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Pattern Bioactive Proteins/Peptides on Hydrogel Using Photochemistry for Biological Applications

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Abstract:	There are many biological stimuli that can influence cell behavior and stem cell differentiation. General cell culture approaches rely on soluble factors within the media to control cell behavior; however, soluble additions cannot mimic certain signaling motifs such as matrix-bound growth factors, cell-cell signaling and spatial biochemical cues, which are common influences to cell. Furthermore, biophysical properties of the matrix such as substrate stiffness plays an important role in cell fate, which is not easily manipulated under conventional cell culturing practices. In this method, we describe a straightforward protocol to provide patterned bioactive proteins on synthetic polyethylene glycol (PEG) hydrogels via photo-chemistry. This platform allows for independent control of substrate stiffness and spatial biochemical cues. These hydrogels can achieve a large range of physiologically relevant stiffness. Additionally, the surface of these hydrogels can be photo-patterned with bioactive peptides or proteins via thiol-ene click chemistry reactions. These methods have been optimized to retain protein function post surface immobilization. This is a versatile protocol that can be applied for any protein or peptide of interest to create a variety of patterns. Finally, cells seeded onto the surface of these bioactive hydrogels can be monitored over time as they respond to spatially specific signals.
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TITLE:
Patterning Bioactive Proteins or Peptides on Hydrogel Using Photochemistry for Biological Applications

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Biomaterials, cell culture scaffold, photopatterning, bioactive hydrogel, thiol-ene bioconjugation, hydrogel

SHORT ABSTRACT:
In this method, we use photopolymerization and click chemistry techniques to create protein or peptide patterns on the surface of polyethylene glycol (PEG) hydrogels, providing immobilized bioactive signals for the study of cellular responses *in vitro*.

LONG ABSTRACT:
There are many biological stimuli that can influence cell behavior and stem cell differentiation. General cell culture approaches rely on soluble factors within the medium to control cell behavior. However, soluble additions cannot mimic certain signaling motifs, such as matrix-bound growth factors, cell-cell signaling, and spatial biochemical cues, which are common influences on cells. Furthermore, biophysical properties of the matrix, such as substrate stiffness, play important roles in cell fate, which is not easily manipulated using conventional cell culturing practices. In this method, we describe a straightforward protocol to provide patterned bioactive proteins on synthetic polyethylene glycol (PEG) hydrogels using photochemistry. This platform allows for the independent control of substrate stiffness and spatial biochemical cues. These hydrogels can achieve a large range of physiologically relevant stiffness values. Additionally, the surfaces of these hydrogels can be photopatterned with

bioactive peptides or proteins via thiol-ene click chemistry reactions. These methods have been optimized to retain protein function after surface immobilization. This is a versatile protocol that can be applied to any protein or peptide of interest to create a variety of patterns. Finally, cells seeded onto the surfaces of these bioactive hydrogels can be monitored over time as they respond to spatially specific signals.

INTRODUCTION:

There are many stimuli that influence cell behavior. Generally, typical cell culturing techniques rely on soluble factors to elicit cellular responses; however, there are limitations to this approach. These methods are unable to accurately display all signaling motifs commonly found *in vivo.* Such signaling mechanisms include sequestered growth factors, cell-cell signaling, and spatially-specific biochemical cues. Furthermore, substrate stiffness can play an important role in cell behavior and stem cell differentiation and is not easily manipulated using common cell culturing practices[1,2]. Biomaterial approaches offer a new platform to begin exploring these signaling mechanisms. In particular, hydrogels are excellent candidates for tuning substrate stiffness[3,4], immobilizing proteins and peptides[5,6], and creating spatially specific patterns[7,8].

Hydrogels are commonly used as scaffolds in tissue engineering due to their biophysical and biochemical commonalities with the extracellular matrix (ECM)[9,10]. Natural polymers are common choices for scaffolds, as they are biocompatible and are found in many tissues of the body. The limitation of using natural polymers as substrates is that they lack easily manipulated chemical moieties for bioconjugation. On the other hand, synthetic hydrogels, as such as PEG, are excellent platforms for targeted chemistries[11,12]. Additionally, PEG hydrogels do not elicit a cellular response and therefore are used as inert backbones for creating bioactive scaffolds.

To create bioactive hydrogels, both photopolymerization and thiol-ene click chemistry reactions are employed. These photoreactions require a photoinitiator and a UV light source. When photoinitiators are introduced to UV light, bonds break to form radicals. Theses radicals are necessary for initiating the reaction but can negatively affect protein bioactivity[12,13]. Therefore, it is crucial to optimize photoinitiator and UV exposure times to maintain protein bioactivity.

In this method, hydrogels are synthesized through acrylate-acrylate chain growth photopolymerization. PEG-diacrylate (PEGDA) monomers react with each other to form branched polymer networks responsible for the structure of the hydrogel. The concentration of PEGDA monomers within the gel precursor solution will control the substrate stiffness. Due to the small pore size of the hydrogel, ECM proteins such as fibronectin can be easily incorporated within the hydrogel for the purpose of cell attachment. Finally, these hydrogels can be surface-patterned with bioactive peptides or proteins via thiol-ene click chemistry reactions. Here, unreacted free acrylates within the hydrogel system will react with free thiols located on the protein or peptide when exposed to UV light. After the proteins or peptides are immobilized on the hydrogel surface, the hydrogel can be stored at 4 °C for several weeks without losing bioactivity. This offers convenience, flexible experimental planning, and the possibility for collaboration between labs. Overall, this platform allows for biomechanical and spatial biochemical control, independent of each other, for the opportunity to influence cellular

behavior.

PROTOCOL:

1. **Preparation of Materials for Hydrogel Synthesis**

1.1. Prepare stock solutions of PEGDA, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and fibronectin under sterile conditions and based on calculations (**Table 1A**).

1.1.1. Weigh out and dissolve compounds in phosphate-buffered saline (PBS). Typically, maintain PEGDA working solution concentrations between 50 and 200 mg/mL (5-20% weight/volume). Pipette the PEGDA solution through a 0.22-µm syringe filter for sterilization.

1.1.1.1. Keep the PEGDA solutions covered with a foil wrapping to protect them from light. Prepare a fresh solution of PEGDA (recommended) for each experiment.

1.1.2. Reconstitute fibronectin protein powder based on the manufacturer's recommendation. Maintain the fibronectin stock solution at 1 mg/mL, aliquot it into 60- to 100-µL aliquots, and store them at -20 °C until use. Defrost the aliquots at 4 °C for several hours or overnight before use. If protein stock is not already provided in sterile conditions, use a 0.22-µm syringe filter for sterilization.

Note: Fibronectin is used for cell attachment. Other ECM proteins can be substituted.

1.1.3. Weigh out LAP for the stock solution and dissolve it in PBS; a typical stock concentration is 25 mg/mL. Sonicate if there is any trouble with dissolving. Pass the solution through a 0.22-µm filter for sterilization. Cover the LAP with a foil wrapping and keep it at 4 °C for up to several months.

Note: LAP is the photoinitiator used for the photochemistry.

1.2. Prepare sterile glass slide molds for hydrogel formation.

1.2.1. Autoclave a polyester sheet; one is needed for each gel mold. Soak two glass slides and three plastic spacers (0.5 mm thick) for each gel mold in 70% ethanol for at least 2 h, but generally allow them to soak overnight. Soak five binder clips for each gel mold in 70% ethanol for 10 min prior to use.

1.2.2. Place the glass slides, spacers, and binder clips onto a small autoclaved sheet in the cell culture hood to allow them to air dry for several hours.

1.2.3. Prepare hydrogel molds by placing plastic spacers around the edge of a glass slide. Place the second glass slide on top. Secure the spacers tightly next to each other with binder clips, two on each long side and one on top.

Note: If this is not properly assembled, the gel solution will leak out.

1.2.4. Surface-sterilize the mold with cell culture hood UV light for 30 min prior to use. Halfway through, flip the mold to expose both surfaces.

2. Modifying Proteins with a Free Thiol

2.1. Prepare stock solutions using calculations from the spreadsheet for converting amine into thiol on proteins (**Table 1A**).

2.1.1. To make the reaction buffer, adjust the PBS to pH 8 and add 5 mM EDTA. Pipette reaction buffer into a 0.22-µm syringe filter for sterilization.

Note: EDTA is important, as it protects thiols from forming disulfide bonds. Thiol groups must remain in their reduced form for the reaction to occur.

2.1.2. Weigh out 2-iminothiolane (Traut's Reagent) and dissolve it in reaction buffer for a stock solution concentration of 2 mg/mL (14 mM). Pass the solution through a 0.22-µm syringe filter for sterilization. Store the stock solution at 4 °C for up to several months.

Note: 2-iminothiolane is the molecule that reacts with solvent-exposed free amines on the proteins and converts them into free thiols.

2.1.3. Reconstitute the protein in reaction buffer at a concentration between 0.1 and 1 mg/mL. Unless previously sterile, pipette this solution through a 0.22-µm syringe filter for sterilization.

Note: This protein is the biochemical signal that will be patterned onto the hydrogel surface. Also, while protein concentrations can vary, higher concentrations are ideal for stronger protein patterns.

2.2. React the protein with 2-iminothiolane by mixing both stock solutions together; use **Table 1B** to calculate the correct volume ratio. Typically, use a molar ratio of 8:1 2-iminothiolane to protein.

Note: For larger proteins or more dilute concentrations, use a higher molar ratio of 2-iminothiolane. Refer to the manufacturer's protocol[15].

2.3. Incubate the reaction for 1 h at room temperature. Use a desalting spin micro-column to remove the thiolated protein product from the remaining reactants, following the manufacturer's protocol[16].

Note: The exclusion limit of this resin is 5 KDa.

2.4. Use Ellman's Assay to quantitatively measure the number of free thiols per protein. Follow the manufacturer's protocol for the assay[17].

3. Hydrogel Formation

3.1. Create the gel precursor solution based on values calculated from **Table 1C**. Mix together the PEGDA, fibronectin, and LAP volumes. Pipette the solution vigorously to ensure a homogenous solution, but avoid creating bubbles. Keep the solution protected from light.

3.1.1. Pipette the gel precursor solution carefully between the two glass slides of the gel mold. Typically, add a volume between 800 and 1,000 µL to the mold. Expose the gel solution in the mold to UV light (wavelength: 365 nm, power: 3-4 mW/cm^2) for 1-2 min to form the hydrogel.

Note: Do not expose the hydrogel to prolonged UV light, as it will limit the surface protein patterning capabilities.

3.2. Take off the binder clips and gently remove top glass slide by applying opposite pressure to the two side spacers.

3.3. Use an appropriately sized biopsy punch to cut out the hydrogel samples. Punch out multiple hydrogels from the gel rectangle to serve as replicates and control samples.

4. **Hydrogel Stiffness Measurements**

4.1. Punch out hydrogels with an 8 mm-diameter biopsy punch for an 8-mm parallel plate rheometer. Load one hydrogel sample into the rheometer (see the **Table of Materials**).

4.2. Lower the parallel plate geometry that is 8 mm in diameter until making contact with the surface of the gel. Keep a gap distance of 0.5 mm for a 0.5 mm-thick hydrogel sample.

4.3. Perform time sweeps for 5 min at 0.1% strain, 0.1 Hz frequency, and 37 °C. Average the G' values across each time sweep. Run independent hydrogels for replicates of each composition.

Note: The G' values should be stable across time points; if they are not, percent strain and frequency sweeps should be performed to select the appropriate values[14].

5. Protein Patterning

5.1. Prepare the desired pattern for the photomask using a computer aided design (CAD) program. Print the photomask on a transparent sheet using a high-resolution printer. Soak the photomask in 70% ethanol for 10 min prior to use. Allow the photomask to air dry in a cell culture hood before use.

5.2. Add the thiolated protein solution prepared in step 2 to the surface of the hydrogel for surface patterning. Pipette 1-2 µL/cm^2 of thiolated protein solution to the surface of each cut-out gel.

Note: It is important to minimize the protein volume; only add enough to evenly cover the entire surface of the hydrogel sample.

5.3. Carefully place the photomask on the surface of the hydrogel. Do not allow air bubbles between the photomask and the hydrogel surface. Gently press down on the mask to remove any bubbles that form.

5.4. Expose the hydrogel to a second round of UV light (wavelength: 365 nm, power: 3-4 mW/cm^2) for 30-60 s.

Note: It is important to exposure the pattern to enough UV light to create a strong pattern without causing the loss of protein function.

5.5. Wash the hydrogels with PBS to remove unreacted species and place each hydrogel within the well plate. Be careful when placing the gels into each well; make sure that the patterned surface is face up. Wash the gels in PBS at 4 °C; gels are stable for at least two weeks at 4 °C.

6. Preparing Hydrogels for Cell Seeding

6.1. Incubate the gels in basal medium for 5-10 min at 37 °C prior to seeding. For human umbilical vein endothelial cells (HUVECs), use EGM-2 medium without growth factors. Minimize the volume of medium added to each well to prevent hydrogel floating. For a 48-well plate, use a 250-µL volume of medium.

6.2. Spin down the plate at 300 x g for 3 min to ensure that the hydrogels are located at the bottom of the well and are not floating. Do this immediately prior to cell seeding.

7. Cell Seeding on Hydrogels

7.1. Use standard sterile mammalian cell culture procedures for cell seeding and experimental procedures. Remove HUVECs from tissue culture flasks using standard cell detachment protocols. Wash the tissue culture dish with sterile PBS and incubate with trypsin for 3-5 min at 37 °C.

7.2. Quench the trypsinized cell suspension with excess cell medium or trypsin neutralizer solution.

7.3. Spin down the cell suspension in the centrifuge at 300 x g for 5 min. Carefully remove the supernatant and keep the cell pellet.

7.4. Resuspend the cell pellet in basal EGM-2 with no growth factors. Use a hemocytometer for cell counting.

7.5. Add 75,000 cells/cm^2 to each hydrogel surface by pipetting slowly into the center of each well so as not to disturb the gels. Place the well plate into a cell culture incubator at 37 °C. For several days, periodically remove the dish for observation.

8. Evaluation of Bioactivity

8.1. Culture *E. coli* overnight in suspension in LB broth in an orbital suspension at 37 °C and 200 rpm. Spin down the *E. coli* culture, decant, and reconstitute the cell pellet in minimal volume of PBS. Weigh out the lysozyme and reconstitute at 1 mg/mL for the stock solution.

8.2. Pipette a small volume (25 µL) of lysozyme stock solution into microcentrifuge tubes. Add varying levels of LAP photoinitiator (0-12 mM) and expose the samples to 1 min of UV light. In a separate group, add the same amount of LAP (2 mM) to lysozyme solution and expose samples to varying UV light times (0-4 min). Include replicates for each treatment.

8.3. Add equal volume of concentrated *E. coli* solution to each lysozyme sample for a final lysozyme protein concentration of 0.5 mg/mL. Incubate the mixed solutions for 4 h at room temperature.

Note: This allows time for functional lysozyme to lyse the bacterial cell wall and release proteins into the solution.

8.4. Use untreated lysozyme incubated with *E. coli* for a positive control; consider this a 100% bioactive measurement. Use lysozyme solution incubated with PBS alone as a negative control.

8.5. Spin down the samples to remove cell debris and keep the supernatant. Run a Bradford assay to quantify the total concentration of protein within the supernatant to measure the amount of bacterial lysate.

8.6. Run a Bradford assay following the manufacturer's protocol[18]. Calculate the fold change in protein concentration compared to the negative control.

REPRESENTATIVE RESULTS:

The protocol to create bioactive patterns on the surface of PEG hydrogels is illustrated in **Figure 1**. A spreadsheet was developed to calculate the volume and concentration for each stock solution (**Table 1A**). Proteins to be immobilized onto the surface of the hydrogel are modified with 2-iminothiolane (**Figure 1B**). This reaction is performed using the volumes from **Table 1B**. The precursor hydrogel solution is prepared with 10% weight/volume of PEGDA with LAP (**Figure 1A**). Various precursor PEGDA concentrations can be used to yield the desired substrate stiffness (**Figure 2A**). Fibronectin is included within this precursor solution for cell attachment purposes. After thorough mixing, this solution is pipetted into the prepared mold and exposed

to UV light (**Figure 1C**). UV light exposure should be minimized; exposure should be just enough to produce a hydrogel. Hydrogel samples are punched out to the appropriate diameter for the desired well plate (**Figure 1C**). For surface patterning, modified protein solution is pipetted onto the surface of a hydrogel and spread evenly. Minimal volume should be used; protein volume should be just enough to cover the entire surface of the hydrogel. The predesigned photomask is placed directly onto the hydrogel surface; air bubbles between the mask and the hydrogel should be avoided. A second round of UV light is used to covalently conjugate UV-exposed proteins to the hydrogel. Hydrogel samples are rinsed to remove unreacted proteins and reveal the immobilized protein pattern (**Figure 1D**).

It is important to minimize photoinitiator concentration and UV exposure time when proteins are present. Using lysozyme bioactivity as an indicator, we found that the LAP photoinitiator concentration should be less than 2 mM (**Figure 2B**) and the UV exposure time should total less than 2 min (**Figure 2C**) to retain a protein bioactivity greater than 80%.

UV exposure time during hydrogel formation and protein patterning are both important parameters for developing a successful protocol (**Figure 3**). First of all, minimizing UV exposure during hydrogel formation is critical to maintaining free acrylate functional groups for subsequent protein immobilization reactions (**Figure 3A**). Hydrogels exposed to UV light for longer than 2 min are unable to create immobilized protein patterns. Additionally, as the UV exposure to the protein pattern increases, more proteins react to the surface (**Figure 3B**).

Finally, cells can be cultured onto these patterned hydrogel substrates to manipulate cell behavior. To show the potential of immobilized patterns on hydrogels, we patterned VEGF, a growth factor important for endothelial cells, and cultured HUVECs on the surface using basal EGM-2 medium (**Figure 4**). HUVECs were uniformly seeded onto the surface of VEGF-patterned PEG hydrogels (**Figure 4A**). Two days after seeding, HUVECs were observed to migrate towards the spatial regions of the hydrogel that contained immobilized VEGF (**Figure 4B, C**). This is one example of a bioactive protein pattern on PEG hydrogels being used to influence cell behavior.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of hydrogel formation and protein patterning. (A) Prepare precursor hydrogel solution with PEGDA monomers, photoinitiator, and extracellular protein for cell attachment. (B) Modify the proteins with free thiol groups by reacting with 2-iminothiolane. (C) Pipette the precursor solution into the prepared mold and expose it to UV light to form the hydrogel. Punch out hydrogel samples of the desired size. (D) Pipette the modified protein solution onto the surface of the hydrogel, place a photomask on the surface, and expose it to a second round of UV light. Rinse the gel to remove unreacted species prior to imaging and cell seeding.

Figure 2. Modulating stiffness and protein bioactivity. (A) Changes in the concentration of PEGDA monomers within the precursor gel solution alter hydrogel stiffness. (B) Increasing the concentration of LAP photoinitiator lowers protein function after 1 min of UV exposure. (C)

Increasing UV exposure time lowers protein function with 2 mM LAP. All error bars represent standard deviation of replicates.

Figure 3. Pattern and hydrogel UV exposure times optimized for protein patterning. (A) Minimizing UV exposure times for hydrogel formation allows for surface patterning. (B) Increasing UV exposure times for surface patterning increases pattern strength. All scale bars represent 200 μm.

Figure 4. Endothelial cells responding to a VEGF pattern. (A) Uniform HUVECs seeded on hydrogels. (B and C) HUVECs sense the VEGF pattern and migrate towards immobilized VEGF. Images were taken two days after seeding in (B) 4x and (C) 10x magnification. All scale bars represent 500 μm. HUVEC-RFPs (red) and VEGF-488 pattern (green) were captured with excitation and emission filters at 528/553 and 465/495, respectively.

Table 1. Calculations for stock and gel precursor solutions. The red box indicates user-defined values, such as molecular weight, desired stock concentrations, and weighed-out masses. Blue boxes represent values that have been calculated based on user-defined values.

DISCUSSION:

This protocol provides a method for creating bioactive protein patterns for biological applications. There are several modifications that can be made to adapt this protocol for different experiments. First, cell attachment requirements will vary for different cell types. If poor cell attachment to the gels is initially observed, increasing the concentration of the ECM protein within the precursor solution is advised. Other ECM proteins can be used instead of fibronectin, including different types of collagen, laminin, or a combination thereof. For each new cell type, cell attachment should be optimized prior to hydrogel patterning. This protocol also allows for user-designed photomasks. Based on the desired application, photomasks can be produced with various feature sizes, shapes, and overall patterns. Uniform immobilization can be achieved in the absence of a photomask.

This protocol has certain limitations. As highlighted in the **Representative Results**, this method is sensitive to the amount of UV light at each step. Overexposure during the hydrogel formation step limits the available acrylate groups for subsequent surface bioconjugation. Therefore, a key step in this protocol is well-managed UV light exposure times for each step. Also, this protocol requires a high concentration protein stock for successful patterning. Low concentrations of protein will result in poor surface patterns. Additionally, photomasks are also limiting in that they can only produce discrete patterns. More complex patterns can be achieved with similar approaches but require a more advanced methodology.

This protocol is significant to existing methods as it provides a simple method for adjusting substrate stiffness and protein patterning. Using acrylate chemistry for the hydrogel formation allows for a magnitude range of substrate stiffness within the physiological range. Simply adjusting the concentration of PEGDA within the precursor solution gives control over the hydrogel stiffness. Additionally, the use of click chemistry for protein patterning allows for rapid

conjugation between hydrogel substrate and thiolated modified protein. This is a key design feature, as it allows this protocol to be applicable to any protein or peptide of interest.

PEG hydrogels are promising biomaterials that can be used to explore new platforms for displaying biochemical cues to biological systems. Whether uniform surface immobilization or spatially specific patterns, these techniques provide novel ways to control cell behavior. Moving forward, the advancement of biomaterial technology will provide new insights into cell behavior and further our abilities to recapitulate *in vivo* signaling motifs within *in vitro* systems. This can be beneficial for stem cell differentiation and modeling developmental signaling within a controlled experimental system.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

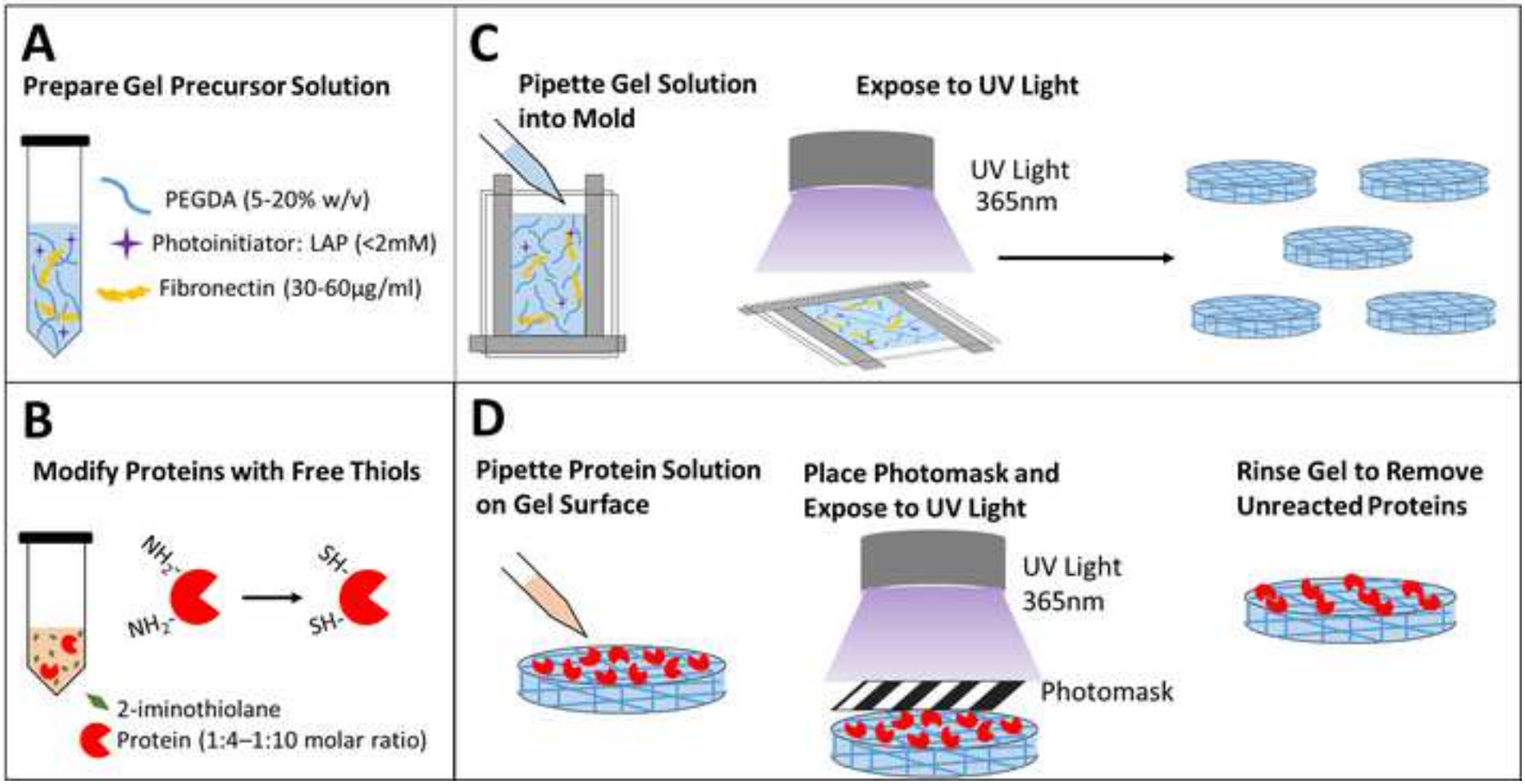

Figure 2

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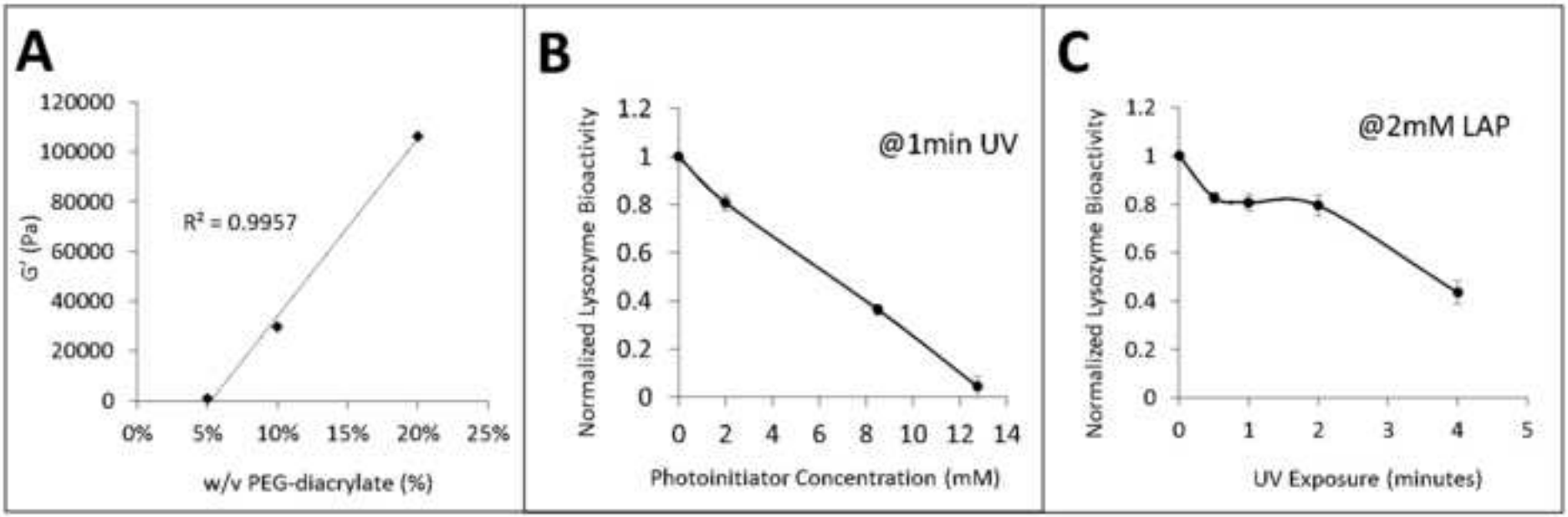

Figure 3

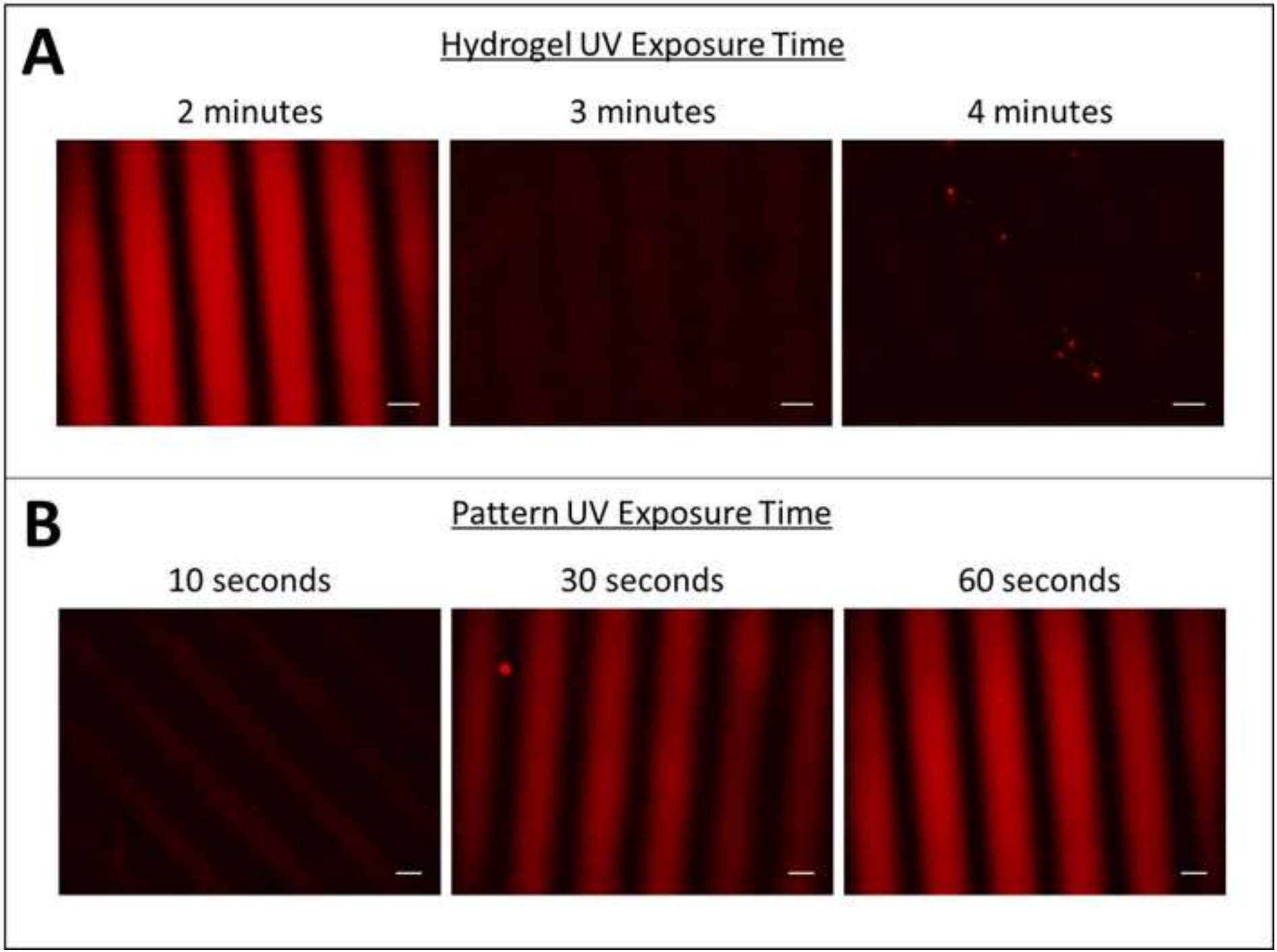

Figure 4

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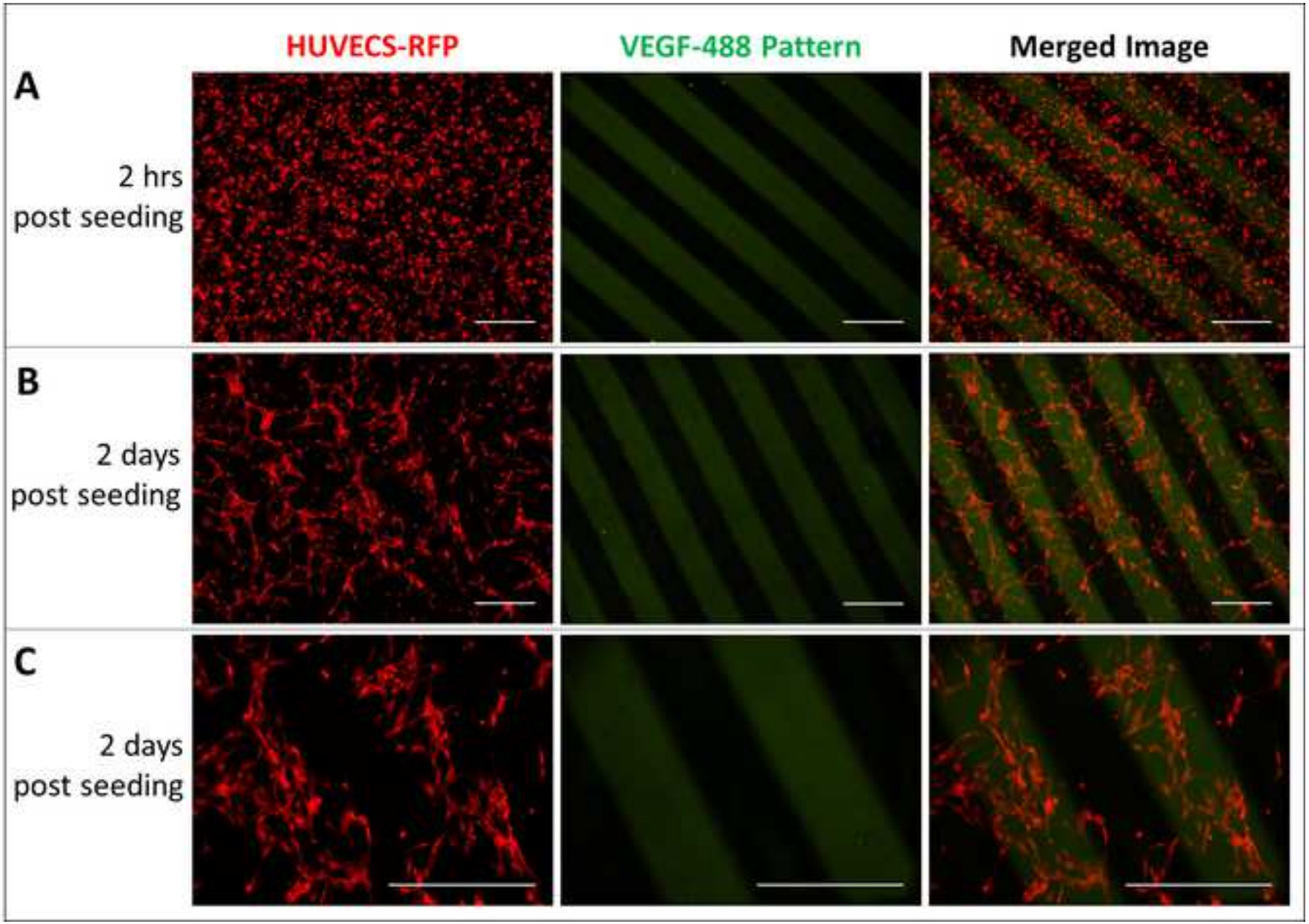

Table 1

Click here to download Table of Materials/Equipment JOVE Manuscript Table 1.xlsx

A. Stock Solutions Calculations

Component	Molecular Weight (kDa)	Stock Concentration (mg/mL)
PEGDA	3400	120
Fibronectin	440,000	1
LAP	293.9	25
2-iminothiolane	137.63	2
Protein (VEGF)	38200	0.1

B. Thiol-Protein Reaction

Reaction Ratio	*8:01*	*2-iminothiolane : Protein*
Component	**Stock Concentration (mg/ml)**	**Volume (µL)**
2-iminothiolane	2	1.44
Protein (VEGF)	0.1	100

C. Gel Precursor Calculations

Desired Gel Volume:	*1000 ul*	
Component	**Stock Concentration**	**Working Concentration**
PEGDA	120 mg/ml (12 w/v%)	10%
Fibronectin	1 mg/ml	60 µg/ml
LAP	25mg/ml (85mM)	2mM
PBS		
		Total Volume:

Mass (mg)	Volume of PBS (ml)
113	0.94
5	5
23.4	0.94
13	6.5
0.1	1

Volume (µL)
833.33
60
24
83.14
1000

Table 2

Click here to download Table of Materials/Equipment JOVE Manuscript Table 2 - Materials List.xlsx

Name	Company	Catalog Number
PEG-diacrylate (PEGDA)	Laysan Bio	ACRL-PEG-ACRL-3400
Lithium Phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)		
Fibronectin	Corning	356008
Phosphate-buffered saline (PBS)	Sigma	D8537-500ML
Photomask	FineLine Imaging	n/a
Binder Clips	Various Vendors	
Compact UV Light Source (365nm)	UVP	UVP-21
2-iminothiolane (Pierce Traut's Reagent)	Thermo Sci.	26101
Ellman's Reagent: DTNB; 5,5-dithio-bis(2-nitrobenzoic acid)	Thermo Sci.	22582
human umbilical vein endothelial cells (HUVECs)	Lonza	
EGM-2 Media	Lonza	CC31-56, CC-3162
0.25% Trypsin EDTA	Life Tech	25200-056
Trypsin Neutralizer	Life Tech	R-002-100
Centrifuge	Various Venders	
Hemocytometer	Hausser Sci. Bright-line	
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E6758
0.22µm filter	Cell Treat	229743
1mL Syringe		
Glass Microscope Slides	Fisher Sci.	12-550C
Plastic spacers	Various Venders	

70% Ethanol	BICCA	2546.70-1
Bio-shield	Bio-shield	19-150-0010
Bradford Reagent	BIO-RAD	
Desalting Resin - Sephadex G-25	GE Healthcare	95016-754
Microspin Columns	Thermo Sci.	PI69725
AR-G2 rehometer	TA Instruments	

Comments
Can also be synthesized or purchased through other venders. Different molecular weights can be used.
Synthesized in lab
Other cell attachment proteins can be used, such as laminin, matrigel
Custom prints on transparent sheets with high resolution DPI.
Other UV light sources can be used, calibration of power is required.
passage number between 6- 10
EGM-2 without growth factors was used in experiments. Full EGM-2 media was used for cell maintainance
0.5mm thickness

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Author(s): Taylor Dorsey, Alexander Grath, Guohao Dai

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Article Title: Pattern Bioactive proteins/peptides on hydrogel using photochemistry for biological applications

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We wish to thank the reviewers and editor for their careful critique of our manuscript. We are encouraged by the positive remarks, and found the criticism very helpful in strengthening the impact of our work and the clarity of our manuscript. We have revised the manuscript to address the reviewers' comments. The changes are highlighted in the revised manuscript. The point-by-point answers to the reviewer comments are provided below:

Changes recommended by the JoVE Scientific Review Editor:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
This has been addressed.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Example NOT in imperative tense: "PEGDA is weighed out and then dissolved in phosphate buffered saline".

Example in imperative tense: "Weight out PEGDA and dissolve it in phosphate buffered saline."
This has been addressed.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.).
1) Line 146: Please provide column MWCO, duration for separation etc.
We have added this information. 5KDa MWCO and a reference.

2) Line 148: Please add a reference for Ellman's assay.
I have added this reference.

3) Line 155: How much solution?
Typically, 800~1000 ul, this has been added to the text.

4) Line 166: Where is the punch hole made?
"Punch out multiple hydrogels from the gel rectangle to serve as replicates and control samples."

5) Line 175: How is the photomask prepared?
I added a step (5.1) to include how to design the photomask and prepare it for use.

6) Line 178: Is the line light the same as in previous steps?

Yes, I have made this clear in the text.

7) Line 188: What is the growth medium used? What volume to you recommend?
Medium is EGM-2 without growth factors at volume of 250ul for a 48 well plate. This has been added to step 6.1

8) Section 6: There are no steps describing cell seeding on the hydrogel, please add them and include cell concentration per hydrogel.
I have added step 7.5 to include this.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
This has been corrected.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps that match the title and abstract. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length (2.75 pages or less). Please see JoVE's instructions for authors for more information.
5) Notes cannot be filmed and should be excluded from highlighting.
I have highlighted sections that is content for filming. Sections: 1, 3, 5, 6, 7

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I have added and edited discussion to address each of these topics listed above.

• **Figures:**
1) Please provide each figure as an individual PDF, TIFF, JPEG or PNG files.
This has been done.

2) Please remove the text "Fig.#...." from all figures.
This has been fixed.

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This has been addressed.

• **Figure/Table Legends:** Please place legends directly below the Representative Results text.
This has been moved.

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This has been completed. Unsure the format to cite manufacture's online protocols (see reference numbers 15-18).

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an in INDIVIDUAL xls/xlsx file. Please include items such as reagents, protein, columns, filters, cells, cell culture media, etc.
This has been provided in the excel spreadsheet.

• Please define all abbreviations at first use (e.g. LAP).
Yes, this has been corrected.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
Yes, this has been corrected

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All figures are original.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This manuscript is well organized and clearly presented. All the steps are described and easy to follow.

Reviewer #2:
Manuscript Summary:
The paper described a photo pattern technique that allowed proteins to be patterned on hydrogel surface.

Major Concerns:
None

Minor Concerns:
1) the full name of PEGDA and LAP should be provided when they are first mentioned in the paper (line 90).
This has been corrected.

2) Line 172, "small volume of protein solution", it's not obvious what protein solution this is. I presume it is 2-iminothiolane-treated solution. It would be much clearer if the authors can specify it.
Yes, this was unclear. I have corrected it to say “pipette a small volume of thiolated protein solution”.

3) There are a few minor grammar mistakes throughout the text. e.g. line 218, "volume is should just", line 225 "UV exposure time for should total ....", line 231, "unable create immobilized...", etc.
I have corrected these grammar mistakes along with others I found during edits for resubmission.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This manuscript describes a protocol to create a layer of patterned protein on biocompatible PEG hydrogel. The PEG gel incorporated fibronectin to support cell proliferation, and the stiffness of the gel is tunable. The patterned protein can potentially modulate the cell migration/differentiation. This work gives a general method to control the cell behavior via applying spatial biochemical cues.

Major Concerns:

Lack of experimental details
We added more details in the revised manuscript.

Minor Concerns:
N/A photochemistry

Additional Comments to Authors:
1. Line 68, the description of "multiple photopolymerization steps" is not accurate, it's photopolymerization followed by click chemistry.
Yes, I agree with this correction of word choice. I corrected the sentence to read “To create bioactive hydrogels, a photopolymerization hydrogel forming step is followed by a thiol-ene bioconjugation reaction.

2. The authors should give more significance in protocol, such as:
a. Line 104, what's LAP's function
I added a sentence to state LAP is the photoinitiator to initiate the polymerization reaction of PEGDA.

b. Line 98, why having fibronectin in precursor solution
I added a sentence to state fibronectin is added to allow cells to attach to the PEG hydrogel.

c. Line 127, what's the sources of sulfhydryl and why it need protection
I changed the diction and added a sentence. EDTA is important as it protects from thiols forming disulfide bonds. Thiol groups must remain in their reduced form to allow for the reaction to occur.

d. Line 171, protein patterning, what's the objective to introduce another protein (i.e., growth factor) besides fibronectin.
I added the following sentence: “Add the thiolated protein to the surface of the hydrogel for surface patterning. The objective of introducing this protein is to examine how cell respond to its embolized spatial signal.”

3. The authors should give more experimental details
a. Line 146, details about purify protein using desalting column
I added the following sentence: “Add the thiolated protein solution, prepared in step 2, to the surface of the hydrogel for surface patterning. The objective of introducing this protein is to examine how cell respond to its spatial signal.”

b. Line 141 and 142, the ratio range 2-20 and 1-8 is kind of too broad, what's the ratio in this work?
The 2-20 ratio is a reference from the manufacturer’s protocol. I have removed that statement and added a reference to the manufacturer’s protocol. I have made it more clear what was used in this work. In this work, 8:1 molar ratio was used.

c. Line 203, seeding density
Yes, I have added the seeding density of 75,000 cells /cm^2.

d. The protocol of getting results of Figure 2 and Figure 3

I have added an additional section for rheology testing of PEG hydrogels (Step 4) and evaluation of bioactivity (Step 8).

4. Line 225 and 226, the content in brackets should be "Figure 2B" and "Figure 2C", respectively.

Yes, this has been corrected.