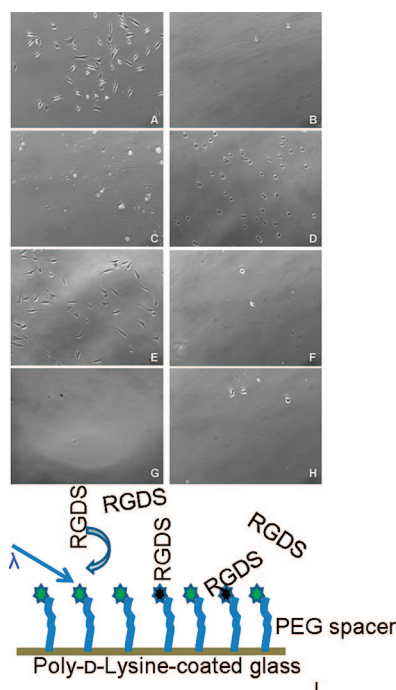


Figure 3. Representative micrographs of 3T3 fibroblast attachment on surface-engineered poly(D-lysine) MatTek dishes from three trials using PEG-FITC-based photoattachment diagrammed in (I). Experimental conditions for each substrate: (A) PEG-FITC and acetylation, RGDS photopatterned; (B) Same as in (A), but in an area where RGDS was not photopatterned; (C) PEG-FITC and acetylation, RGDS passively adsorbed; (D) No PEG-FITC, acetylation, RGDS passively adsorbed (cells are rounded); (E) No PEG-FITC, no RGDS (native surface); (F) PEG-FITC, no acetylation, no RGDS; (G) PEG-FITC and acetylation, bleached 1 h prior to cell adsorption, no RGDS; (H) PEG-FITC and acetylation, bleached 3 h. prior to cell adsorption, no RGDS. Cells in (A) and (E) showed similar viability of seeded cells as indicated by trypan blue exclusion (not shown). (50 $\times$ magnification).

utilized for efficient, facile conjugation of proteins to material surfaces (13), resulted in a higher mAb coupling efficiency (4.2 pmol/cm²). Although the chemical crosslinking technique was capable of immobilizing a higher quantity of mAb, further optimization of experimental conditions may produce higher yields for visible light photoconjugation, such as buffer strength, reaction volume, PEG molecular weight, and reagent concentrations. Furthermore, photopatterning offers control over site specificity of coupling, which in itself may warrant its future implementation.

The light patterning properties of the confocal microscope facilitated the efficient photoattachment of antibodies on the biosensor surface. With a fixed laser intensity (full output), the number of bleach iterations within the FRAP software module controlled the coupling efficiency of FITC-mAb. Furthermore, the ROI can be readily defined within the software to pattern microscale regions. The patterning of antibodies upon groups of four capacitors was achieved within 5 min, and real-time fluorescence intensity measurements within the software confirmed successful photobleaching. The confocal microscope should facilitate the implementation of photoattachment methods in various laboratories in biological and biomedical sciences, where they are readily available. Since these laboratories often do not have access to cleanroom facilities needed for photolithography, photoattachment chemistry may constitute a viable alternative to the in-house development of custom molecular and cellular arrays as diagnostic or biological study tools.

In addition to extending techniques related to the coupling of dye-conjugated biomolecules on surfaces, we also hypothesized that biomaterial substrates could be engineered to bear the photoreactive groups, thus obviating the need for bioconjugating proteins, a step which can often compromise native function. Poly(ethylene glycol) (PEG) has long been utilized for reducing protein adsorption to biomaterial surfaces, is biocompatible, and is amenable to the grafting of functional groups (14). NHS-PEG-FITC is a commercially available heterobifunctional reagent used to detect PEGylation efficiency in bioconjugate chemistry, and combines the properties of PEG with the bioconjugation moiety NHS (amine-reactive) for immobilization on a variety of substrates, as well as a FITC dye, to enable detection of the PEGylated species *in vitro* or *ex vivo*. We observed that pipetting an RGDS peptide solution over the PEG-FITC-functionalized glass substrate, followed by photobleaching of the dye groups, resulted in site-specific immobilization of the adhesion peptide, as indicated by fibroblast binding exclusively on regions of irradiation (Figure 3a). Therefore, the use of this reagent in the development of biosensor arrays affords the resistance of the coating to nonspecific adsorption of proteins.

Dye photobleaching generally involves the energy transfer from the dye in the excited state to molecular oxygen in the ground state (15–17). As a result of photobleaching, singlet molecular oxygen is produced, leaving the fluorophore with an unpaired electron. While the process of visible light coupling of dye-conjugated species has not been completely elucidated, it is possible that the unpaired electron on the dye serves as a binding site to an electron-rich substrate. Our data provide further evidence for the photobleached fluorophore as the

coupling moiety. When including 1 mg/mL sodium fluorescein (NaFI) as a free dye in the initial mAb coupling solution, we observed a decrease in fluorescence signal from the virus-associated Cy5 secondary antibody, indicating that the coupling technique was associated with fluorophore photobleaching (Figure 2b). In previous work, oxygen depletion in the coupling medium was associated with significantly lower photoattachment efficiency (*I*), which is consistent with our report that photobleaching chemistry is essential for the coupling reaction. Provided that photobleaching events drive the coupling of biomolecules onto the surfaces tested in this work, it is important to take necessary precautions when carrying out individual reactions. Samples were only exposed to ambient laboratory light for durations of <5 min, to minimize premature photobleaching due to the photolability of fluorescein.

This work expands upon previous approaches to achieve photopatterning of biomolecules, through adapting a confocal microscope to perform automated patterning of microscale regions, and by expanding the scope of surfaces which are candidate substrates for this technique. Future work will focus on the controlled orientation/deposition of cells on adhesion peptide-patterned surfaces. Such a feature would be useful in the development of arrays which incorporate cells as biosensors, or in the synthesis of 3-D tissue constructs, in which hierarchical organization of cells and proteins elicit specific cell functions (18). Our technique features the microscale resolution necessary to construct such devices, and nanoscale resolution is potentially achievable with this technique, when used in conjunction with techniques to overcome diffraction barriers, such as stimulated emission depletion fluorescence microscopy (STED) (19, 20). While fluorescein was used as the coupling moiety in this work, it has been demonstrated that phototattachment strategies can be extended toward the patterning of multiple dye-labeled species, such as Alexa Fluor-labeled proteins (1).

4. CONCLUSION

Our studies have extended the utility of visible light photoattachment chemistry in creating biomolecular and/or cellular arrays in well-defined microscale regions on diverse substrates. The method is rapid and can be easily controlled on a spatial and temporal scale. Furthermore, protein activity is retained, and the high-efficiency conjugation of proteins using light patterning may facilitate the development of high-sensitivity screening platforms. Additionally, we have shown that a photoreactive substrate can be the mediator for photocoupling, eliminating the need for bioconjugation of species prior to patterning. In forthcoming applications which utilize cells as biosensors, this technique may be useful for controlling cell orientation and spreading. This rapid, flexible technique is readily accessible and can be implemented in a number of biomedical applications.

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Supporting Information Available: Additional information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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