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Photoactivatable Adhesive Ligands for Light-guided Neuronal Growth

Aleeza Farrukh[a,b], Wenqiang Fan[c], Shifang Zhao[a,e], Marcelo Salierno[c,d], Julieta I. Paez*[a],

Aránzazu del Campo*[a,e]

(a) INM – Leibniz Institute for New Materials, Campus D2 2, 66123, Saarbrücken, Germany
(b) Max Planck Graduate Center, Forum Universitatis 2, Building 1111, 55122 Mainz, Germany
(c) Institute of Physiological Chemistry, University Medical Center Johannes Gutenberg University, Hanns-Dieter-Hüsch-Weg 19, D-55128 Mainz, Germany
(d) Focus Program Translational Neuroscience, Johannes Gutenberg University, 55131 Mainz, Germany
(e) Saarland University, Chemistry Department, 66123 Saarbrücken, Germany

(*)Corresponding authors:

Prof Dr A. del Campo: Tel: +49(0)681-9300-510, Fax +49(0)681-9300-223, e-mail: aranzazu.delcampo@leibniz-inm.de ORCID: 0000-0001-5725-2135

Dr J. Paez: Tel: +49(0)681-9300-369, e-mail: julieta.paez@leibniz-inm.de ORCID: 0000-0001-9510-7254

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Abstract

Neuroregeneration after trauma requires growth and reconnection of neurons to reestablish information flow in appropriate direction across the damaged tissue. To support this process, biomaterials for nerve tissue regeneration need to provide spatial information to adhesion receptors on the cells membrane and provide directionality to growing neurites. Here photoactivatable adhesive peptides based on the CASIKVAVSADR laminin-peptidomimetic are presented and applied to spatiotemporal control of neuronal growth to biomaterials *in vitro*. The introduction of a photoremovable group (NVOC, DMNPB, or HANBP) at the amine terminal group of the K residue temporally inhibited the activity the peptide. The bioactivity was regained by controlled light exposure. When used in neuronal culture substrates, the peptides allowed light-based control of the attachment and differentiation of neuronal cells. Site-selective irradiation activated adhesive and differentiation cues and guide seeded neurons to grow in predefined patterns. This is the first demonstration of ligand-based light-controlled interaction between neuronal cells and biomaterials.

Keywords: phototriggered cell adhesion, laminin peptidomimetics, neuronal adhesion, IKVAV, guided axon growth

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Introduction

Nerve tissue engineering requires synthetic scaffolds that support the attachment, development and connection of neurons to reestablish information flow in particular directions. *In vivo,* adhesion and directionality for the growth of neuronal processes is provided by the spatial arrangement of extracellular adhesive proteins and membrane receptors of other cells (i.e. glia) along which neurons migrate and extend their processes.[1] *In vitro*, spatial organization of neuronal growth has been mainly realized using surface patterning and microstructuring techniques. Relevant examples are aligned electrospun nanofibers,[2] micro-contact printed substrates,[3] aligned peptide amphiphile nanofibers,[4] or magnetically aligned composite hydrogels.[5]

An interesting strategy to guide positioning and migration of cells in desired directions, not yet realized with neurons, is by using photoactivatable cell adhesive ligands and light to site-selective activate molecular interaction with cell membrane receptors.[6] In this approach, the adhesive ligand is modified with a photolabile moiety (a "caging group") that inactivates recognition by the adhesive receptor at the cell membrane.[6] Biomaterials modified with the caged ligands are initially not able to interact with the cells. Light exposure cleaves the chromophore and switches on the latent bioactivity on the biomaterial. This strategy has enabled spatiotemporal control of ligand-mediated cell adhesion *in vitro* [6b, 7] and *in vivo*.[8] It requires previous identification of adhesive molecules able to support effective attachment of the particular cell type and development of photoactivatable variants.

The protein laminin is an abundant adhesive protein in neural tissue and plays a fundamental role in neural attachment, migration and differentiation.[9] Different adhesive membrane receptors of the integrin family can bind to laminin, such as $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_1$.[10] Laminin is commonly

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used as adhesive protein in *in vitro* neural cell cultures to facilitate adhesion of neuronal cells and neurite out-growth on biomaterials. Laminin peptidomimetics containing the YIGSR, IKVAV, or RKRLQVQLSIRT sequences have also been used as laminin alternatives.[10b] These are small molecules and allow better control over binding chemistry and coating densities on biomaterials than the bulky laminin. Among them, IKVAV containing peptides have been demonstrated to stimulate neurite growth, branching and maturation when coupled to biomaterials, by interacting with the integrin receptors $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$.[11] The pentamer IKVAV is highly hydrophobic and shows low solubility. In this sequence, the isoleucine and lysine amino acids in the IKVAV core have been found crucial for recognition of $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$ integrin.[12] The commercially available 19-mer peptide CSRARKQAAS**IKVAV**SADR (IK-19) is the most widely used IKVAV variant.[13] In IK-19 the biologically active IKVAV sequence is flanked by positively charged tails that enhance the water solubility of the hydrophobic IKVAV sequence and prevent formation of aggregates.[14] In addition, the positively charged amino acids support cellular attachment by ionic interaction with the negatively charged cell membrane. The 12-mer AASIKVAVSADR has also been reported as shorter alternative to IK-19.[12] The shorter sequence simplifies the synthesis and eventually improves specificity in the control of neuron-substrate interactions.

In this manuscript, photo-triggerable variants of the CAS**IKVAV**SADR (IK-12) laminin mimetic peptide are developed and used to functionalize hydrogels and direct neuronal growth on synthetic biomaterials (Figure 1). The photoactivatable IK-12 peptides contain a photolabile group at the amine side chain of the Lys rest. Optoregulated attachment, development and directional migration of neural progenitor cells (NPCs) on derivatized hydrogels is demonstrated. Site-selective irradiation activated the laminin-mimetic peptide and provided spatial adhesive

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and differentiation guidance to seeded neurons. This is the first demonstration of a molecularly controlled, light-guided neuronal growth, in contrast to other attempts using a laser beam as a physical guiding cue to redirect axon growth.[15] This simple and flexible approach for neuronal guidance can be extended to different material types, and eventually 3D constructs.

Results and discussion

The development of photoactivatable peptides requires careful selection of the molecular site at which the photoremovable chromophore can be introduced in order to effectively inhibit bioactivity. Reported data have demonstrated that the isoleucine and lysine amino acids of the IKVAV sequence are crucial for integrin recognition.[12] The amine side chain of the lysine is an appropriate site for binding a photocleavable group, since amine groups offer multiple possibilities for functionalization.[6a] An important issue to consider with IKVAV containing peptides is the low solubility of IKVAV sequence, which mainly contains hydrophobic residues. The introduction of chromophores at the amine group of the Lys rest is expected to further decrease solubility.

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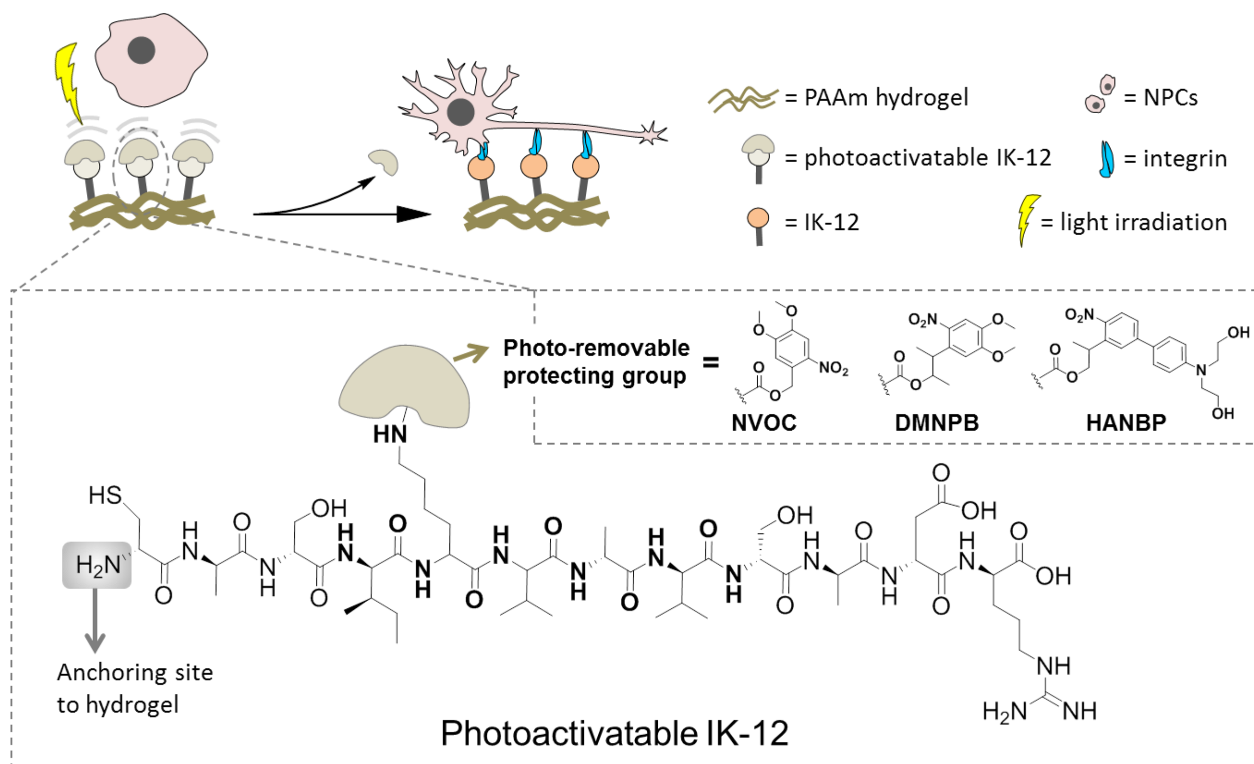

Figure 1. Schematic representation of the use of phototriggerable laminin-mimetic peptides to optoregulate neuronal attachment and differentiation on synthetic biomaterials. The hydrogel functionalized with photoactivatable IK-12 peptide does not interact with the neural progenitor cells (NPCs). Light exposure activates IK-12 by removing the chromophore, providing an adhesive and differentiation cue to NPCs. The chemical structure of the synthesized photoactivatable variants of IK-12 is also shown.

Three different variants of photoactivatable Lys were synthesized by introducing a photoremovable protecting group (i.e. "cage") on the amine side chain. The selected chromophores were: 6-nitroveratryl alcohol (NVOC)[6a], 3-(4,5-dimethoxy-2-nitrophenyl)-2-butanol (DMNPB),[6b, 7d, 8] and 2,2'-((3'-(1-hydroxypropan-2-yl)-4'-nitro-[1,1'-biphenyl]-4-yl)azanediyl)bis(ethan-1-ol) (HANBP)[16] (Fig. 1). This selection was based on different reasons. NVOC is a commercially available photocleavable of the *o*-nitrobenzyl family that can be

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readily incorporated to Lys in one step reaction. This chromophore was used to work out optimum reaction conditions for the caged peptide synthesis. The DMNPB caging group is an *o*-nitrophenethyl derivative that showed good photolysis efficiency and better hydrolytic stability than NVOC in previous cell experiments in our group.[7d] The photolabile HANBP group, a new variant of the *o*-nitrobiphenyl donor–acceptor family, is expected to show improved photoresponse (due to its extended conjugated structure) and better water solubility than DMNPB.[16] Additionally, *o*-nitrobiphenyls show good two-photon absorption cross-section and could be good candidates for photoactivation in 3D.[16] The chromophores DMNPB and HANBP were synthesized in 4- and 8-step synthetic routes. DMNPB was synthesized according to reported protocols.[7d] The synthesis of HANPB-Lys required the adaptation of a published protocol[16] by rearranging the order of the synthetic steps (Scheme 1). In our case the hydroxy group of the pinacolato borate intermediate (**1**) was activated with triphosgene to get the chloroformate (**2**) and coupled to the amine group of Lys, followed by Suzuki coupling to iodo-N-phenyldiethanolamine (**4**). The introduction of the free dihydroxyethylamine group at this point avoided additional (de)protection steps. Additionally, the free –OH groups of HANBP proved orthogonal during subsequent solid-phase peptide synthesis, which again avoided the use of a protecting group at the chromophore. The final HANBP-Lys (**5**) and well as NVOC- and DMNPB-variants were obtained at 350-750 mg scale in good yields (see Supporting Information for synthetic and characterization details).

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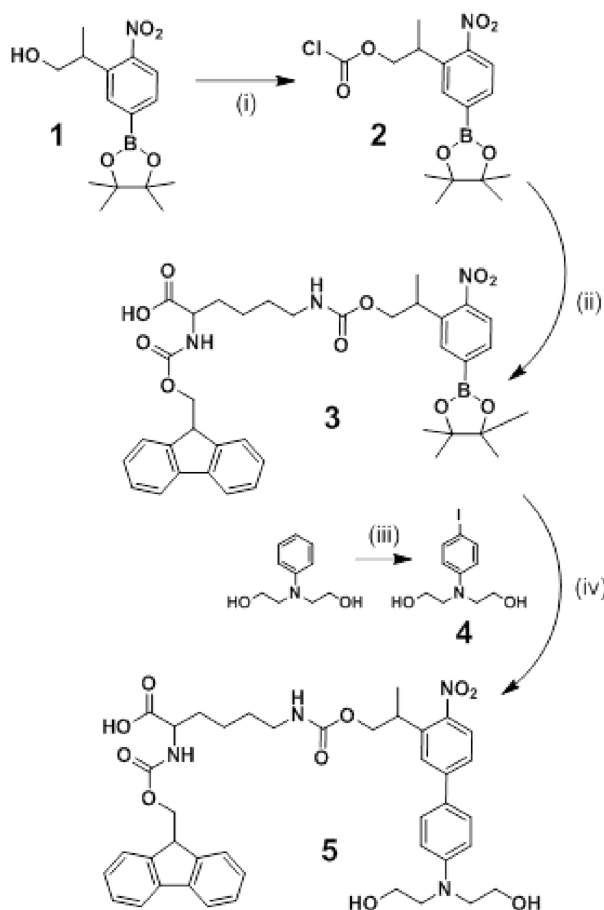

Scheme 1. Synthesis of Fmoc-Lys(HANPB)-OH Reagents and conditions: (i): triphosgene, K_2CO_3, toluene, 8h; (ii) Fmoc-Lys-OH, water:dioxane (1:1), Na_2CO_3, 24h; (iii) iodine, pyridine:dioxane (1:1), 24h; (iv) Bu_4NBr, $Pd(OAc)_2$, K_2CO_3, ethanol:water (2:1), microwave, 150°C, 10 min.

IK-12 and the photoactivatable derived peptides IK-12(NVOC), IK-12(DMNPB) and IK-12(HANBP) were obtained by solid-phase peptide synthesis, purified by HPLC and characterized by mass spectrometry. The three phototriggerable peptides showed reduced water solubility (ca. 0.75 mg mL^{-1}) in relation to IK-12 (1 mg mL^{-1}), but high enough to be used in the subsequent steps. The photolytic removal of the chromophore from the caged peptides at the corresponding λ_{max} (345 nm for NVOC, 365 nm for DMNPB, and 420 nm for HANBP, see

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reactions in Fig. S1, Supporting Information) was studied by UV-Vis spectroscopy of 1mM solutions after irradiation for increasing times. HPLC characterization of the irradiated solutions demonstrated successful cleavage of the chromophore and generation of IK-12 in all three cases (Fig. S2). The measured photolysis efficiency for uncaging increased in the order NVOC < DMNPB < HANBP (15%, 12% and 48 % respectively as calculated from HPLC profiles after 30 min exposure at λ_{max}). The photolysis mechanism of the HANBP chromophore is shown in Fig. S1d.

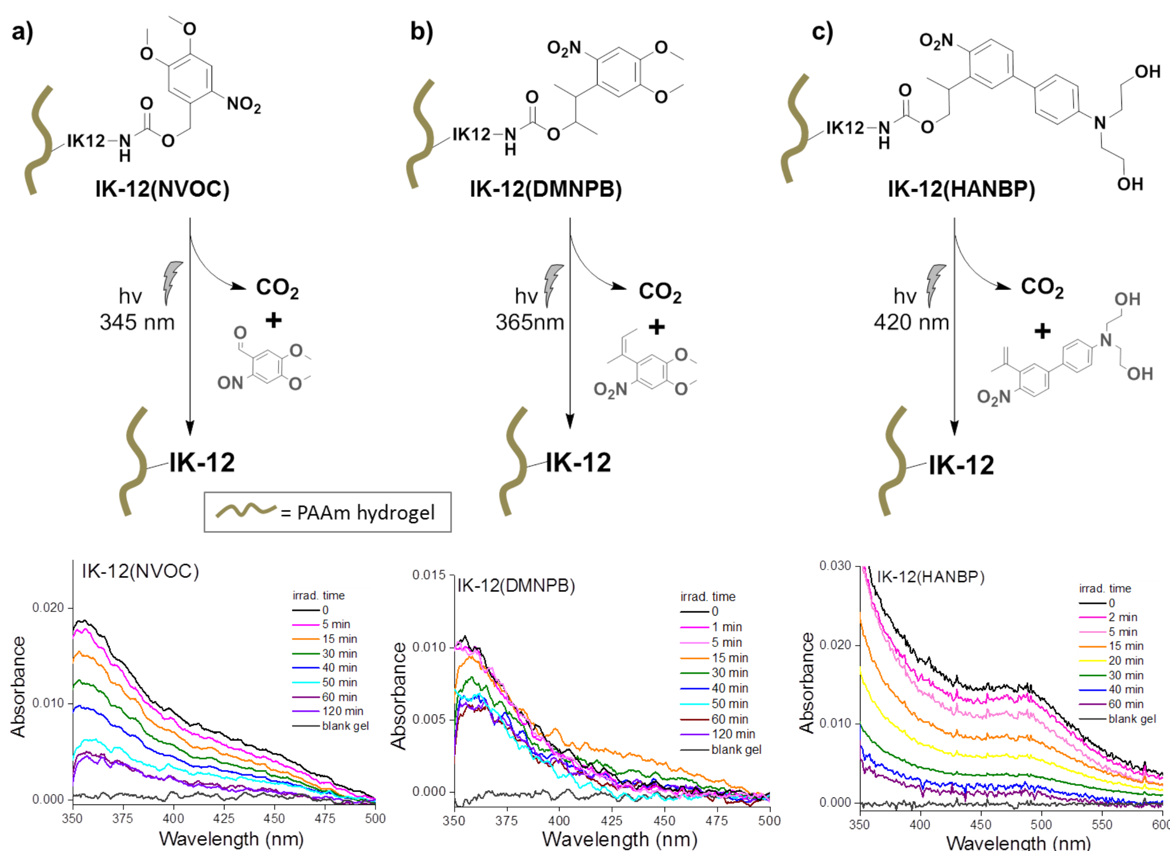

Figure 2. Photocleavage of IK-12(NVOC)/(DMNPB)/(HANBP) variants. Photolysis reactions (a-c), followed by UV-Vis of hydrogel films functionalized with the photoactivatable peptides, after irradiation and washing. UV-Vis spectra were recorded after light exposure for increasing

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times. Irradiation was performed at λ_{max}: 345 nm for NVOC,[6a] 365 nm for DMNPB,[6b] and 420 nm for HANBP (based on a previous report[16]).

The photoactivatable IK-12 peptides were covalently bound to poly(acrylamide-*co*-acrylic acid) P(AAm-AA) hydrogel films. These gels have been recently demonstrated as valuable platforms for study of neuron differentiation and maturation.[17] Gels of 2-kPa stiffness were selected for the neuronal cultures based on the current understanding that neurons prefer soft substrates for development *in vitro*.[18] The obtained peptide concentration, as calculated from UV spectra of the modified hydrogel films after washing (see Experimental Section for details), was similar for all three phototriggerable variants (IK12-NVOC: 0.39 mM, -DMNPB: 0.33 mM, -HANBP: 0.36 mM under specified coupling conditions). The similar coupling efficiency found may be related to the comparable water solubility of the three peptides. The photoactivation of the hydrogel-bound ligands was followed by measuring UV-Vis spectra of the hydrogel films after increasing exposure times and washing (Figure 2). The photolysis cleaves the chromophore from the caged IK-12 peptide and the washing step removes it from the gel. Decay in the UV-Vis spectra was observed in all cases, indicating effective photocleavage of the chromophore from the hydrogel upon light irradiation. The measured photolysis efficiencies for uncaging followed the same trend as observed in the solution experiments (uncaging ratios of 17%, 14% and 48% for IK12-NVOC, -DMNPB and -HANBP respectively after 15 minutes of exposure at λ_{max}).

Preliminary experiments were performed to assess the activity of plain IK-12 and the expected inhibition by introducing modifications at the Lys rest. For this purpose, gels were functionalized with IK-12 and also with the sequence CASI**E**VAVSADR (IE-12), where the Lys rest was

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substituted by a glutamate rest (E).[12] Neural progenitor cells (NPCs) from mouse embryonic cortex (E14.5) were seeded on the IK-12 and IE-12 functionalized gels. NPCs are very active, and the current benchmark for the study of neural functionality. Cells on IK-12 showed high viability, underwent neuronal differentiation and developed elongated axon and dendrites, branches and dendritic filopodia within 24h at comparable levels to laminin (LN), or laminin/poly-D-lysine (LN/PDL) functionalized hydrogels typically used for neuronal cultures (Figure 3 and Table S2 for quantification).[19] On the contrary, hydrogels functionalized with IE-12 failed to attach NPCs or support differentiation. Neurons did not develop long process and exhibit low cell viability on the IE-12 substrates (Figure 3 and Table S2). These results indicate that changes in the K residue of IKVAV impair the bioactivity of IK-12 and confirm the suitability of this position for caging.

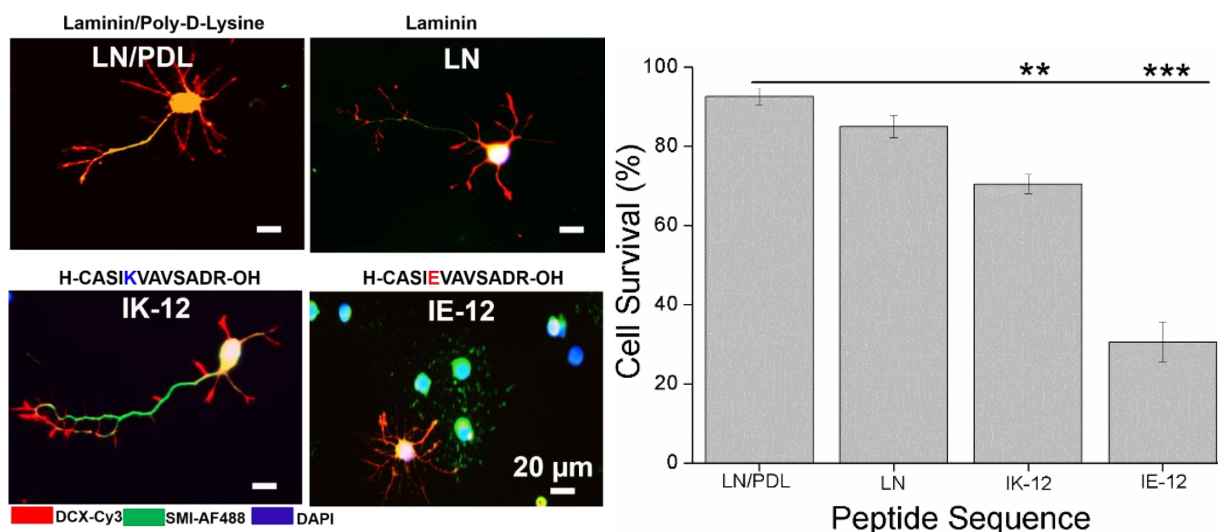

Figure 3. Neural progenitor cells (NPCs) from embryonic cortex (E-14.5) on IK-12 and IE-12 modified 2-kPa P(AAm-AA) hydrogels after 24 h culture. (a) Immunofluorescence images of cells showing the neuronal features. The DCX stains cell body, SMI marks axonal filament and nucleus is stained by DAPI. (b) Quantification of cell viability after 24h of cell culture. The

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significance of data obtained for the different samples was analysed by Tukey- test (mean ± SD, ANOVA, ** $p < 0.01$, *** $p < 0.001$) and compared to LN/PDL and LN controls.

Hydrogels functionalized with the photoactivatable peptides IK-12(NVOC), IK-12(DMNPB) and IK-12(HANBP) were then tested. Only few cells attached on caged substrates, confirming the inhibition of the bioactivity of the peptides as a consequence of the introduction of the chromophore in the Lys rest. Cells retained a rounded morphology, show poor cell viability (19-28%) similar to the values observed on hydrogels modified with the IE-12 peptide (27%), and did not develop processes (Figure 4a, non-irradiated samples and Figure S3). When IK-12 (NVOC)/ (DMNPB)/(HANBP) modified substrates were pre-irradiated before cell seeding, cells attached and developed neuronal morphologies (Figure 4a, pre-irradiated) like those observed on IK-12 (Figure 3) and remained viable over 4 days (Figure S3). These results confirm that the photolysis reaction on the gel-immobilized peptide was successful and the activity of photoactivatable IK-12 could be restored after exposure.

Neuronal growth on the photoactivated IK-12 hydrogels was quantified by analyzing the number of processes and secondary branches, the length of axon, and the number of dendritic filopodia (Figure 4 and Table S2 in Supporting Information). All pre-irradiated substrates promoted the growth of neurites, branches and dendritic filopodia of cells during 2 days culture. A 4-5 fold increase in the number of process, a 8-13 fold increase in the number of branches and a 10-15 fold increase in the number of dendritic filopodia was observed on irradiated vs. non-irradiated photoactivatable IK-12 modified hydrogels. It should be noted that dendritic filopodia could be the morphological precursors of neuronal spines,[20] which are required for formation of

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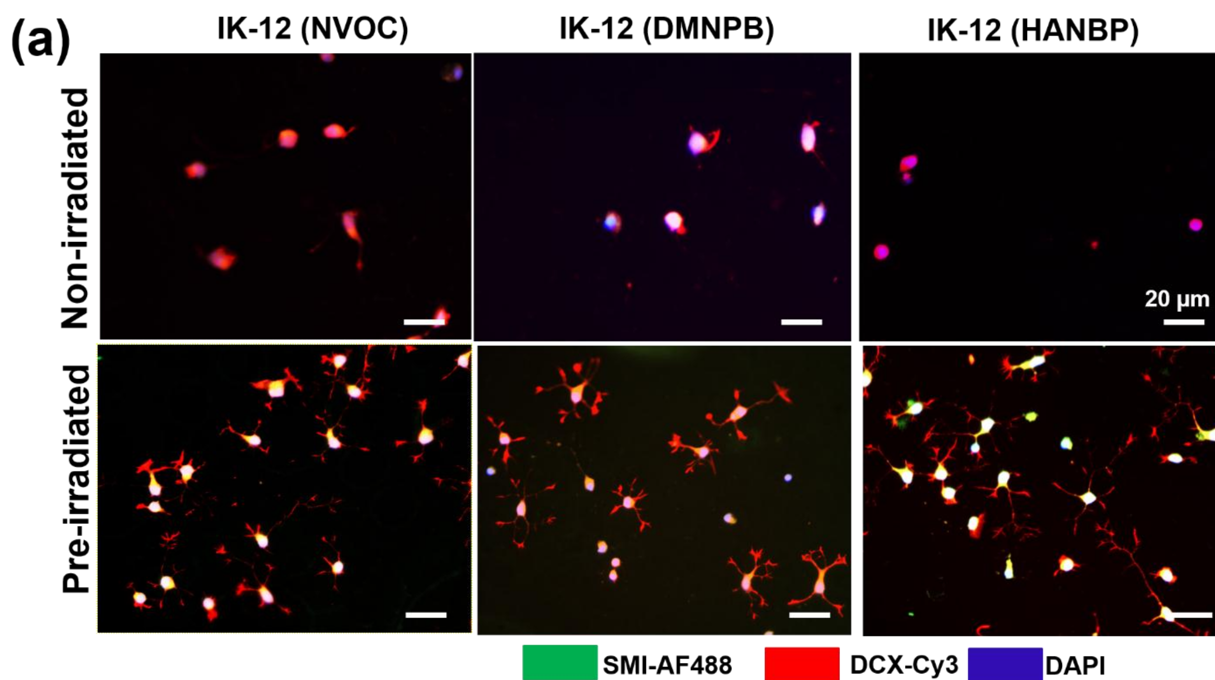
synapses.[21] Moreover, the developed axon displayed a 4-6 fold increase in length on pre-irradiated substrates. This effect was more pronounced on pre-irradiated IK-12(HANBP) gels and attributed to higher uncaging efficiency at given conditions.



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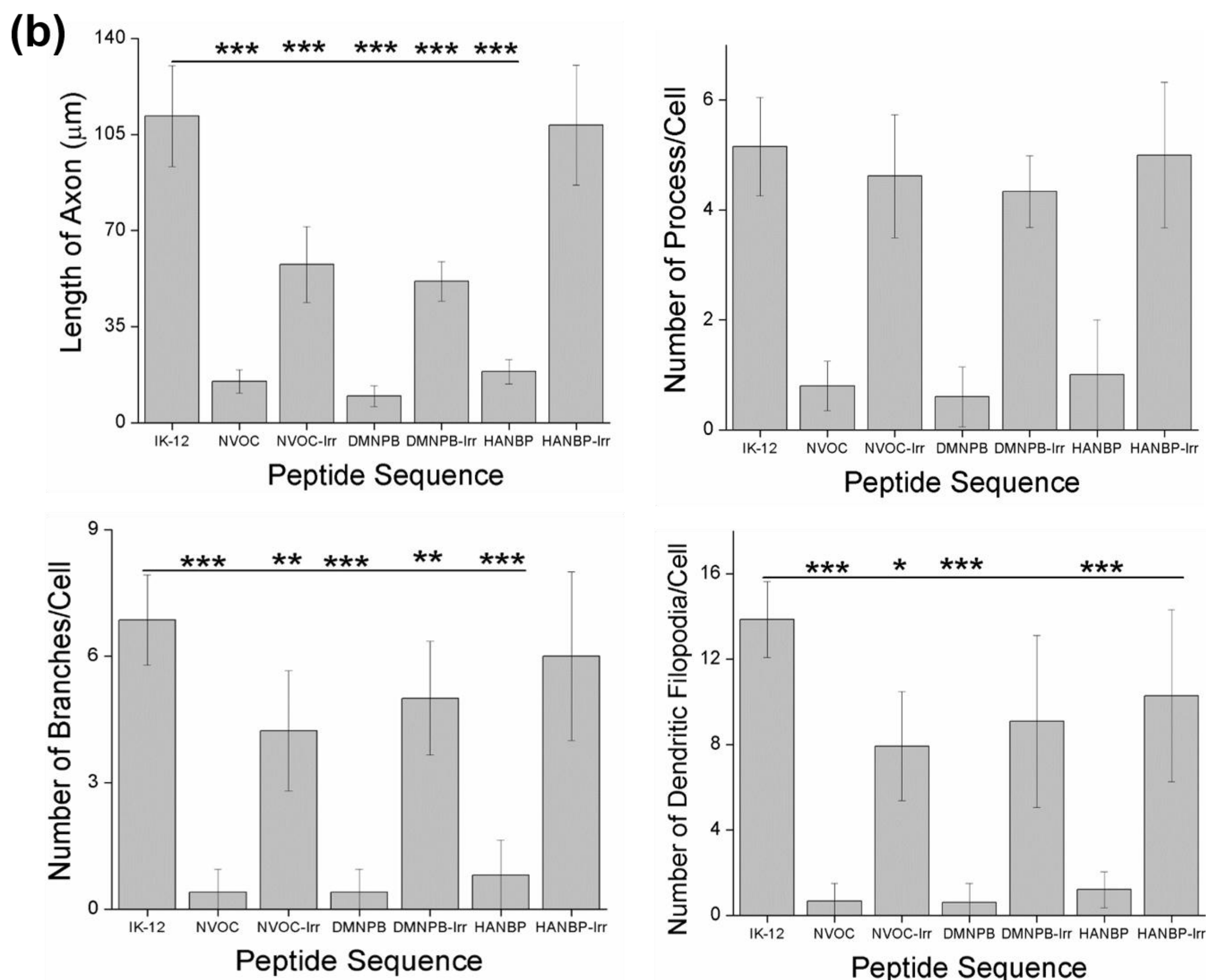

Figure 4. Behavior of neural progenitor cells seeded on non-irradiated and pre-irradiated hydrogels modified with photoactivatable IK-12 variants. (a) microscopy images showing the morphology of cells before and after light exposure, 48 h after seeding and (b) quantification of axonal length, number of neurites, branches and dendritic filopodia developed in neurons after 48 h. Pre-irradiation was performed for 15 min at λ_{max} 345 nm for IK-12(NVOC), 365 nm for IK-12(DMNPB), and 420 nm for IK-12 (HANBP). The significance of samples was analyzed by Tukey- test (mean ± SD, ANOVA, ** p <0.01, *** p <0.001) in comparison to IK-12.

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In summary, the three variants of photoactivatable IK-12 peptide can mediate photo-triggerable neuronal differentiation, being IK-12(HANBP) the one that performs best. Uncaging efficiency, hydrolytic stability, water solubility, and synthetic effort are all parameters to be considered for the final performance of a photo-activatable probe on biomaterials. Here, the three phototriggerable peptides showed similar water solubility and appropriate hydrolytic stability (at least 48 h in cell culture medium). The higher photolysis efficiency of the HANBP photocleavable group (2.8 - 3.4 folds *vs.* other variants) conferred this peptide a superior performance in our experiments.

The ability of the photoactivatable peptides to guide the position of neurons on the gel surface upon light exposure was then tested. IK-12(HANBP) modified hydrogels were pre-irradiated through a mask to generate a micropattern of activated and non-activated IK-12(HANBP) modified regions (Figure 5). Neural stem cells (NSCs) dissociated from neurospheres were seeded on modified hydrogels. Neurospheres are a source of undifferentiated stem cells, frequently used to follow differentiation of neurons in biomaterials[22] and easier to handle than NPCs. In control experiments, dissociated NSCs showed no attachment to non-irradiated IK-12(HANBP) modified gels. However, attachment and growth of neurites and branches on pre-fully irradiated samples was observed. Cell survival and growth on fully illuminated substrate were comparable to control IK-12 substrate, and cells differentiated into neurons, as established by SMI and DCX staining (Figure 5). On mask-illuminated samples, cells followed the irradiated pattern and specifically attached and spatially aligned on the photoactivated IK-12 regions of the substrate. The observed neuronal patterns on IK-12(HANBP) modified gels were maintained for at least 4 days, demonstrating that the photoactivatable peptide is hydrolytically stable in cell

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culture conditions. Cells were found exclusively on the illuminated area demonstrating the specificity of the interactions, and did not cross to neighbor illuminated stripes (at the chosen pattern dimensions, see Experimental Section for details). Our results demonstrate the possibility of spatial guidance of neuronal attachment, migration and differentiation on IK-12(HANBP) modified hydrogels.

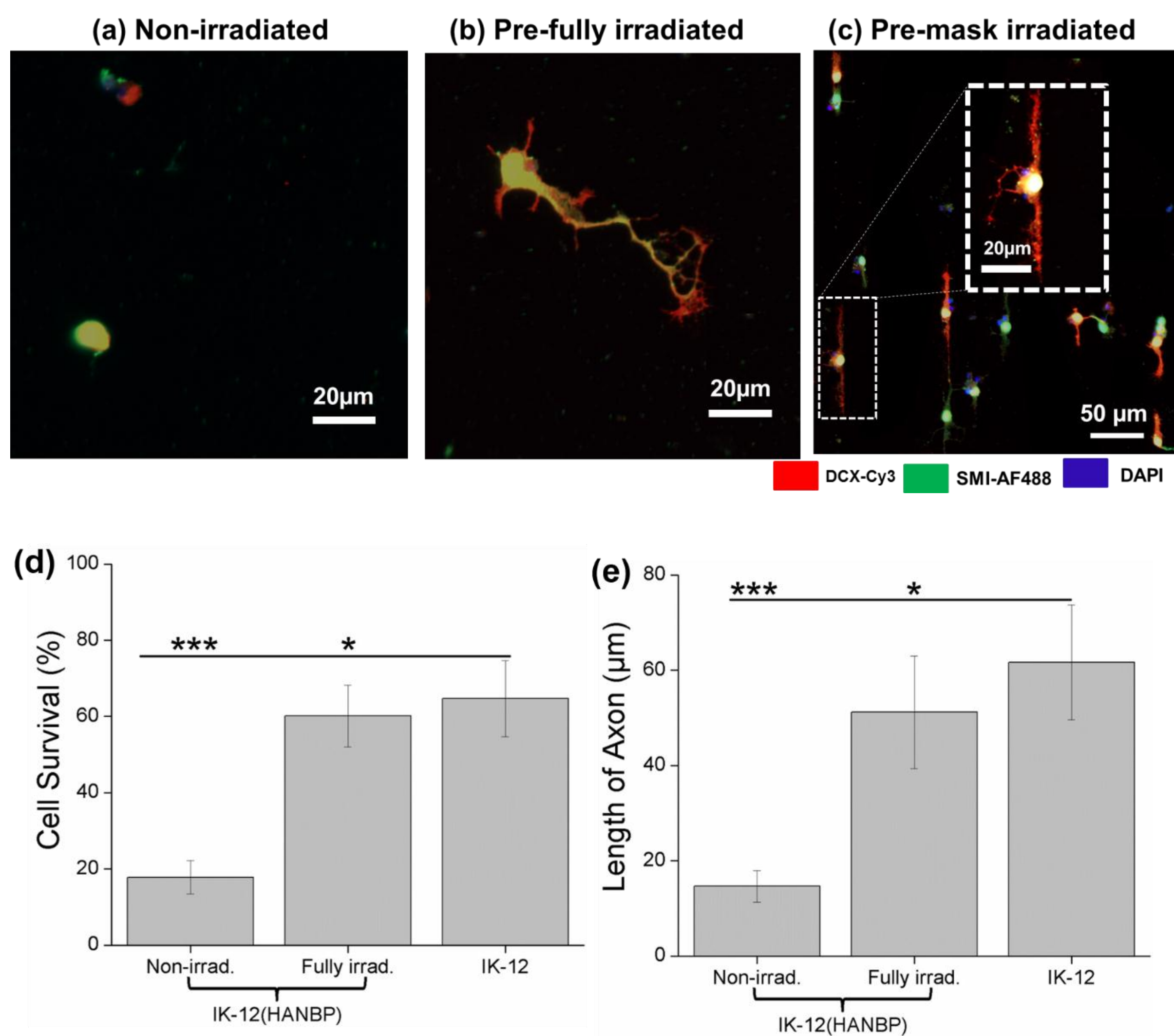

Figure 5. Dissociated NSCs from neurosphere (P-3) seeded on hydrogels functionalized with IK-12(HANBP) peptide, 24 h after seeding. Substrates were (a) non-irradiated, (b) pre-fully

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irradiated, or (c) pre-mask-illuminated (mask: 10µm strips separated by a 100 µm gap). Cell survival and axon length are also represented (d-e). Immunofluorescence images of cells showing the neuronal features, DCX stains cell body, SMI marks axonal filament and nucleus is stained by DAPI. The graph shows cell survival ratio and length of axon with statistical significance analyzed by Tukey- test in comparison to IK-12 (mean ± SD, ANOVA, ** $p < 0.01$, *** $p < 0.001$).

Conclusion

Three different photo-activatable short laminin-mimetic peptides were developed to support spatiotemporal control of neuronal attachment and differentiation to biomaterials. The introduction of a photoremovable protecting group at the Lys rest of the IKVAV active sequence allowed for optoregulated bioactivity of the peptidomimetic. Hydrogels functionalized with the photoactivatable peptides allowed light-mediated cell attachment and neuronal development to grades comparable to standard laminin or laminin/polylysine modified substrates. IK-12(HANBP) showed the best performance in terms of photochemical efficiency and bioactivity. This is a general strategy biomaterials strategy to optoregulate site-selective neurite outgrowth on any material of choice and points to the possibility to light-directed axonal growth on customized materials and geometries.

Experimental Section

Materials

All chemicals were purchased from commercial sources (Sigma Aldrich, Acros, or Novabiochem Merck) and used without purification. Fmoc-solid-phase peptide synthesis (Fmoc-SPPS) was

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performed manually following the procedure described in the Supporting Information. Details on the syntheses of chromophores and photoactivatable peptides are described in the Supporting Information.

Hydrogel preparation and functionalization

Gel Preparation Protocol

Poly(acrylamide-*co*-acrylic acid) P(AAm-AA) gels were prepared and characterized by following reported procedures.[23] Briefly, acrylamide (60 mg) was dissolved in PBS (1 mL). Acrylic acid (6 μL) and N,N-methylene-bis-acrylamide (0.4 mg) were added and the pH of solution was adjusted to pH 8 by using 0.1M NaOH aq. solution. The solution was degassed to remove oxygen and the free radical initiator ammonium persulfate (10% solution, 1/100 of total volume) and *N,N,N′,N′*-tetramethylethylenediamine catalyst (1/1000 of total volume) was added. 10 μL drops of the monomer solution were placed on hydrophobic Sigmacote-coated glass slides and covered with 3-acryloxypropyl-trimethoxysilane (APTS) functionalized coverslips. Samples were left for polymerization during 5 minutes, subsequently peeled-off from the hydrophobic slide and kept immersed in MilliQ water until use. The obtained thin film gel had a stiffness of 2 kPa as characterized by dynamic mechanical analysis following the procedure described in our previous report.[23c]

Functionalization of hydrogels with IK-12 derivatives

IK-12 peptide and its derivatives were coupled to P(AAm-AA) gels after EDC/NHS activation of the carboxylic groups in the gel and coupling to the amine group of the IK-12 peptide. Gels were covered with 100 μL of aqueous solution of EDC (0.2M) and NHS (0.1M) in MES buffer (0.1M, containing 0.5 M NaCl for 15 min. Gels were washed with water and incubated with one drop

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(30 µL) of peptide solution (0.5 $mgmL^{-1}$ in PBS, pH 7.4) for 1 hour at room temperature and then washed 3 times with water.

Photolysis of IK-12(NVOC)/(DMNPB)/(HANBP) variants bound to thin film hydrogels

Gels functionalized with caged IK-12 variants were irradiated at the corresponding λ_{max} for the different chromophores (i.e. 345, 365 and 420 nm for NVOC, DMNPB, and HANBP, respectively). A Xe-lamp coupled to a monochromator (Polychrome V, TILL Photonics GmbH, Gräfelting, Germany) was used for irradiation. The irradiance values were comparable at the different wavelengths: 0.28 mW at 345 nm, 0.22 mW at 365 nm, and 0.34 mW at 420 nm. Substrates were illuminated for 15 min, as this was the shortest illumination time required for activation in all the three gels. At shorter irradiation times (5-15 min) no response from the DMNPB and NVOC variants was observed; while longer illumination times (20-40 min) resulted in topographical defects on the hydrogels due to drying.

Patterned irradiation was performed by placing a quartz mask on top of the substrate during exposure. The mask was patterned with 10µm glass while 100µm chrome stripes. The IK-12 caged (NVOC/DMNPB/HANPB) functionalized gels were irradiated at respective wavelengths for different time intervals, followed by washing with PBS to remove photolyzed by-product. A 10 µm width of illuminated stripes proved the best to accommodate the body of a single cell. The optimal separation among illuminated lines was 100 µm. At lower separation (e.g. 50 µm), cells attached on one illuminated line were able to cross to the neighbor irradiated line.

Calculation of peptide density and binding efficiency on gels by UV-vis spectroscopy

The concentration of photoactivatable ligand in the hydrogel films was calculated from the UV absorbance (Figure 2) at λ_{max} according to Beer-Lambert law:

$$C = \frac{A}{l\varepsilon}$$

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The extinction coefficient ε_{max} value was taken from literature (NVOC: 6210 $M^{-1}cm^{-1}$ at 365 nm[24], DMNPB: 4100 $M^{-1}cm^{-1}$ at 346 nm [6b], and HANBP: 7500 $M^{-1}cm^{-1}$ at 397 nm [16]), the absorbance value A at respective λ_{max} was determined from UV-vis spectra of the films and the path length l corresponded to the swollen thickness of hydrogel film (~ 0.007 cm) and was obtained for each film from fluorescence correlation spectroscopy.[17] The binding efficiency was calculated by the ratio between the absorbance of peptide solutions, before (Ai) and after (Aa) coupling to hydrogel, according to following equation:

$$\text{binding efficiency (\%)} = \frac{Ai - Aa}{Ai} x100$$

Cell culture experiments

Embryonic cortical progenitors

All animal procedures were carried out in accordance with the Policies on the Use of Animals approved by the Institute of Physiological Chemistry, University Medical Center, Johannes Gutenberg University Mainz. Cerebral cortex of embryonic mouse (E14.5) obtained from C57BL/6 mice was digested in 0.5% trypsin EDTA (GIBCO) for 15 min at 37 ºC. Then trypsin was inactivated by the plating DMEM medium (GIBCO) and 10% FBS (Hyclone) and gently triturated with a 5-ml disposable pipette to get single cells. Centrifugation at 1000 rpm was performed for 5 min, cell pellets were re-suspended in 1 ml differentiating medium DMEM/F12 (GIBCO) and 2% B27(Invitrogen) and cell density was counted. Substrates functionalized with different ligands were sterilized with ethanol, washed with PBS and placed in 24-well cell culture plate. $5x10^4$ cells were seeded in each well, cell growth was followed by regular intervals and cells were fixed after 24-48 hours.[17]

Neurosphere Culture

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The cells isolated from cerebral cortex of embryonic mouse (E14.5) obtained from C57BL/6 mice as described above, were centrifuged at 1000 rpm for 5 min and cell pellet was resuspended in DMEM/F12 media containing 1% N2 (Invitrogen), 0.1% Heparin, 20 ng/ml basic fibroblast growth factors (bFGF, Invitrogen), 20 ng/ml epidermal growth factors (EGF, Invitrogen) and penicillin-streptomycin (N2 complete medium).[22d, 25] Dissociated cells were seeded in 6-well plate and maintained as undifferentiated neurospheres. The neurospheres were gathered and dissociated mechanically with 1 ml pipette. Then medium was changed from N2 complete media to differentiation media (DMEM-F12/glutamax, 2% B27, penicillin-streptomycin). Finally, dissociated neural stem cells were seeded (50,000 cells per well in 24-well plate) on IK-12 patterned hydrogel and maintained for 2-4 days. The absence of growth factors and addition of differentiation medium (B27) supported neuronal cells survival in comparison to glial cells.

Immuno-staining

Cells were fixed with 4% PFA solution (paraformaldehyde) for 10 min and washed 3 times with PBS pH 7.4 followed by blocking and permeabilization by incubating for 45 min with bovine serum albumin BSA 2% and Triton 0.2% in PBS at room temperature. Cells were incubated for 2 hours at room temperature with 1:500 DCX (guinea pig, Dianova) to stain cell body and 1:800 SMI-312 (mouse, Biolegend) to stain axon. Cells were washed 3 times with PBS followed by incubation for 2 hours at room temperature with 1:500 Cy3 (anti-guinea pig) and 1:800 (alexa 488 anti-mouse). The samples were mounted with immune select Antifading Mounting Medium (Dianova) containing DAPI for nucleus staining by using following standard protocols. Images were taken with Zeiss Axio Observer microscope at 0.42 μm per px. Cell viability and proliferation was calculated from time-lapse videos. Length of axons was measured using FIJI software, while number of neurites, dendrites and dendritic filopodia were counted manually.[17]

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Statistical Analysis

Data were expressed as mean ± standard deviation. For each condition, a minimum of three independent experiments were performed with sample size larger than 25 fields in all cases. The value of $p < 0.05$ was used for statistical significance. A one-way ANOVA with a Tukey test of the variance was used to determine the statistical significance between groups. The statistics was performed for non-irradiated and fully irradiated samples in comparison to IK-12 samples, and significance difference was set to* $\alpha < 0.05$, **$\alpha < 0.01$, ***$\alpha < 0.001$.

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Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Synthesis and characterization of photoactivatable lysine and peptide variants, photo-irradiation studies in solution and detailed quantification of cell culture results after 48 h.

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References

[1] W. Lee, C. W. Frank, J. Park, *Advanced Materials* **2014**, *26*, 4936-4940.

[2] aH. Koh, T. Yong, C. Chan, S. Ramakrishna, *Biomaterials* **2008**, *29*, 3574-3582; bB. Mammadov, R. Mammadov, M. O. Guler, A. B. Tekinay, *Acta biomaterialia* **2012**, *8*, 2077-2086; cC. R. Wittmer, T. Claudepierre, M. Reber, P. Wiedemann, J. A. Garlick, D. Kaplan, C. Egles, *Advanced functional materials* **2011**, *21*, 4232-4242.

[3] aR. Fricke, P. D. Zentis, L. T. Rajappa, B. Hofmann, M. Banzet, A. Offenhäusser, S. H. Meffert, *Biomaterials* **2011**, *32*, 2070-2076; bA. C. Greene, C. M. Washburn, G. D. Bachand, C. D. James, *Biomaterials* **2011**, *32*, 8860-8869; cY.-P. Lu, C.-H. Yang, J. A. Yeh, F. H. Ho, Y.-C. Ou, C. H. Chen, M.-Y. Lin, K.-S. Huang, *International journal of pharmaceutics* **2014**, *463*, 177-183.

[4] E. J. Berns, S. Sur, L. Pan, J. E. Goldberger, S. Suresh, S. Zhang, J. A. Kessler, S. I. Stupp, *Biomaterials* **2014**, *35*, 185-195.

[5] J. C. Rose, M. Cámara-Torres, K. Rahimi, J. Köhler, M. Möller, L. De Laporte, *Nano Letters* **2017**, *DOI: 10.1021/acs.nanolett.7b01123*.

[6] aV. San Miguel, C. G. Bochet, A. del Campo, *Journal of the American Chemical Society* **2011**, *133*, 5380-5388; bS. Petersen, J. M. Alonso, A. Specht, P. Duodu, M. Goeldner, A. del Campo, *Angewandte Chemie* **2008**, *47*, 3192-3195.

[7] aJ. Iturri, L. García-Fernández, U. Reuning, A. J. García, A. Del Campo, M. J. Salierno, *Scientific reports* **2015**, *5*; bM. J. Salierno, A. J. García, A. Del Campo, *Advanced functional materials* **2013**, *23*, 5974-5980; cM. J. Salierno, L. García-Fernandez, N. Carabelos, K. Kiefer, A. J. García, A. del Campo, *Biomaterials* **2016**, *82*, 113-123; dM. Wirkner, S. Weis, V. San Miguel, M. Álvarez, R. A. Gropeanu, M. Salierno, A. Sartoris, R. E. Unger, C. J. Kirkpatrick, A. del Campo, *ChemBioChem* **2011**, *12*, 2623-2629.

[8] T. T. Lee, J. R. García, J. I. Paez, A. Singh, E. A. Phelps, S. Weis, Z. Shafiq, A. Shekaran, A. del Campo, A. J. García, *Nature Materials* **2015**, *14*, 352-360.

[9] aP. Liesi, *The EMBO journal* **1985**, *4*, 1163; bL. A. Flanagan, L. M. Rebaza, S. Derzic, P. H. Schwartz, E. S. Monuki, *Journal of neuroscience research* **2006**, *83*, 845-856.

[10] aC. D. Weaver, C. K. Yoshida, I. De Curtis, L. F. Reichardt, *Journal of Neuroscience* **1995**, *15*, 5275-5285; bA. Domogatskaya, S. Rodin, K. Tryggvason, *Annual review of cell and developmental biology* **2012**, *28*, 523-553; cB. L. Patton, J. M. Cunningham, J. Thyboll, J. Kortesmaa, H. Westerblad, L. Edström, K. Tryggvason, J. R. Sanes, *Nature neuroscience* **2001**, *4*, 597-604.

[11] aJ. E. Frith, R. J. Mills, J. E. Hudson, J. J. Cooper-White, *Stem cells and development* **2012**, *21*, 2442-2456; bG. A. Silva, C. Czeisler, K. L. Niece, E. Beniash, D. A. Harrington, J. A. Kessler, S. I. Stupp, *Science* **2004**, *303*, 1352-1355.

[12] M. Yamada, Y. Kadoya, S. Kasai, K. Kato, M. Mochizuki, N. Nishi, N. Watanabe, H. K. Kleinman, Y. Yamada, M. Nomizu, *FEBS letters* **2002**, *530*, 48-52.

[13] aD. Thid, M. Bally, K. Holm, S. Chessari, S. Tosatti, M. Textor, J. Gold, *Langmuir* **2007**, *23*, 11693-11704; bD. Thid, K. Holm, P. S. Eriksson, J. Ekeroth, B. Kasemo, J. Gold, *Journal of Biomedical Materials Research Part A* **2008**, *84*, 940-953; cH. Kleinman, R. C. Ogle, F. Cannon, C. Little, T. Sweeney, L. Luckenbill-Edds, *Proceedings of the National Academy of Sciences* **1988**, *85*, 1282-1286; dK.-i. Tashiro, G. C. Sephel, B. Weeks, M. Sasaki, G. R.

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Martin, H. K. Kleinman, Y. Yamada, *Journal of Biological Chemistry* **1989**, *264*, 16174-16182.
[14] J. P. Ranieri, R. Bellamkonda, E. J. Bekos, J. A. Gardella, H. J. Mathieu, L. Ruiz, P. Aebischer, *International Journal of Developmental Neuroscience* **1994**, *12*, 725-735.
[15] aA. Ehrlicher, T. Betz, B. Stuhrmann, D. Koch, V. Milner, M. Raizen, J. Käs, *Proceedings of the National Academy of Sciences* **2002**, *99*, 16024-16028; bB. J. Black, L. Gu, S. K. Mohanty, *PloS one* **2014**, *9*, e86292; cB. Black, V. Vishwakarma, K. Dhakal, S. Bhattarai, P. Pradhan, A. Jain, Y.-t. Kim, S. Mohanty, *Scientific reports* **2016**, *6*; dJ. Q. Zheng, *Nature* **2000**, *403*, 89-93.
[16] L. Donato, A. Mourot, C. M. Davenport, C. Herbivo, D. Warther, J. Léonard, F. Bolze, J. F. Nicoud, R. H. Kramer, M. Goeldner, *Angewandte Chemie International Edition* **2012**, *51*, 1840-1843.
[17] A. Farrukh, J. I. Paez, M. J. Salierno, W. Fan, B. Berninger, A. del Campo, *Biomacromolecules* **2017**, *18*, 906–913.
[18] aA. M. Hopkins, L. De Laporte, F. Tortelli, E. Spedden, C. Staii, T. J. Atherton, J. A. Hubbell, D. L. Kaplan, *Advanced functional materials* **2013**, *23*, 5140-5149; bY. B. Lu, K. Franze, G. Seifert, C. Steinhäuser, F. Kirchhoff, H. Wolburg, J. Guck, P. Janmey, E. Q. Wei, J. Käs, A. Reichenbach, *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 17759-17764; cJ. Lantoine, T. Grevesse, A. Villers, G. Delhaye, C. Mestdagh, M. Versaevel, D. Mohammed, C. Bruyère, L. Alaimo, S. P. Lacour, L. Ris, S. Gabriele, *Biomaterials* **2016**, *89*, 14-24.
[19] W. Ma, T. Tavakoli, E. Derby, Y. Serebryakova, M. S. Rao, M. P. Mattson, *BMC developmental biology* **2008**, *8*, 90.
[20] L. E. Dansie, I. M. Ethell, *Developmental neurobiology* **2011**, *71*, 956-981.
[21] R. Yuste, T. Bonhoeffer, *Nature Reviews Neuroscience* **2004**, *5*, 24-34.
[22] aG. Wang, Q. Ao, K. Gong, A. Wang, L. Zheng, Y. Gong, X. Zhang, *Acta biomaterialia* **2010**, *6*, 3630-3639; bY.-J. Ren, Z.-Y. Zhou, F.-Z. Cui, Y. Wang, J.-P. Zhao, Q.-Y. Xu, *Journal of Bioactive and Compatible Polymers* **2009**, *24*, 56-62; cJ. R. Thonhoff, D. I. Lou, P. M. Jordan, X. Zhao, P. Wu, *Brain research* **2008**, *1187*, 42-51; dM. R. Costa, R. Jagasia, B. Berninger, *Protocols for Neural Cell Culture: Fourth Edition* **2010**, 29-49; eL. Pacey, S. Stead, J. Gleave, K. Tomczyk, L. Doering, *Nat Prot. doi* **2006**, *10*.
[23] aO. F. Zouani, J. Kalisky, E. Ibarboure, M.-C. Durrieu, *Biomaterials* **2013**, *34*, 2157-2166; bP. Moshayedi, L. da F Costa, A. Christ, S. P. Lacour, J. Fawcett, J. Guck, K. Franze, *Journal of Physics: Condensed Matter* **2010**, *22*, 194114; cA. Farrukh, J. I. Paez, M. Salierno, A. del Campo, *Angewandte Chemie* **2016**.
[24] F. Buhr, J. Kohl ‐ Landgraf, C. Hanus, D. Chatterjee, A. Hegelein, E. M. Schuman, J. Wachtveitl, H. Schwalbe, *Angewandte Chemie International Edition* **2015**, *54*, 3717-3721.
[25] H. Azari, S. Sharififar, M. Rahman, S. Ansari, B. A. Reynolds, *JoVE (Journal of Visualized Experiments)* **2011**, e2457-e2457.

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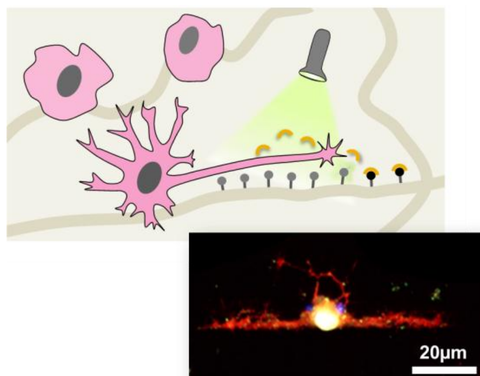

Text Suggestion for the Table of Contents:

On the light spot: Soft hydrogels functionalized with photo-activatable laminin-mimetic peptides provide a strategy to optoregulate the interaction of neuronal cells with a biomaterial. Site-selective irradiation activated the laminin-mimetic peptide and provided spatial adhesive and differentiation guidance to seeded neurons.

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