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Photobleaching-activated micropatterning on self-assembled monolayers

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Abstract

Functional chemical micropatterns were fabricated by exploiting the photobleaching of dye-coupled species near methacrylate self-assembled monolayers. Using this approach we have demonstrated that multiple chemistries can be coupled to the monolayer using a standard fluorescence microscope. The surface bound functional groups remain active and patterns with feature sizes down to 3 μm can be readily achieved with excellent signal-to-noise ratio. Control over the ligand binding density was demonstrated to illustrate the convenient route provided by this platform for fabricating complex spatial gradients in ligand density.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Surfaces bearing spatially localized chemical or biological functionalities have, in recent years, become essential tools in the fields of life science and bio-nanotechnology. Such substrates have found a home in a diverse range of applications including chemical and biosensors [1, 2], and as a means of producing tailored environments for the study and manipulation of cells and tissue [3–6]. Alongside this rapidly increasing demand for chemically defined surfaces, the opportunities as well as the need for accessible, inexpensive, robust patterning techniques are clear.

The promise of engineered surfaces has driven an impressive collaboration between many scientific disciplines. A diverse toolbox for surface patterning of proteins has been established which includes powerful techniques such as microcontact printing (μCP) [2, 7], dip pen nanolithography (DPN) [8], micellar nanolithography [9] and more recently thermochemical nanolithography (TCNL) [10]. High-resolution patterning techniques like these, however, often require dedicated lithographic equipment, which while necessary for nanopatterning, presents a hurdle for the adoption of micropatterning where lower resolution is required. Here we present a facile approach to achieve relatively sophisticated micropatterning that relies on tools and chemistries commonly available in life science laboratories.

Recently, it has been shown that the photobleaching of fluorescent dyes can facilitate the deposition of dye-coupled functional groups onto protein-coated surfaces [11, 12]. Photobleaching-activated patterning (PAP) of dye-coupled functional groups works through the free radicals generated during photobleaching, which become covalently bound to methacrylate groups on a nearby surface. This is an appealing approach given the commercial availability and diversity of dye conjugates, and because it requires little investment in either dedicated lithographic equipment or custom chemistry. Other photonic micropatterning approaches exist [13, 14], but they typically require UV sources with specialized optics, or custom-made chemistries such as caged ligands [15, 16]. PAP has the further appeal that it is relatively straightforward to spatially vary the ligand density by tuning the total integrated energy applied during photobleaching—a capability not easily realized by most other lithographic techniques. The one practical drawback of the PAP thus far, has been the lack of background passivation in the non-patterned areas. The use of an adsorbed protein layer, bovine serum albumin (BSA), as the substrate for deposition severely restricts the ability to engineer the properties of the surface, and the chemistries to which the surface can be exposed post-patterning.

Here, we demonstrate that PAP can be used to pattern self-assembled methacrylate monolayers to produce surfaces

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with well-defined, addressable functionalities at all locations. Further, we demonstrate that PAP possesses a straightforward capability to tune the ligand density on the micron scale on SAMs. Since the absorption of many common fluorescent dyes is in the visible spectrum, patterning can be achieved using a standard fluorescence microscope and a bright fluorescence lamp [11]. In the simplest realization, patterns are defined by placing an appropriate mask at the microscope's field aperture. PAP is then used to produce micropatterns of the desired chemical linker. Once the micropatterning is completed, the unreacted methacrylate groups provide a chemical handle for further surface modification, allowing the properties of the surface to be controlled. In the system presented here, outlined in figures 1 and 2, we show that the surface can be modified with both biotin and PEG-amine groups and that these groups can be addressed specifically by appropriate labels. A thiol-linked polyethylene glycol (thiol-PEG) is used to render the surface resistant to protein adsorption through a thiol-acrylate addition reaction. To demonstrate this method's powerful but simple approach to tune the local ligand density, the surface bound intensity versus exposure time is measured for both bound biotin and bound PEG-amine, and gradients on the micron scale are demonstrated.

2. Methods and materials

2.1. Preparation of methacrylate self-assembled monolayer

Unless otherwise stated, materials were acquired from Sigma-Aldrich. Glass cover slides (#1.5) (VWR) were cleaned by sonication for 15 min in 2% Hellmanex, rinsed extensively in pure water and dried under nitrogen. Prior to deposition of the self-assembled monolayer (SAM), substrates were piranha cleaned in concentrated sulfuric acid and hydrogen peroxide (3:1) for 45 min, after which they were rinsed, first in pure water, and then in ethanol before being dried at 75 °C in a convection oven. Self-assembled monolayers were formed by immersing the glass slides in acetone containing 1% 3-(trimethoxysilyl)propyl methacrylate under a nitrogen atmosphere for 90 min. Prepared surfaces were washed by sonication in acetone and ethanol for 5 min each and the slides were dried under a stream of nitrogen.

2.2. Surface patterning by photobleaching

Biotin functionalized patterns were produced using biotin-4-fluorescein (B4F) prepared in phosphate buffered saline (PBS) at 100 μg ml^{-1}. Prior to use the B4F stock was mixed with 20 kDa polyethylene glycol (PEG) solution (20% w/v) or 70 mM of Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) to give a final B4F concentration of 50 μg ml^{-1}. The B4F/PEG solution was then sealed between the activated glass slide and a clean microscope slide. *Amine functionalized patterns* were produced using fluorescein–PEG-NH_2 dissolved in PBS at 600 μg ml^{-1}, which was sealed between the activated glass slide and a clean microscope slide.

Prepared slides were placed on the motorized stage (H117 prior scientific) of a Nikon TE2000 fluorescence microscope and the methacrylate surface was patterned through a 100× 1.4 numerical aperture (NA) objective lens and a Nikon Intensilight. Masks printed at 1200 dpi on transparencies using an inkjet printer were positioned in the field diaphragm and imaged on to the slide's surface. After patterning, the slides were washed by sonication in pure water for 10 min. Large area patterning was achieved with a step-and-repeat approach using a macro written in the Nikon Elements software. The Nikon Perfect Focus System was used to maintain the focal plane of the microscope objective and the surface of the sample.

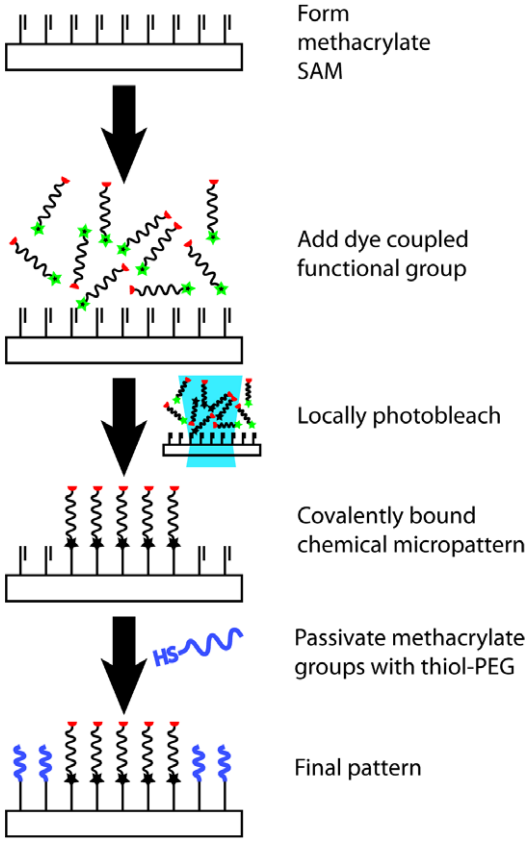

Figure 1. (a) Schematic for the photobleaching-activated surface functionalization. Functional groups coupled via a linker to a fluorescent dye become covalently bound to methacrylate surface groups when the dye is photobleached. Patterning is achieved by using a mask to selectively illuminate the surface. The properties of the surface can be modified using thiol-coupled reagents to target the remaining methacrylates; in this case a thiol-PEG was used to produce a surface that is resistant to non-specific adsorption.

2.3. Passivation of non-patterned areas

Methacrylate functional groups remaining on the surface after patterning were exposed to a 1 mg ml^{-1} solution of thiol-PEG (*O*-[2-(3-mercaptopropionylamino)ethyl]-*O*′-methylpolyethylene glycol, molecular weight 5000 Da) in PBS for 2 h to minimize non-specific protein adsorption and suppress cell interactions with non-patterned areas. The patterned surfaces consisting of covalently coupled biotin or PEG-amine with PEG were then cleaned by sonication in pure water for 10 min.

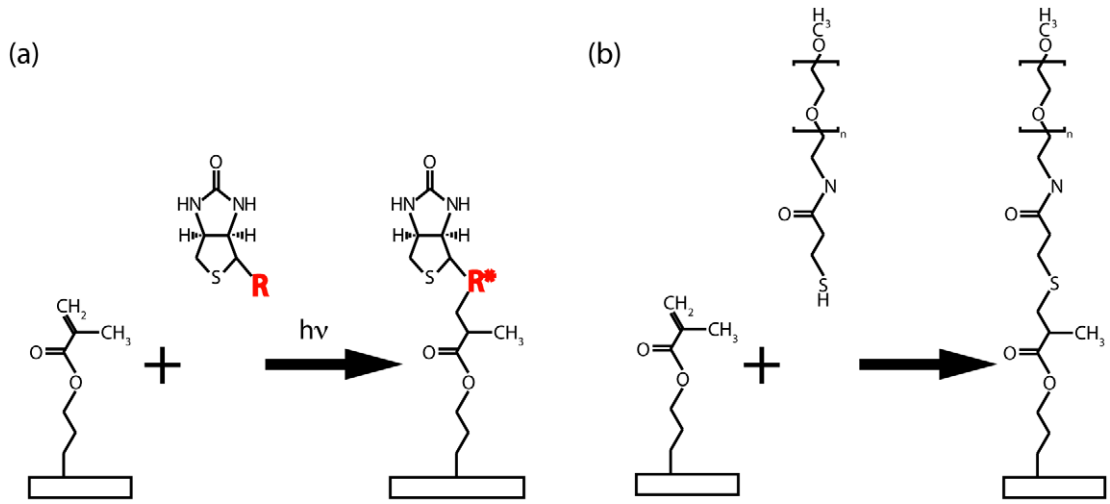

Figure 2. (a) and (b) show detailed schematics of the chemistries for the functionalization and passivation steps of the patterning process respectively. (a) Functional groups, such as biotin (pictured) or amines are coupled to the methacrylate surface groups through the photobleaching of the linked fluorescent dye R to the radical R^*. (b) The surface methacrylate groups and thiol-PEG react through a Michael type addition reaction in the presence of a primary amine catalyst.

2.4. Microscopy

All microscopy was performed on a Nikon TE2000 Microscope, equipped with a Perfect Focus System, running Nikon Elements software. A Nikon Intensilight provided fluorescence illumination and images were acquired on a Roper Cascade 512B camera. Further details of the exposure conditions are given in the text.

3. Results and discussion

A platform based on a methacrylate self-assembled monolayer (SAM) combined with PAP provides two orthogonal chemical routes to achieve the combined goals of (i) localized chemical and/or bio-functionalization and (ii) global passivation in the non-patterned regions. The first route utilizes photobleaching-activated deposition of dye-labeled molecules (PAP) to locally address the SAM and covalently bind chemical functionalities. The second route implements a thiol-acrylate Michael addition reaction to modify the general properties of the surface.

PAP applied to methacrylate SAMs generates localized micro-functionalization of the surface by a covalently bound chemical linker of choice. Local photobleaching of the dye-linked functional groups is achieved by imaging a mask placed in the back of the field diaphragm onto the methacrylate surface. The radicals generated by the photobleaching diffuse through the solution until they are either quenched or react with the methacrylate surface where they become covalently immobilized. This approach is demonstrated by micropatterning two common chemical moieties, fluorescein-coupled biotin (B4F) and fluorescein–PEG-amine, onto the surface from solution as shown in figure 3.

The activity of the photobleach-activated radicals before they bind to the surface determines the fidelity of the final photopatterned area compared with the mask. To achieve optimal resolution and minimize unwanted background deposition of these small and therefore rapidly diffusing molecules, it is important to limit the lifetime of the photo-induced radicals in solution. This insures that only radicals generated near the surface have the chance to interact with the

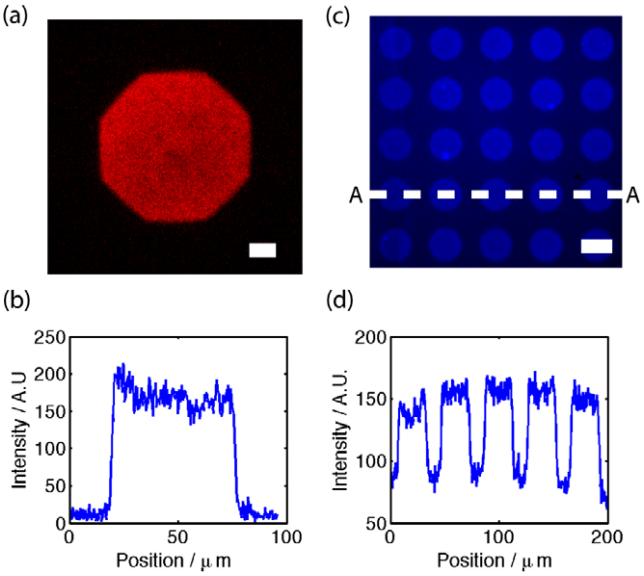

Figure 3. (a) A self-assembled methacrylate monolayer was covered with biotin-4-fluorescein solution and the solution photobleached at 488 nm through an octagonal mask imaged on to the surface. The result is localized deposition of biotin functional groups on to the methacrylate surface. The remaining methacrylate groups were reacted with thiol-PEG and the octagonal biotin region was specifically labeled with CY5-streptavidin. Scale bar 10 μm. (b) The plot shows the intensity profile across the micropatterned octagon. (c) Self-assembled methacrylate monolayers selectively functionalized with amine groups by depositing fluorescein–PEG-NH2 from solution. The amine groups were selectively labeled with AlexaFluor 350-NHS. Scale bar 25 μm. (d) The plot shows the intensity profile across section AA indicated in the fluorescence image.

methacrylate and contribute to the surface functionalization, minimizing the diffusion of radicals outside of the illumination pattern. To achieve this, we add polyethylene glycol (PEG, 20 kDa) to the patterning precursor in order to increase the solution's viscosity and provide a physical barrier to diffusion. Additionally, water soluble vitamin E (Trolox), a radical scavenger, can be used as a buffer against excess radical diffusion. The average displacement of the radical is then limited by the probability of encountering and being quenched by a scavenger. It has also been shown in separate work that using highly oxygenated solutions [11], speeds up the process by increasing the advent of free radical formation.

Using this approach, chemical micropatterns such as the biotinylated octagon (50 μm diameter) shown in figure 3(a) are generated. Visualized with fluorescently labeled streptavidin, intensity variations in such microstructures are typically below 25% of the mean intensity within the functionalized region. Due to the solution-based approach to patterning, the edges of the patterns tend to be slightly brighter than the inner areas. This results from the higher probability that unbleached species will diffuse into the illumination area during an exposure from the edge, become bleached and then stick locally as opposed to diffuse into the middle of the pattern. This effect can be minimized with lower exposure times and by patterning small areas.

We have also demonstrated the deposition of PEG-linked amine groups from a solution of fluorescein–PEG-amine, as shown in figure 3(c). The presence of the free amines on the surface was confirmed with fluorescence microscopy following a staining of the amines via reaction with an AlexaFluor-NHS ester. Figure 3(d) shows a line profile across the array of 25 μm diameter circles, revealing good uniformity both within and between the patterns. All amine patterns demonstrated in this paper were created from solutions containing no PEG/Trolox (since the moderately large fluorescein–PEG-amine diffuses more slowly than the B4F) and imaging was performed without passivation.

The resolution of the PAP technique was measured using a photomask with lines of varying thicknesses and a solution of pure fluorescein–PEG-amine (figure 4(a)). The thinnest lines produced have a FWHM of 3.1 μm as shown in figure 4(b). The same resolution is seen at the 'edges' of the micropatterns such as the octagons (figure 3(b)) and circles (figure 3(d)). Thus, even under conditions with no added PEG for increased viscosity or radical-scavengers (Trolox), the typical line width is comparable to that achieved in earlier work, $\sim$1.2 μm, based on a laser scanning system with similar optics [12]. There are two caveats for direct comparison between this study and the scanning laser work: the laser deposited molecules were BF4 rather than fluorescein–PEG-amine. The slower diffusion of the larger molecules increases the theoretical resolution possible relative to the smaller species. On the other hand, working with a laser allows for working at optimal intensities so that exposure time is minimal and excess diffusion and smearing at the borders limited. In any case, wide-field PAP is capable of producing highly resolved features relevant for a diverse set of applications in biological and bioengineering assays. Further, if slightly higher resolution is required,

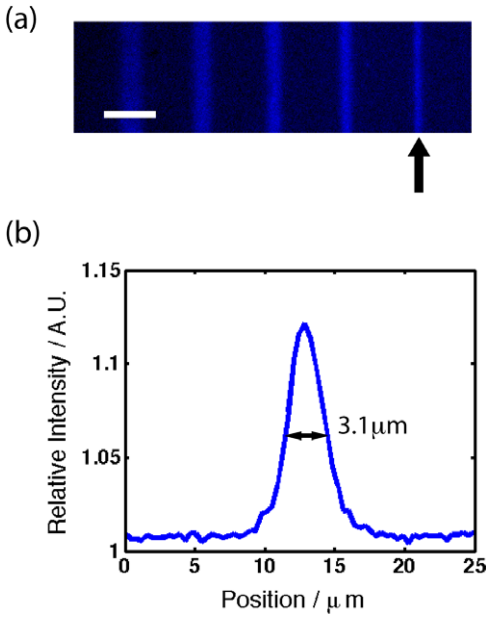

Figure 4. A series of PEG-amine lines patterned on a methacrylate SAM was used to test the resolution of the system. (a) A fluorescence image of the lines, labeled with AlexaFluor 350-NHS. Scale bar is 20 μm. (b) Shows the intensity profile across the final line in the series as indicated by the arrow in (a). The FWHM of this line is 3.1 μm.

implementation of laser-assisted PAP on a SAM would allow for the advantage of passivation or if desirable, the background of some other molecule of interest. Other simple improvements are possible with the use of a brighter illumination source, such as that achieved with a laser-based epi-fluorescence microscope. This would allow for shorter photobleaching times, which would decrease the blurring at the edges of the intended patterns and increase resolution.

Robust passivation is an equally important aspect of producing ideal micropatterned substrates for cell-interface studies. Here, we demonstrate the utility of this platform for the fabrication of patterned, bioactive surfaces passivated by polyethylene glycol (PEG)—a standard choice for minimization of non-specific adsorption of proteins to surfaces. Passivation typically precedes bio-functionalization of the arrays to minimize non-specific binding to the SAM. In this step, thiol-PEG (PEG, MW 5000) is covalently bound to the acrylate groups via a sulfo-ester bond. Fluorescence microscopy was used to quantify the non-specific binding of proteins to the PEG passivated SAMs. For the fluorescence analysis, the passivated surfaces were incubated with Cy5-streptavidin and then rinsed prior to imaging. Several areas across the surface were imaged at random and the mean pixel intensity calculated. Background intensity levels at each region were determined by photobleaching the Cy5. The mean background level was then subtracted from the mean intensity prior to photobleaching to determine the relative quantity for surface bound protein. As can be seen in figure 5, the surface exposed to thiol-PEG has significantly lower levels of bound protein than the methacrylate surface.

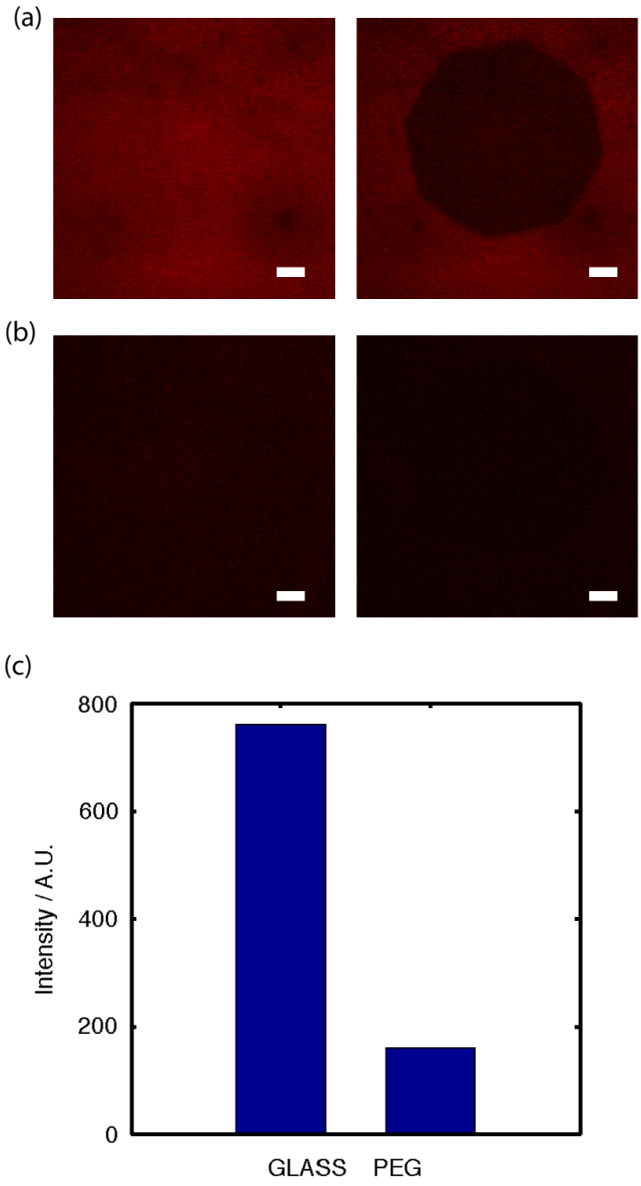

Figure 5. Non-specific protein binding to (a) methacrylate and (b) PEG5000 surfaces was evaluated using fluorescence microscopy. Surfaces were incubated with 10 μM Cy5-streptavidin dissolved in PBS for 1 h before they were rinsed for 1 h in PBS. Binding was evaluated by first imaging the surface fluorescence (left), the background level for each image was then determined by bleaching the Cy5 through a mask for 15 min (right). (c) To evaluate the level of non-specific binding the background fluorescence was subtracted from the initial intensity. The same imaging conditions were used throughout and experiments on methacrylate and PEG5000 were performed consecutively. Images shown were subject to identical image processing and the scale bars are 20 μm.

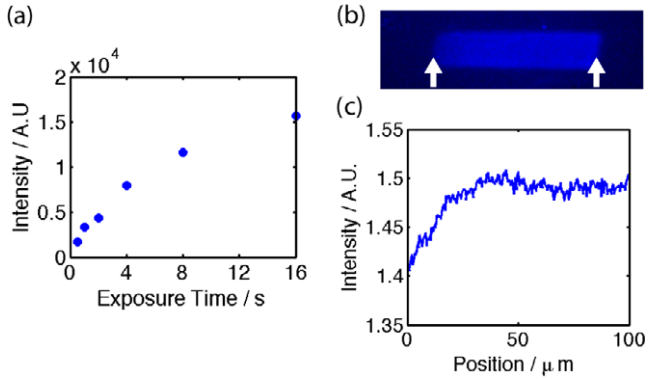

Figure 6. A gradient of PEG-amine was deposited on a methacrylate SAM. (a) The plot shows the effect of increased photobleaching time on the intensity of Cy5 fluorescence when B4F in deposited from solution. (b) Fluorescence image of the gradient labeled with AlexaFluor 350-NHS. Arrows indicate where the microscope's aperture clipped the gradient mask. The arrow separation is 100 μm. (c) Intensity profile of the gradient taken between the arrows shown in (b).

The combined technology of PAP with SAMs presents several simple options for tuning the ligand concentration on the surface. Changes made to either the chemical protocol or the illumination protocol can control the total deposition and the surface density of bound ligands. It has been shown that simply varying the solution concentration of the dye-conjugated ligand under the same PAP conditions leads to surface binding which is consistent with a Langmuir isotherm [12]. An alternative approach is to chemically tune the binding site density in the SAM by diluting the silane-acrylate component of the SAM with a filler molecule [17–19].

Both of these chemical approaches, however, are limited in their scope because they do not provide a reasonable strategy for creating spatial patterns comprised of varying different ligand densities. Changing the duration of the exposure time, as shown in figure 6(a), during photobleaching leads to a linear increase in surface bound biotin at lower exposure times (assuming a linear relation between intensity and surface concentration), while at longer exposure times, the surface deposition begins to saturate. Saturation likely arises from reduction of the number of free binding sites on the surface.

A second approach to tune the ligand density is to adjust the intensity of the light source, as shown in figure 6(b). In this example, rather than discretely varying the lamp illumination, a photomask printed with a linear gradient was created to produce a corresponding PEG-amine gradient. A line profile of the fluorescently labeled PEG-amine groups along the gradient is shown in figure 6(c). The two arrows indicate where an external aperture cropped the pattern. A clear modulation of the surface intensity is apparent, although the change in intensity is not linear as one might expect given the photomask. Instead, we see a relatively linear region from 0 to 30 μm, which then begins to plateau for the rest of the stripe until the aperture is reached at $\sim$110 μm. The gradient was created with a time exposure of 20 s still below the measured saturation time for this molecule (data not shown). It is doubtful that saturation is reached under these conditions, since it was observed that the transmission was reduced by approximately 30% in the non-printed regions alone due to the transparency itself. The plateau more likely arises from the quality of the photomask and the poor representation of the more white regions of the gray scale ($\sim$150–255) rather than from saturation. Further optimization of the materials and the printing technique will develop this proof of principle approach into a full-fledged protocol for designing complex

micropatterns with spatially varying ligand density using a standard epi-fluorescence microscope. Usage of a brighter intensity source or a laser-based epi-fluorescence microscope would increase the range of tunable ligand densities available for patterning, which has been shown to be over three orders of magnitude on BSA-coated surfaces [12].

The ability to pattern gradients on the micrometer length scale in an arbitrary, non-monotonic manner is a unique feature of PAP. Few other techniques can create spatially varying ligand densities with such little effort. Most gradient generating techniques, such as cross-diffusion of different alkanethiols [20], microfluidics approaches [21, 22] and gradient micellar nanolithography [23] produce gradients over relatively large scales. Further, with these techniques it is tricky or impossible to control the second spatial derivative of the ligand density—the local variation of the gradient itself. Reversing the gradient's slope, as far as we know, can only be accomplished with the PAP or other analogous photonic techniques. The relative sophistication garnered by PAP should enable a new class of cell assays, with particularly interesting opportunities in the area of cell adhesion and cell migration.

The simplicity of the PAP approach makes useful extensions of the technique quite feasible. For example, one or more chemical linkers with non-overlapping fluorescent dyes can be serially deposited from the same solution onto the surface. Simultaneous, multi-species deposition is possible if multiple wavelengths can be selected simultaneously (i.e. dual pass filter) and independently patterned using a color mask. Whatever PAP strategy is employed, the linkers and their conjugated dyes can be selected from numerous choices often already found in a life sciences laboratory. Further, while passivation may be desirable in many applications, the non-patterned SAM areas can be filled with thiolated compounds of interest other than thiol-PEG.

4. Conclusions

In summary, we have fabricated functional chemical micropatterns by exploiting the photobleaching of dye-coupled functional groups near a methacrylate self-assembled monolayer. Multiple chemistries can be applied to the surface, so long as they do not interfere with the thiol-based chemistry that is used to directly modify the surface acrylate groups in the post-patterning stage. Well-localized surface functionalization can be achieved and used as a platform for the construction of bioactive surfaces. While dye-enabled deposition was first observed with protein-coated surfaces, the PAP platform based on self-assembled monolayers has significant advantages. It allows for the surface density of binding sites to be chemically tuned, for the application of specific chemistries that would be impossible with a bound protein layer due to their free functional groups, and the underlying self-assembled monolayer itself can be addressed chemically to modify its properties. Arbitrary, non-monotonic spatial variations in ligand density on the methacrylate SAMs are possible using a photomask approach, requiring little dedicated technical expertise. The accessibility and familiarity of PAP's reagents and the patterning tools (fluorescence microscope) should make this rather sophisticated but straightforward micropatterning technique amenable to the growing demands on micropatterning tools by life science research and bio-nanotechnology.

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