Video Article

# Photopatterning Proteins and Cells in Aqueous Environment Using $TiO_2$ Photocatalysis

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## Abstract

Organic contaminants adsorbed on the surface of titanium dioxide ($TiO_2$) can be decomposed by photocatalysis under ultraviolet (UV) light. Here we describe a novel protocol employing the $TiO_2$ photocatalysis to locally alter cell affinity of the substrate surface. For this experiment, a thin $TiO_2$ film was sputter-coated on a glass coverslip, and the $TiO_2$ surface was subsequently modified with an organosilane monolayer derived from octadecyltrichlorosilane (OTS), which inhibits cell adhesion. The sample was immersed in a cell culture medium, and focused UV light was irradiated to an octagonal region. When a neuronal cell line PC12 cells were plated on the sample, cells adhered only on the UV-irradiated area. We further show that this surface modification can also be performed *in situ*, *i.e.*, even when cells are growing on the substrate. Proper modification of the surface required an extracellular matrix protein collagen to be present in the medium at the time of UV irradiation. The technique presented here can potentially be employed in patterning multiple cell types for constructing coculture systems or to arbitrarily manipulate cells under culture.

## Video Link

The video component of this article can be found at http://www.jove.com/video/53045/

## Introduction

Semiconductor lithography processes and its derivatives — such as photolithography[1,2], electron-beam lithography[3-6], and microcontact printing[7-10] — have now become an established tool in cell biology to grow living cells in a defined position and geometry. The patterning method relies on the use of microfabricated substrates, consisting of micro-island of cell permissive coating in a non-permissive background. Such substrate serves as a template to pattern the cells. These technologies have provided us the novel methods to engineer cells and their function at a single- and multi-cellular level, to extract the intrinsic properties of cells, and to increase the throughput of cell-based drug screening[11].

The degree-of-freedom in cell patterning would greatly increase if the template pattern geometry could be altered *in situ*, *i.e.*, while cells are cultured on the surface. The conventional methods for pattern formation cannot be directly applied here, since they process samples in atmosphere or in vacuum. Therefore various new surface modification techniques have been proposed, which are based, *e.g.*, on photoreactive compounds[12,13] or laser ablation[5,14], just to name a few. The proposed methods have been nicely reviewed by Robertus *et al.*[15], and more recently by Choi *et al.*[16] and by Nakanishi[17].

Here in this article, we describe a novel protocol of *in-situ* surface modification, which takes advantage of photocatalytic decomposition of organic molecules on a titanium dioxide ($TiO_2$) surface[18,19]. In this method, a $TiO_2$ film is inserted between the glass substrate and the organic film that interfaces the cells, and the organic film is decomposed *in situ* by locally irradiating ultraviolet (UV) light to a region of interest ($\lambda < 388$ nm). We show that the new protocol can be used to create micropatterns of extracellular matrix proteins and living cells both *ex situ* and *in situ*. $TiO_2$ is biocompatible, chemically stable, and optically transparent, features of which makes it friendly to introduce in cell-culture experiments. This protocol provides a materials science-based alternative for modifying cell-culture scaffolds in cell-culture environment.

## Protocol

# 1. Preparation of $TiO_2$-coated Glass Coverslip

1. Number the coverslips using a diamond scriber. This helps not only to keep track of each coverslips but also to ensure that the correct side of the sample is facing up. Clean the coverslips, first under running $ddH_2O$, then by immersing them in piranha solution ($H_2SO_4$:$H_2O_2$ = 4:1). After 10 min, rinse the coverslips thoroughly, 8 times in $ddH_2O$. Dry the coverslips under $N_2$ flow.
2. Set $TiO_2$ target in the radio-frequency (RF) sputtering system. Attach the coverslips onto a sample holder of the sputtering equipment using a polyimide tape. Place the sample holder in the sputtering chamber. Evacuate the chamber until the pressure reaches $2.0\times10^{-4}$ Pa.
3. Introduce Ar gas into the chamber and set the deposition pressure to 4.0 mTorr. While keeping the shutter closed, gradually increase the RF power to 70 W.
4. Open the shutter and sputter for 15 min to obtain a film with a thickness of 120-150 nm (**Figure 1**: Step A). Growth rate of the film needs to be derived for each machine.

# 2. Surface Coating with Cell-repellent Film

1. Hydrophilize the $TiO_2$ surface by treating the sample with $O_2$ plasma, following instructions provided by the manufacturer of the plasma reactor. We treat the sample for 5 min at 200 W with $O_2$ flow of 100 sccm. Immerse the sample in $ddH_2O$ and confirm that the surface is superhydrophilic. Dry the surface thoroughly under $N_2$ flow.
2. Prepare 1 mM octadecyltrichlorosilane (OTS) solution by adding 39.6 µl OTS to 100 ml toluene. Immerse the sample in the solution for 1 hr at RT. Conduct this step inside an $N_2$-filled glove bag (**Figure 1**: Step B).
3. To remove physisorbed molecules, sonicate the sample in toluene, acetone, ethanol, and $ddH_2O$ for 5 min each, in that order. Rinse the sample four times in fresh $ddH_2O$ and dry the surface under $N_2$ flow. The surface should be hydrophobic with a contact angle of 100-110°.

# 3. *Ex-Situ* Surface Patterning

1. Working in a laminar flow hood, draw several scratch marks with a diamond scriber on the surface. The marks help in keeping track of the processed regions and also bringing microscopes into focus. Sterilize the OTS-coated $TiO_2$ by immersing the sample in 70% ethanol for 5 min. Then rinse the sample twice in sterilized $ddH_2O$.
2. Place the sample in a 35-mm dish, and add 2 ml of PC12 growth medium (see Step 4.2). Incubate for over 3 hr in a $CO_2$ incubator (37 °C). This procedure is intended to let serum albumins absorb onto the surface. Adsorbed albumins inhibit subsequent adsorption of other proteins and cells (**Figure 1**: Step C).
3. While waiting, set up the inverted fluorescence microscope.
    1. Turn on the arc lamp, insert the UV filter cube, and set the objective lens to 20X.
    2. Measure light intensity $I$ (W $cm^{-2}$) using an UV-intensity meter, and calculate irradiation time $t$ (in seconds) for a dose of $d$ (in J $cm^{-2}$) as: $t = d/I$.
    For instance, to irradiate at a dose of 200 J $cm^{-2}$ using a light source of 600 mW $cm^{-2}$, 333 sec of irradiation is required.
    3. Use a stage micrometer and close the field diaphragm to set the size of the region to be irradiated, *e.g.*, 200 µm.
4. After the 3 hr incubation, supplement the medium with 200 µl of 3.0 mg $ml^{-1}$ type-IV collagen (Col-IV; final concentration 300 µg $ml^{-1}$).
5. Transfer the 35-mm dish to the microscope stage. Find the scratch mark, focus the microscope onto the sample surface, and irradiate UV-light at a dose of 200 J $cm^{-2}$ (**Figure 1**: Step D). The area of UV irradiation can be altered either by adjusting the opening of the field diaphragm or by replacing the field diaphragm with a metal mask of arbitral geometry.
6. Replace the medium with a fresh growth medium (without Col-IV), and place the sample back in the incubator.

# 4. Cell Culture

1. Routine culture of PC12 cells is carried out on a collagen-coated plastic dish.
    1. To coat a 60-mm tissue-culture dish with Col-IV, first wet the surface with $ddH_2O$ and aspirate all $ddH_2O$.
    2. Prepare 300 µg $ml^{-1}$ Col-IV by diluting the original solution (3 mg $ml^{-1}$) 10x with $ddH_2O$.
    3. Add 200 µl of 300 µg $ml^{-1}$ Col-IV per 60-mm dish. Let the solution spread through the surface.
    4. Dry the dish in a laminar flow hood for approximately 1 hr.
    5. Rinse the surface twice with 4 ml of Dulbecco's phosphate-buffered saline (D-PBS). When not using the coated dish immediately, rinse the dish once with 4 ml of $ddH_2O$. After aspirating $ddH_2O$, store the dish in the incubator. Try not to keep it stored for more than two weeks.
2. PC12 cells are grown in a growth medium that consists of: Dulbecco's modified Eagle's medium (low glucose) + 10% fetal bovine serum + 5% horse serum + 1% penicillin-streptomycin solution. Incubate the cells in a $CO_2$ incubator (37 °C, 5% $CO_2$), and replace half of the medium every other day. Passage cells before they reach confluence.
    1. Aspirate the growth medium, and add 2 ml of $PBS^+$ (D-PBS + 10 mg $ml^{-1}$ bovine serum albumin (BSA) + 10 mM EDTA) prewarmed to 37 °C. Incubate for 5 min at 37 °C. Gently tap the dish to detach all cells.
    2. Collect cells in a 15 ml conical tube. Rinse the dish with 3 ml of fresh D-PBS, prewarmed to 37 °C.
    3. Centrifuge the tube at 150 × g for 4 min.
    4. Aspirate the supernatant, and add 1 ml of the growth medium.
    5. For routine culture, split the cells 1:3 to 1:5.

3. To plate cells on the UV-modified OTS/$TiO_2$ sample, count cell density and add $3.0\times10^5$ cells in a 35-mm dish (**Figure 1**: Step E). Incubate the dish in the humidified incubator (37 °C, 5% $CO_2$) for 1-2 days. Both naive and nerve growth factor (NGF)-differentiated PC12 cells can be used for the patterning experiments. For NGF differentiation, 100 ng $ml^{-1}$ of 7S-NGF is added to the growth medium several days before plating the cells onto the sample. NGF-differentiation seems to increase the adhesibility of the PC12 cells and makes handling easier, especially in the *in-situ* patterning experiments.

## 5. *In-Situ* Surface Patterning

1. After 1-2 days of culture on the *ex-situ* modified surface, confirm that the cells are attaching and growing only on the UV-irradiated region. Set up the microscope, as described in Step 3.4.
2. Transfer the sample to a new 35-mm tissue culture dish containing the growth medium, 100 ng $ml^{-1}$ NGF (in the case of using NGF-differentiated cells), and 100 µg $ml^{-1}$ Col-IV.
3. Place the dish on the microscope stage. Find the appropriate position, and irradiate UV light at a dose of 200 J $cm^{-2}$ (**Figure 1**: Step F, G). The newly created permissive region is indicated with an asterisk in **Figure 1**: Step G.
4. Try to complete processing of a single sample within 30 min. After irradiation, transfer the sample back into the medium without Col-IV.
5. Be extremely careful when carrying the dish with cultured cells and transferring the sample from dish to dish to prevent detaching of the patterned cells.

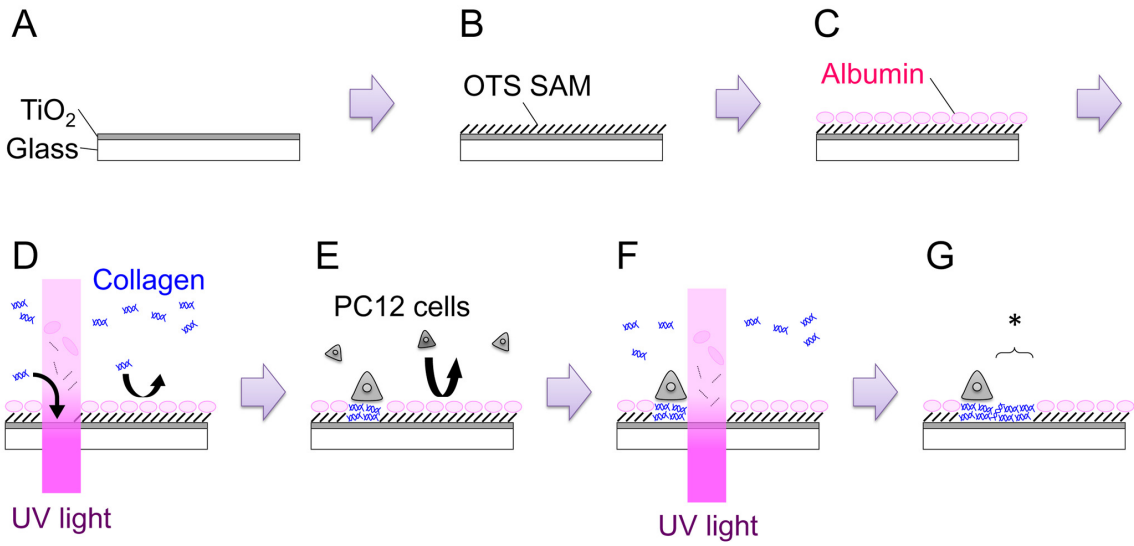


**Figure 1. Schematic illustration of the overall process.** See text for details. Please click here to view a larger version of this figure.

## Representative Results

**Figure 2A** shows a cross-sectional scanning electron microscopy (SEM) image of the sputter-deposited $TiO_2$ film. From the observation, thickness of the film was estimated to be approximately 150 nm. Noticeable here is the flatness of the deposited $TiO_2$ film. Further analysis by atomic force microscopy (AFM) revealed that the root-mean-square (rms) roughness of the surface was 0.2 nm (**Figure 2B**).

When the $TiO_2$ surface is modified with OTS and then immersed in a serum-containing growth medium, serum albumins adsorb onto the OTS surface and inhibit subsequent protein adsorption and cell adhesion[18,20]. Subsequently, when focused UV light is irradiated to the surface, reactive oxygen species (ROS) are generated at the $TiO_2$ surface[21], which decompose the OTS self-assembled monolayer (SAM) on $TiO_2$ and remove the SAM and the adsorbed albumins from the surface. This renders the irradiated region hydrophilic and permissive to protein adsorption. Direct interaction of the ROS with albumins might be occurring simultaneously, but the decomposition of OTS should be the main reaction, since OTS SAM is interfacing albumin and $TiO_2$. In the current protocol, an extracellular matrix protein Col-IV is supplemented in the medium during irradiation, and Col-IV adsorbs preferentially to the UV-irradiated region where albumins are removed (**Figure 3A**). Other proteins such as fibronectin can be patterned in the same protocol (**Figure 3B**), indicating the versatility of this approach.

Serum albumins, which are also present in the medium at the time of UV irradiation, should also adsorb onto the irradiated region, but the results suggest that the amount is not high as that on the intact OTS SAM. To confirm this, we investigated the difference in the affinity of BSA to UV-irradiated and intact OTS SAM by fluorescence microscopy of dye-labelled BSA. For this experiment, focused UV light was irradiated to the OTS/$TiO_2$ sample that has not been immersed in the serum-containing medium, and then the sample was immersed in a solution of fluorescein-labeled BSA. **Figure 3C** shows the adsorption pattern of BSA. Brighter fluorescence in the intact OTS SAM region than the UV-irradiated area indicates that the amount of adsorbed BSA is higher in the intact region. The mechanism of preferential adsorption can be understood by the difference in hydrophobicity of the two regions. The amount of BSA adsorption is known to be higher on hydrophobic surfaces[22], and the intact OTS SAM region is more hydrophobic than the UV-irradiated region[18].

The pattern of Col-IV thus created serves as a scaffold for cell adhesion. **Figure 4A** shows NGF-differentiated PC12 cells cultured on an *ex-situ* patterned region for 1 day. Next, UV light was irradiated to the side of a micropattern, and the cells were cultured for additional 5 days. As shown

in **Figure 4B-D**, cells further migrated to the modified region, indicating that cell affinity of the surface was successfully modified in cell-culture environment.

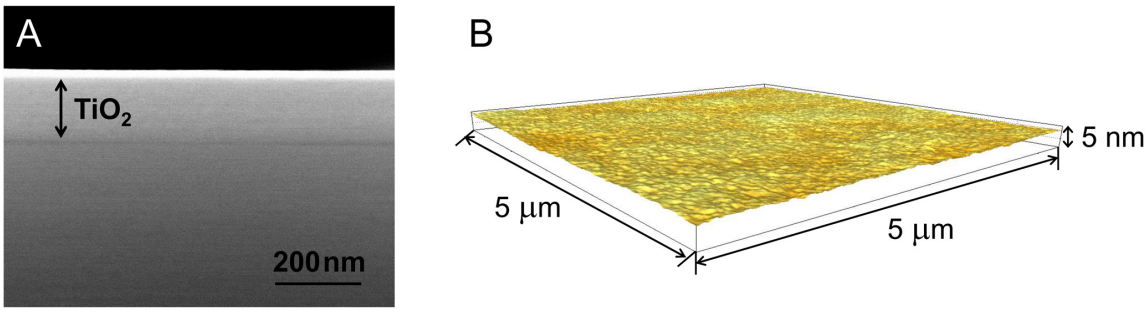


**Figure 2. Characterization of the $TiO_2$ film.** (**A**) Cross-sectional SEM image of the sample. (**B**) AFM image of the $TiO_2$ surface. Please click here to view a larger version of this figure.

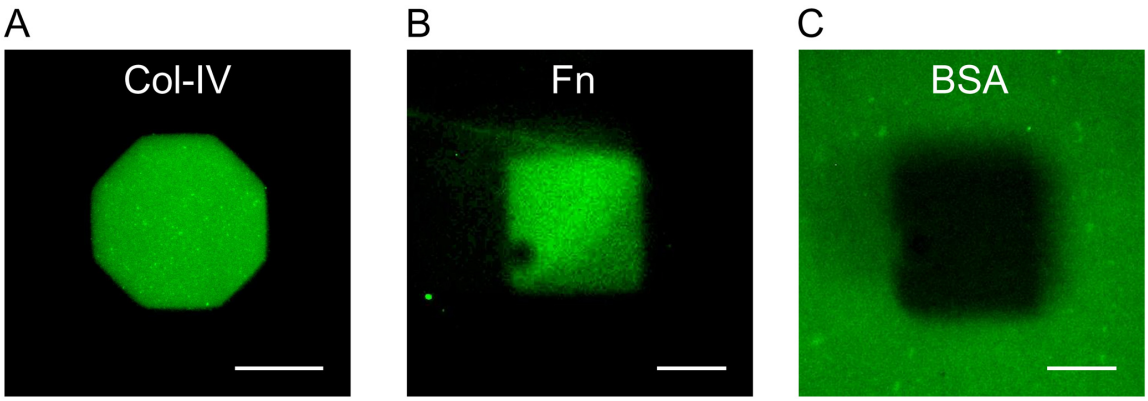


**Figure 3. Site-selective adsorption of proteins onto the UV-irradiated region.** (**A**) Fluorescein-labelled collagen (200 µm octagonal pattern) and (**B**) rhodamine-labelled fibronectin (100 µm square pattern). Preferential adsorption of these proteins relies on the absence of albumins that block protein adsorption (**C**). Note that in (**C**), OTS SAM was not immersed in serum-containing medium prior to application of fluorescein-labelled BSA. Scale bars, 100 µm (**A**) and 50 µm (**B**, **C**). Please click here to view a larger version of this figure.

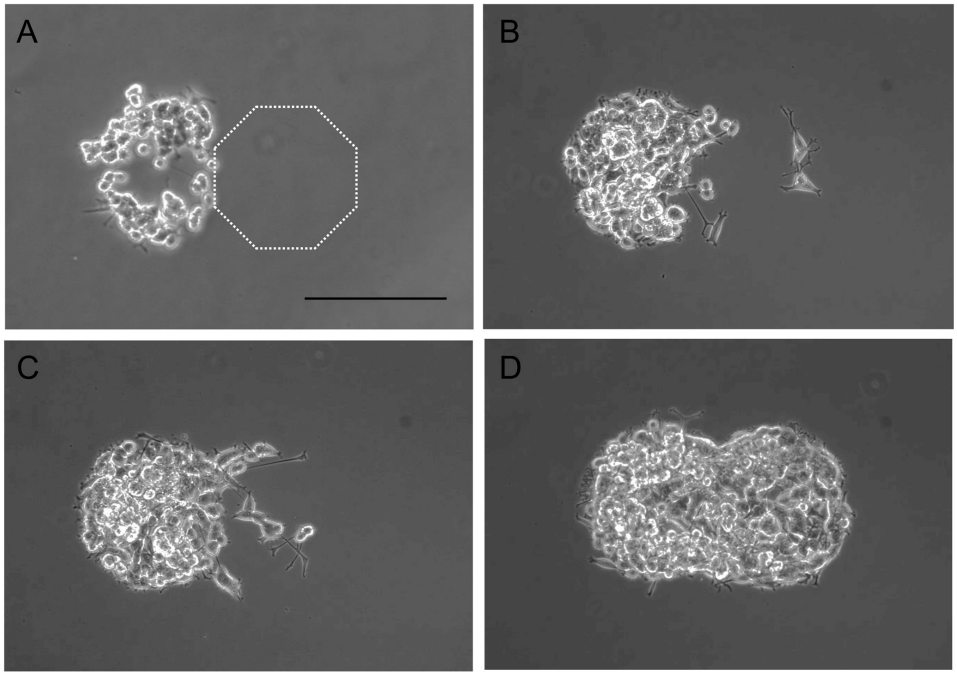


**Figure 4. *Ex-situ* and *in-situ* patterning of PC12 cells.** (**A**) Adhesion of PC12 cells onto an *ex-situ* patterned region. Dotted octagon indicates the site of *in-situ* UV irradiation. (**B**-**D**) Migration of PC12 cells to the *in-situ* patterned region, at 2 days (**B**), 3 days (**C**), and 5 days (**D**) after irradiation. Scale bar, 200 µm. Modified with permission from Yamamoto *et al.*[18]. Please click here to view a larger version of this figure.

## Discussion

In our current protocol, $TiO_2$ film was formed by RF-magnetron sputtering. We favor this method of deposition since it allows us to reproducibly prepare a photocatalytic $TiO_2$ film with a sub-nm roughness. Although sputter deposition processes are familiar to materials scientists and electronic engineers, it may not be quite accessible to biologists. In that case, spin-coated $TiO_2$ film would be an alternative choice[23]. In this method, $TiO_2$ nanoparticles dissolved in a solvent is spread out on a substrate surface by centrifugal force. Special equipment required is a spin coater, which is much affordable than a sputtering system. We have used for ourselves a spin-coated $TiO_2$ nanoparticle film and have confirmed that the film can also be used to carry out similar experiments (data not shown). The surface of the spin-coated $TiO_2$ film is comparatively rough with an rms roughness of several tens of nm, and it takes some practice to uniformly coat the substrate reproducibly especially when multiple spin coatings are needed to obtain thick films. Nevertheless, the method might be the first candidate for biologists.

Thus far, we have utilized this method in patterning not only the PC12 cells but also a human pancreatic cancer cell line AsPC-1 and primary neurons obtained from embryonic rat hippocampi[19]. Adhesibility of AsPC-1 cells are much stronger than that of PC12 cells or hippocampal neurons, and supplementation of scaffolding molecules, such as collagen, was not required in patterning them. We yet note that non-specific adhesion of the cells to the unirradiated region was occasionally observed, which is probably due to defects in the OTS SAM.

Patterning primary neurons were much more challenging since their adhesibility is weak. In fact, the critical step of the current process lies in the proper adsorption of scaffolding molecules. A simple supplementation of collagen or laminin was insufficient to increase affinity of the modified region for the neurons. Conventional culture of hippocampal neurons relies on positively-charged peptide poly-lysine to coat the surface of the coverslip[24]. In this context, inducing selective attachment of poly-lysine to the UV-irradiated area is desired, but was impossible since poly-lysine adsorbed on the intact OTS SAM region as well. We tried other surface coatings, such as polyethylene glycol-silane SAMs and perfluoro-silane SAMs, to find that none of these were anti-fouling to poly-lysine. To solve this issue, we very recently showed that a cross-linking molecule could be used to induce active adsorption of the scaffolding molecules onto the UV-irradiated region[19]. Such selection of scaffolding molecules and optimization of its concentration should be carried out for each experiment.

UV irradiation dose should also be optimized since it strongly affects the amount of protein adsorption. In our case, fluorescence intensity measurement was used to derive an optimal UV dose of 200 J $cm^{-2}$ [18]. The value is likely to vary depending on experimental parameters such as the thickness and the crystallinity of the $TiO_2$ film.

Among various techniques of *in-situ* surface modification developed so far, the major advantage of the present method is that it does not require organic synthesis of specialized molecules for the surface processing. The method presented here is, in principle, compatible with organic films prepared from the vast library of commercially-available polymers, peptides, and SAM precursors. It is also favorable in a sense that the surface-modification process can be carried out using conventional lab equipment, such as a wide-field fluorescence microscope equipped with a mercury arc lamp, as was shown here. Using an UV laser as the light source, subcellular resolution down to ~3 μm can be achieved (data not shown).

Some limitations of the current method include the irreversibility of the surface modification, need for supplementing scaffolding molecules in the medium, and generation of ROS. The process described here is applicable to non-permissive to permissive conversion of the surface, but the conversion is one-way and cannot be reversed. For experiments that require reversal conversion of cell affinity, other methods[15] need to be considered. In addition, our process requires scaffolding molecules such as collagen to be bath-applied to the cell culture medium. Since the population of pre-patterned cells would inevitably be exposed to the molecules, this may limit some application of this process. Photocatalyzed generation of ROS, such as hydroxyl radicals, at the UV-irradiated region may be toxic to nearby cells, but, at least, this effect was not observed in our experiments. Even if this becomes prominent for other applications, this problem should be solved by supplementing oxygen scavengers in growth medium. Since the oxygen scavenging molecules would not be located between the $TiO_2$ and the non-permissive molecules on its surface, they are expected to eliminate excessive ROS without affecting the patterning process kinetics.

## Disclosures

The authors have nothing to disclose.

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## References

1. Hughes, M.A., Brennan, P.M., Bunting, A.S., Shipston, M.J., Murray, A.F. Cell Patterning on Photolithographically Defined Parylene-C: $SiO_2$ Substrates. *J. Vis. Exp.* **85**, e50929, doi:10.3791/50929, (2014).
2. Kleinfeld, D., Kahler, K.H., Hockberger, P.E. Controlled Outgrowth of Dissociated Neurons on Patterned Substrates. *J. Neurosci.* **8**, 4098 - 4120 (1988).
3. Pensen, D., Heinz, W.F., Werbin, J.L., Hoh, J.H., Haviland, D.B. Electron Beam Patterning of Fibronectin Nanodots that Support Focal Adhesion Formation. *Soft Matter.* **3**, 1280 - 1284, doi:10.1039/b710659a, (2007).

 

4. Tanii, T., *et al.* Application of Organosilane Monolayer Template to Quantitative Evaluation of Cancer Cell Adhesive Ability. *Jpn. J. Appl. Phys.* **50**, 06GL01, doi:10.1143/jjap.50.06gl01, (2011).
5. Yamamoto, H., *et al.* In-Situ Guidance of Individual Neuronal Processes by Wet Femtosecond Laser Processing of Self-Assembled Monolayers. *Appl. Phys. Lett.* **99**, 163701, doi:10.1063/1.3651291, (2011).
6. Yamamoto, H., *et al.* Differential Neurite Outgrowth is Required for Axon Specification by Cultured Hippocampal Neurons. *J. Neurochem.* **123**, 904 - 910, doi:10.1111/jnc.12001 (2012).
7. Shen, K., Qi, J., Kam, L.C. Microcontact Printing of Proteins for Cell Biology. *J. Vis. Exp.* **22**, e1065, doi:10.3791/1065, (2008).
8. Johnson, D.M., LaFranzo, N.A., Maurer, J.A. Creating Two-Dimensional Patterned Substrates for Protein and Cell Confinement. *J. Vis. Exp.* **55**, e3164, doi:10.3791/3164, (2011).
9. Singhvi, R., *et al.* Engineering Cell Shape and Function. *Science.* **264**, 696 - 698, doi:10.1126/science.8171320, (1994).
10. Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., Ingber, D.E. Geometric Control of Cell Life and Death. *Science.* **276**, 1425 - 1428, doi:10.1126/science.276.5317.1425, (1997).
11. Degot, S., *et al.* Improved Visualization and Quantitative Analysis of Drug Effects using Micropatterned Cells. *J. Vis. Exp.* **46**, e2514, doi:10.3791/2514, (2010).
12. Nakanishi, J., *et al.* Photoactivation of a Substrate for Cell Adhesion under Standard Fluorescence Microscopes. *J. Am. Chem. Soc.* **126**, 16314 - 16315, doi:10.1021/ja044684c, (2004).
13. Kim, M., *et al.* Addressable Micropatterning of Multiple Proteins and Cells by Microscope Projection Photolithography Based on a Protein Friendly Photoresist. *Langmuir.* **26**, 12112 - 12118, doi:10.1021/la1014253, (2010).
14. Deka, G., Okano, K., Kao, F.-J. Dynamic Photopatterning of Cells In Situ by Q-Switched Neodymium-Doped Yttrium Ortho-Vanadate Laser. *J. Biomed. Opt.* **19**, 011012, doi:10.1117/1.jbo.19.1.011012, (2014).
15. Robertus, J., Browne, W.R., Feringa, B.L. Dynamic Control over Cell Adhesive Properties using Molecular-Based Surface Engineering Strategies. *Chem. Soc. Rev.* **39**, 354 - 378, doi:10.1039/b906608j, (2010).
16. Choi, I., Yeo, W.-S. Self-Assembled Monolayers with Dynamicity Stemming from (Bio)chemical Conversions: From Construction to Application. *ChemPhysChem.* **14**, 55 - 69, doi:10.1002/cphc.201200293, (2013).
17. Nakanishi, J. Switchable Substrates for Analyzing and Engineering Cellular Functions. *Chem. Asian J.* **9**, 406 - 417, doi:10.1002/asia.201301325, (2014).
18. Yamamoto, H., *et al.* In Situ Modification of Cell-Culture Scaffolds by Photocatalytic Decomposition of Organosilane Monolayers. *Biofabrication.* **6**, 035021, doi:10.1088/1758-5082/6/3/035021, (2014).
19. Sekine, K., Yamamoto, H., Kono, S., Ikeda, T., Kuroda, A., Tanii, T. Surface Modification of Cell Scaffold in Aqueous Solution using $TiO_2$ Photocatalysis and Linker Protein L2 for Patterning Primary Neurons. *e-J. Surf. Sci. Nanotech.* **13**, 213 - 218, doi: 10.1380/ejssnt.2015.213, (2015).
20. Arima, Y., Iwata, H. Effects of Surface Functional Groups on Protein Adsorption and Subsequent Cell Adhesion using Self-Assembled Monolayers. *J. Mater. Chem.* **17**, 4079 - 4087, doi:10.1039/b708099a, (2007).
21. Fujishima, A., Zhang, X., Tryk, D.A. $TiO_2$ Photocatalysis and Related Surface Phenomena. *Surf. Sci. Rep.* **63**, 515 - 582 (2008).
22. Sigal, G.B., Mrksich, M., Whitesides, G.M. Effect of Surface Wettability on the Adsorption of Proteins and Detergents. *J. Am. Chem. Soc.* **120**, 3464 - 3473, doi:10.1021/ja970819l, (1998).
23. Zhang, X., *et al.* A Transparent and Photo-Patternable Superhydrophobic Film. *Chem. Commun.* **2007**, 4949-4951, doi:10.1039/b713432k, (2007).
24. Kaech, S., Banker, G. Culturing Hippocampal Neurons. *Nat. Protoc.* **1**, 2406 - 2415, doi:10.1038/nprot.2006.356, (2006).


 