Rapid multicomponent optical protein patterning

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Cells sense spatial distributions of molecules which trigger signal transduction pathways that induce the cell to migrate or extend by remodelling the cytoskeleton. However, the influence of local and small variations of extracellular protein concentration on chemotaxis is not fully understood, due in part to the lack of simple and precise methods to pattern proteins *in vitro*. We recently developed a new technology to fabricate such patterns which relies on photobleaching fluorophores to adsorb proteins on a cell culture substrate: *laser-assisted protein adsorption by photobleaching* (LAPAP). Here we report several key improvements to LAPAP: we created arbitrary patterns made of several different proteins simultaneously, we reduced the fabrication time more than one order of magnitude and we used secondary antibodies to significantly enlarge the spectrum of proteins that can be employed. As a result, multicomponent protein gradients can be produced using reagents that are typically available in life science research laboratories on a standard inverted microscope equipped with a camera port.

Introduction

Spatial distributions of proteins are essential to the normal development and life of organisms; central nervous system wiring,[1,2] muscle formation,[3] and defence against pathogens[4] are examples where protein distributions play a fundamental role. Indeed, understanding the function of these spatial distributions in cellular mechanisms is critical to achieve major advances in regenerative medicine. For example, a profound comprehension of how molecular cues are read by axonal growth cones is a prerequisite for repairing nerve damage after injury, which is one of the ultimate challenges of neuroscience.[5,6] Growth cones integrate information from different molecules in the environment to extend in the correct direction and to connect with their appropriate targets[7] during development. The arrangement of chemoattractants and chemorepellants encountered by growth cones dictate the reorganization of microtubules and actin filaments to steer the axons.[8,9] Therefore, the need for a precise and reproducible *in vitro* assay to create protein patterns is vital for a deeper understanding of axonal guidance. Moreover, a low-cost and user-friendly technology is required to allow an increasing number of research groups to carry out studies leading to major advances in the field.

Laser-Assisted Protein Adsorption by Photobleaching (LAPAP) was recently introduced in an attempt to overcome most of the limitations that constrained the applications of protein patterning.[10] Simplicity and robustness make LAPAP an attractive alternative to existing techniques[11] in obtaining protein gradients and even arbitrary spatial distributions of molecules. LAPAP-made protein gradients are more stable in time and reproducible as compared to micropipette puffed generated gradients[12,13] and unlike patterns generated by microcontact printing,[14,15] the protein concentration can be continuously varied over three orders of magnitude. LAPAP is relatively straightforward as compared to microfluidic implementations[16–18] as it has a very simple setup, high spatial resolution and even the ability to be implemented on commercial confocal microscopes. Finally, the use of low power visible lasers and all commercially available reagents is simpler than UV illumination.[19,20]

Briefly, in LAPAP, biotin-4-fluorescein (B4F) molecules are photobleached by a blue laser in order to adsorb them to a glass surface.[10] The photobleaching of fluorescein creates free radicals[21] that react and bind these molecules that are in close proximity to the surface. To produce on-demand arbitrary patterns, the laser is focused at the glass surface and scanned across the substrate, while varying the illumination intensity and the scanning velocity.[10] In the second step, streptavidin is incubated which binds B4F and finally, biotinylated proteins or antibodies can interact with streptavidin.

Three fundamental improvements are presented in this work. First, we describe how to bind several different molecules with full control of their individual concentrations and spatial distributions. The level of complexity of the protein patterns that can now be achieved is highly improved. Secondly, in order to further simplify the method and reduce the time required to produce patterns, we introduce widefield illumination LAPAP. This novel setup can be implemented using any standard inverted microscope equipped with a camera port. Finally, we illustrate how to carry out the patterning protocol using chemical reagents that are usually in stock in most cellular biology research laboratories. Primary and fluorescently tagged secondary antibodies can be used to link most proteins of interest.

Overall, we extended LAPAP to achieve protein patterns of high complexity while simultaneously simplifying and accelerating the fabrication time.

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Results

Widefield illumination LAPAP

The widefield illumination setup that we implemented (Fig. 1a) is an add-on device that can be coupled to the camera port of any standard inverted microscope. Such a device makes use of a light spatial filter located at the image plane of the microscope that is here used as an illumination port instead of an imaging port. Illuminating this spatial filter produces its image on the focal plane of the objective. Thus, instead of scanning a laser to tailor a desired pattern, the same pattern can be achieved by widefield illumination. Such illumination modality speeds up the process, does not require automation software and almost no opto-mechanical elements are needed.

To obtain a perfectly focused image of the spatial filter on the sample, the spatial filter must be precisely placed at the position where the CCD chip would normally be located if that port was equipped with a camera instead of the patterning setup. An extended light source is ideal for this application with light-emitting diodes and lamps probably being the best options. Several objects can be used as spatial filters, but photography slides and liquid crystal displays (LCD) are the most convenient for this application. The advantage of LCDs is that they can be controlled using a standard VGA or SVGA computer output that allows a very simple setup and no need for specialized software. An example of how flexible protein patterns can be is shown in Fig. 1b, where streptavidin-Cy5 was used to reveal the pattern.

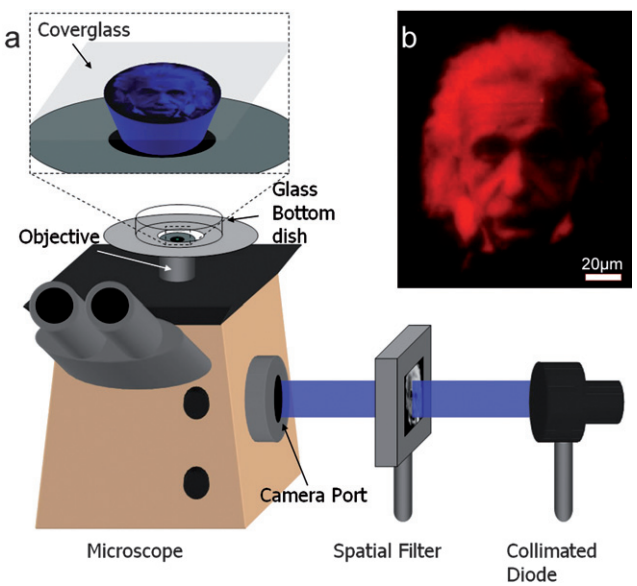

Fig. 1 Widefield illumination LAPAP. (a) A collimated blue diode (470 nm) illuminates a spatial filter which is positioned at the image plane of the camera port. This scheme produces an image of the spatial filter at the focal plane of the objective which is focused at the top surface of a glass-bottom dish. Biotin-4-Fluorescein or FITC conjugated antibodies are then photobleached following the pattern represented in the image sent to the spatial filter. In this case, a grayscale image of Einstein is recreated using blue light on the top surface of the coverglass. (b) The Einstein protein pattern made using a 60× objective and B4F was later revealed with streptavidin-Cy5 and imaged by fluorescence microscopy.

Using widefield illumination LAPAP, two options are readily available to vary the protein concentration: using a greyscale image as a spatial filter or using binary black and white image series. In order to characterize the dynamic range of both possibilities, a pattern of 12 squares was fabricated by generating a greyscale image as a filter where each square had a linearly increasing intensity from 21 to 255. We show that the amount of protein can be modulated by 1 order of magnitude only by changing the pixel value within the image on the spatial filter (Fig. 2a), where 0 is black and 255 is white.

Similarly, a white square filter was used to subsequently fabricate 12 square patterns using a 60× 1.35 NA objective with various exposure times for each square ranging from 5 s to 85 min at a constant power after the objective of 15.5 μW. This gives the possibility of changing the protein concentration by 50-fold (Fig. 2b).

To evaluate the amount of protein bound as a function of the exposure time, the bound B4F was revealed using streptavidin-Cy5 and the fluorescence intensities were plotted in Fig. 2a and Fig. 2b respectively. The experimental details including protein concentrations and incubation times can be found elsewhere.[10]

Finally, to assess the resolution that can be obtained by widefield illumination LAPAP, a protein pattern of a modified USAF resolution target was produced using a 60× objective yielding 1 μm as a result (Fig. 2c and d).

Fluorescently tagged secondary antibody patterns

In order to decrease the number of incubation steps required to produce full protein patterns, we developed a protocol to directly bind fluorescently conjugated antibodies to the glass surface by either standard or widefield illumination LAPAP. In this case, fluorescently tagged secondary antibodies replace B4F as the molecule that first binds to the substrate by photobleaching the fluorophores. Subsequently, primary antibodies and full proteins are bound to create patterns.

We produced patterns of various concentrations to characterize the technique using rabbit anti-laminin antibodies following the binding of FITC goat anti-rabbit IgG to the glass substrate. The antibody pairs were revealed using Cy5 conjugated antibodies. Different concentrations were obtained by either changing the laser intensity or the scanning velocity of the laser focus. For both methods, we show that the dynamic range obtained is approximately one order of magnitude (Fig. 3).

Single step multicomponent antibody patterns

Multicomponent protein patterns can be obtained by combining multiple laser wavelengths and different fluorophores. We demonstrate that it is possible to tailor superposed gradients of two different antibodies. Provided that the absorption maxima of the two fluorophores are distinct (so that each laser line photobleaches only one specific molecule), the binding of each species can be controlled by the intensity of the corresponding wavelength, thereby controlling their individual concentrations.

In Fig. 4c one two-component sample consisting of four different gradients is shown. The top-right region appears yellow as FITC goat anti-rabbit IgG (green) and Cy5 goat anti-mouse IgG (red) gradients are precisely superposed. The bottom-left

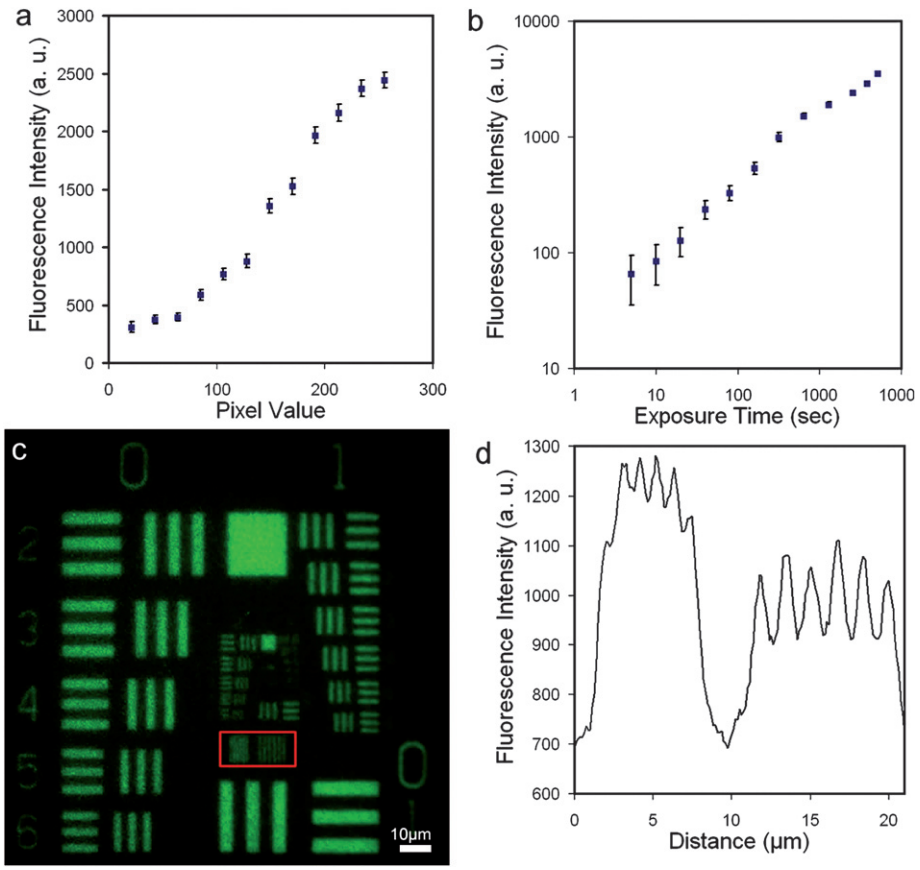

Fig. 2 Characterisation of widefield illumination LAPAP. (a) Fluorescence intensity as a function of the spatial filter's pixel value. Our results show that the amount of bound protein can be changed by one order of magnitude. (b) Protein quantity as a function of exposure time was modulated by 50-fold using exposure times ranging from 5 s to 85 min. (c) Image of a resolution target produced by widefield illumination LAPAP with a 60× objective (1.35 NA). The red rectangle shows a region where 6 lines (on the left) were separated by 1 μm and the other 6 lines (on the right) by 1.5 μm. (d) Average linear profiles of this region show that it is possible to resolve individual lines separated by 1 μm (left) and 1.5 μm (right).

pattern shows gradients of opposing slopes therefore appearing half-green and half-red with fading intensity toward the middle. The remaining two patterns are both made of one type of antibody and appear red and green respectively. It is important to state that some level of cross talk from one component to the other was observed. In the location where only the blue laser is on (top left corner of Fig. 4c), a relatively small amount of Cy5 goat anti-mouse IgG is adsorbed and where only the red laser is on (bottom right corner of Fig. 4c), FITC goat anti-rabbit antibodies are also adsorbed. This non-specific binding is approximately 10% for FITC and 30% for Cy5; control experiments were performed to assess the origin of such cross-talk.

Multicomponent patterns using subsequent illumination

Subsequent widefield illumination LAPAP steps with different FITC conjugated antibodies have lead to the production of multicomponent patterns avoiding cross-talk from one component to the others. We were able to combine three different molecules on the same pattern by photobleaching them sequentially and changing the solutions between each exposition: two different primary antibodies (mouse anti-myc and rabbit anti-thy1) as well as streptavidin were patterned. A glass-bottom culture dish was placed on the microscope positioned at the focal plane of the objective and three circles were patterned by widefield illumination. The dish was clamped to the microscope to avoid possible displacement during the iterative illumination and rinsing steps. The result obtained is shown in Fig. 5 where the streptavidin is revealed by B4F (blue), mouse anti-myc (9E10) by TRITC-goat anti-mouse IgG (green) and rabbit anti-thy1 by Cy5-goat anti-rabbit IgG (red). Seven regions are clearly distinguishable in the image where the different proteins are combined.

Experimental

Widefield illumination LAPAP

An extended light source is ideal for this application and we used a 470 nm 380 mW blue collimated light-emitting diode (LED) (Thorlabs, NJ). We used an 800 × 600 translucent liquid crystal microdisplay (LCD) (Holoeye, Berlin, Germany) combined with two linear polarizing filters placed on each side with a polarization efficiency of 95% (Edmund Optics, NJ).

The LCD was positioned at the right side port of an Olympus IX71 microscope and since it followed the C-mount standard, the LCD was placed 17.5 mm from the flange of the camera port. Patterns of 12 squares were fabricated by generating an 8-bit greyscale image as a filter where each square had a linearly

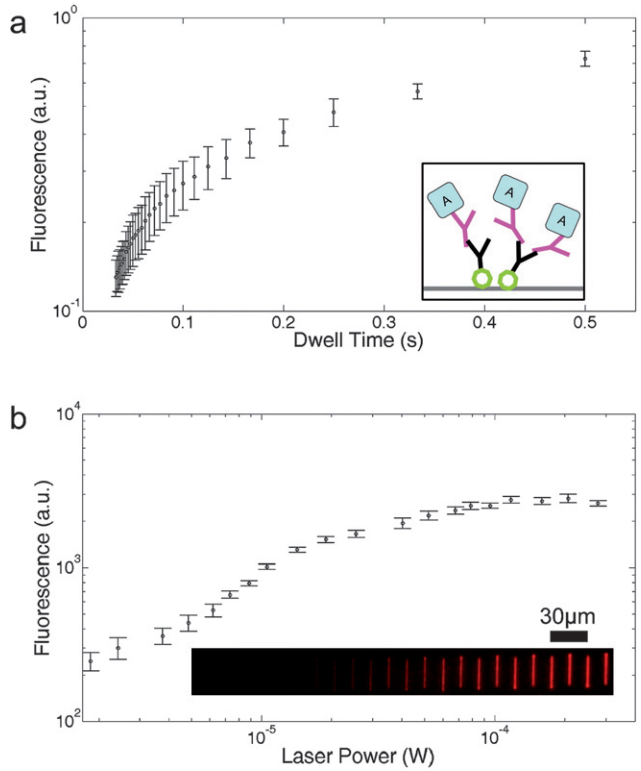

Fig. 3 Patterns of antibodies produced by LAPAP. FITC conjugated goat anti-rabbit IgGs were directly bound to a glass substrate by a focused 473 nm laser. Rabbit anti-laminin, biotin conjugated goat anti-rabbit and streptavidin-Cy5 were subsequently incubated on the pattern for visualization. (a) Semi-log plot of antibody concentration as a function of dwell time shows a dynamic range close to one order of magnitude. The inset shows how a specific protein pattern is made using a fluorescently conjugated secondary antibody (in black), a primary antibody (in pink) and the protein of interest (rounded square). (b) Log-log plot of bound antibody concentrations as a function of laser power shows a one order of magnitude dynamic range. The inset shows one of the 10 patterns used to obtain this graph.

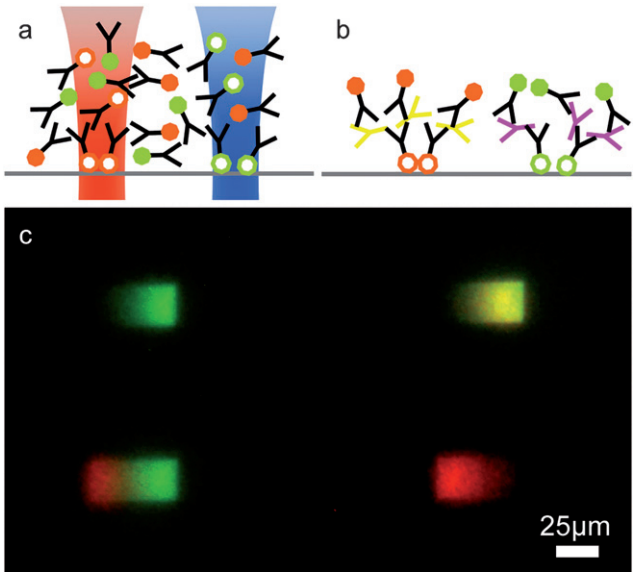

Fig. 4 Two-component patterns by photobleaching FITC and Cy5 conjugated antibodies simultaneously illuminated by 473 nm and 671 nm lasers. (a and b) Schematic representations of antibody patterns fabricated using two lasers. The blue (473 nm) and red (671 nm) lasers in the scheme are not superposed for better clarity. The red laser photobleaches Cy5 goat anti-mouse IgG to adsorb them onto the surface, which later binds to mouse anti-myc (9E10) shown in yellow and finally Cy5 goat anti-mouse IgG is again used to reveal the pattern. The blue laser photobleaches FITC goat anti-rabbit IgG, which binds to rabbit anti-laminin (in pink) and FITC goat anti-rabbit IgG finally reveals the pattern. (c) For this image, FITC is shown in green and Cy5 is shown in red. The bottom-left pattern was obtained by scanning 50 lines by increasing the laser intensity from left to right for the blue laser and by decreasing the intensity for the red laser. The top-right pattern was produced by increasing both lasers in the same direction given precisely superposed red and green gradients, therefore appearing yellow. The top-left gradient was obtained using only the blue laser and the bottom-right one with the red laser alone.

increasing intensity from 21 to 255. This image file sent to the LCD as a spatial filter was generated by a custom program in Matlab (MathWorks, MA). A B4F (50 μg/ml) solution was exposed for 30 min using a 20× 0.75 NA objective and the pattern was later revealed by streptavidin-Cy5 (5 μg/ml). Both solutions were prepared using 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS).

An 800 × 600 pixels image of the USAF resolution target was produced on the LCD spatial light modulator (Fig 2c). We also modified the resolution target by adding 12 white lines of a single pixel thickness, 6 of them separated by 1 black pixel and the remaining 6 by 2 black pixels.

Patterns using fluorescently tagged antibodies

In order to minimize non-specific protein adsorption, a blocking solution (1% goat serum and 1% BSA in PBS) was incubated for 30 min on a 14 mm microwell culture dish (MatTek Corporation, MA). A 20 μL drop of 2 mg/mL of FITC goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA) in 2% BSA and 80% goat serum was placed onto the coverglass of the dish. Patterning was then performed by moving the focus of a 473 nm laser diode across the sample. Rabbit anti-laminin (5 μg/mL), biotinylated goat anti-rabbit IgG (5 μg/mL) and streptavidin-Cy5 (5 μg/mL) all in 3% BSA were subsequently incubated for 30 min each to reveal the pattern obtained by FITC goat anti-rabbit IgG

The dynamic range obtained with the antibody was characterized in terms of laser dwell time and power using the standard LAPAP setup.[10] For dwell time characterisation, lines at beam focus velocities from 30 μm/s to 1 μm/s were scanned at a constant 160 μW laser power and after all the incubation steps, streptavidin-Cy5 fluorescence was measured and assumed to be proportional to the bound FITC goat anti-rabbit IgG concentration. The laser power characterisation was performed by scanning lines at a constant 1 μm/s velocity and by increasing laser power from 1.8 μW to 277.2 μW and by measuring the streptavidin-Cy5 fluorescence after the last incubation step.

Single step multicomponent antibody patterns

The blocking solution was incubated for 30 min on a glass-bottom culture dish. Then, a drop of FITC goat anti-rabbit IgG

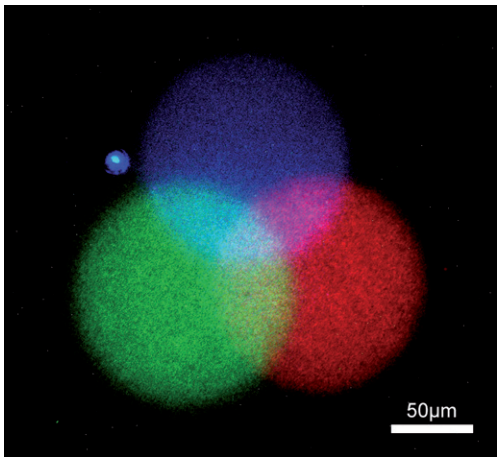

Fig. 5 Three-component protein pattern. Using widefield illumination LAPAP, an image of a circle was positioned at top-center of the spatial filter while illuminating the sample containing a drop of B4F to adsorb it to the glass surface. The sample was then rinsed and a drop of FITC goat anti-rabbit IgG was placed on the sample to be illuminated by a bottom-right positioned circle. The sample was again rinsed and a drop of FITC goat anti-mouse IgG was illuminated by a circle positioned at the bottom left of the image. The sample was rinsed and then incubated with a solution containing streptavidin, mouse anti-myc (9E10) and rabbit anti-thy1. To reveal the pattern, the sample was incubated with B4F (blue cicle), TRITC goat anti-mouse IgG (green circle) and Cy5 goat anti-rabbit IgG (red circle).

and Cy5 goat anti-mouse IgG (each 2 mg/mL in BSA 4% and goat serum 60%) was placed on the coverglass and was scanned simultaneously by the moving focal spot of two diode lasers (473 nm and 671 nm, Fig. 4a). A total of four patterns were produced by scanning 50 lines 25 μm long and by increasing the laser intensity every next line between 0.2 μW to 90 μW for the 473 nm laser and by increasing the intensity between 0.2 μW to 430 μW for the 671 nm laser. One pattern was made by increasing both laser lines in the same direction, giving two superposed gradients. Another one was drawn with the lasers increasing their intensity in opposite directions giving two gradients of opposing slopes. The remaining two patterns were obtained by scanning only one laser line for each of them to assess the presence of non-specific binding. After laser illumination, two incubation steps of 30 min were required to reveal the patterns: rabbit anti-laminin (5 μg/mL) and mouse anti-myc (9E10) (5 μg/mL) in 3% BSA in PBS followed by FITC goat anti-rabbit IgG (5 μg/mL) and Cy5 goat anti-mouse IgG (5 μg/mL) in 3% BSA in PBS (Fig. 4b).

Multicomponent patterns using subsequent illumination

A solution of BSA 3% was incubated on a microwell culture dish for 30 min and was later rinsed with PBS. The culture dish was then placed on the microscope stage with the top surface of the coverglass positioned at the focal plane of the objective. The dish was tightly fixed with clamps to avoid displacement during the subsequent illumination and rinsing steps. A drop of B4F (50 μg/mL) in BSA 3% was then placed on the coverglass and exposed by a top-center circle using widefield illumination LAPAP for 15 min. The sample was then rinsed several times with PBS while maintained at a fixed position and next a drop of FITC goat anti-rabbit IgG (2mg/mL in BSA 2% and goat serum 80%) was placed on the coverglass. The sample was again exposed with the blue LED, but this time with an image of a bottom-right circle for 30 min. The sample was again rinsed while kept in a fixed position for the final exposure step where the blue light image of a bottom-left circle adsorbs FITC goat anti-mouse IgG (2 mg/mL in BSA 2% and goat serum 80%) onto the coverglass for 30 min. A mix of streptavidin (5 μg/mL), mouse anti-myc (9E10) (5 μg/mL) and rabbit anti-thy1 (5 μg/mL) was then incubated on the pattern for 30 min. In order to reveal the pattern, B4F (5 μg/mL), TRITC-goat anti-mouse IgG (5 μg/mL) and Cy5-goat anti-rabbit IgG (5 μg/mL) were finally incubated on the sample.

Discussion

The improvement made to LAPAP[10] by implementing the technology using a standard microscope equipped with a camera port highly simplifies the method with the aim of making the technique amenable to be used in a standard life science lab. The time needed to produce a pattern was significantly reduced as the Einstein miniature (Fig. 1b) only took 5 minutes of exposure compared to 80 minutes needed for similar patterns produced with original LAPAP.[10] Furthermore, there is no sophisticated control and automation software required for producing these patterns.

Compared with standard LAPAP,[10] the results we obtained (Fig. 2a) using widefield illumination shows a reduced dynamic range. This is not a limitation of the new illumination method itself, but is mainly due to the poor polarization efficiency of the low-cost polarizers we used, which reduced the contrast ratio of our spatial filter. Nevertheless, the dynamic range we obtained is enough for a large fraction of possible biological applications.

Regarding the resolution obtained (Fig. 2c and 2d), 1 μm is considerably above the diffraction limit for a 1.35 NA objective using 470 nm light. One should consider that the smallest feature that can be patterned *via* this setup is basically limited by the pixel size of the spatial filter. The size we measured approximately matches what was expected given the characteristics of the LCD we used and the magnification of the microscope objective.

The use of antibodies yields the ability to produce patterns of several specific proteins, therefore allowing improved *in vitro* studies of cellular response. Furthermore, this change also reduces the number of intermediate molecules required to pattern the desired protein.

It is important to note that the molecular mass of tagged antibodies being roughly 200 times the mass of B4F, an IgG concentration of 10 mg/ml should be used to obtain a similar amount of fluorescent molecules per volume unit. However, significant non-specific binding was obtained at this concentration during preliminary experiments and the final concentration was decreased by 5-fold to obtain optimum results. Even if the final concentration we used is much larger than what is currently utilized for immunostaining, commercially available lyophilized antibodies can be used to prepare the solution while maintaining a remarkably low cost per sample. The results obtained showed that it is possible to vary the amount of adsorbed antibodies by one order of magnitude (Fig. 3).

Regarding multicomponent protein patterns, this is, to our best knowledge, the first technique that is capable of providing such patterns with micron resolution and a total control of the local spatial concentration. The limitation to the number of molecules that can be mixed is determined by the width of the absorption spectra. It is challenging to find more than two molecules whose absorption spectra do not overlap significantly. Indeed, despite the direct visual results obtained by this technique (Fig. 4c), the quantitative analysis of the profile of each fluorescence channel showed relatively small cross talk. Control experiments were then performed to understand the binding of Cy5 goat anti-mouse IgG with the 473 nm laser and of FITC goat anti-rabbit IgG with the 671 nm laser. We found that the 671 nm laser was not able to bind FITC goat anti-rabbit IgG when it was not mixed in solution with Cy5 goat anti-mouse IgG. The most probable explanation for the cross-talk binding of FITC-goat anti-rabbit IgG illuminated by 671 nm laser is the non-specificity of the chemical reaction that allows single Cy5 goat anti-mouse IgG to covalently bind to the surface and while also linking FITC-goat anti-rabbit IgGs. For the binding of Cy5-goat anti-mouse IgG with the 473 nm laser, we found that approximately one third of the cross talk is due to direct photobleaching of Cy5 by the 473 nm laser. This cross-talk became significant when a third fluorescently tagged antibody was mixed and a 532 nm laser was added to the setup. The undesirable mixing of molecular species when adding TRITC conjugated antibodies yielded serious cross talk with the two other tagged IgGs.

To circumvent this cross talk problem and be able to add a third protein, we designed an assay with subsequent illumination steps. Simply using FITC conjugated antibodies against different antigens opened the possibility to obtain three component patterns (Fig. 5). In this case, the limitation in the number of different molecules that can be attached is their availability on the market. Since most secondary antibodies bind mouse and rabbit immunoglobins, the possibility of further expanding the number of proteins requires the use of chemical reagents that are not of general use.

Conclusion

The novel technology we presented made it possible to pattern different proteins on the same substrate. We can now fabricate multi-protein patterns at high levels of resolution. Two and three different molecules were bound to glass substrates using very simple technology and commercially available reagents. Furthermore, simple improvements to the illumination setup allowed the fabrication of micron resolution protein patterns using a standard microscope. We designed a device that mounted on the camera port of an inverted microscope would render substrate bound protein patterning simple, inexpensive and user-friendly.

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