Optoregulated Protein Release from an Engineered Living Material

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Abstract: Developing materials to encapsulate and deliver functional proteins inside the body is a challenging yet rewarding task for therapeutic purposes. High production costs, mostly associated with the purification process, short-term stability *in vivo*, and controlled and prolonged release are major hurdles for the clinical application of protein-based biopharmaceuticals. In an attempt to overcome these hurdles, herein, we demonstrate the possibility of incorporating bacteria as protein factories into a material and externally controlling protein release using optogenetics. By engineering bacteria to express and secrete a red fluorescent protein in response to low doses of blue light irradiation and embedding them in agarose hydrogels, living materials are fabricated capable of releasing proteins into the surrounding medium when exposed to light. These bacterial hydrogels allow spatially confined protein expression and precisely dosed protein release, regulated by the area and extent of light exposure. The possibility of incorporating such complex functions in a material using relatively simple material and genetic engineering strategies highlights the immense potential and versatility offered by living materials for protein-based biopharmaceutical delivery.

1. Introduction

Engineered living materials are an emerging class of materials that combine synthetic architectures with living organisms to incorporate programmable functionalities.[1] Self-healing fabrics responding to mechanical tearing,[2] humidity-responsive self-ventilating fabrics,[3] self-healing concrete[4] or self-renewing metal sequestration membranes[5] are a few examples of novel applications explored in this field. The living components in these materials are usually genetically modified microbes that respond to external cues and perform pre-programmed functions. Implementing genetic engineering possibilities into synthetic materials opens new possibilities to materials design with properties and functions well beyond those of non-living matter.[6]

The incorporation of programmed bacteria into biomaterials and their envisioned applications in the fields of regenerative medicine and drug delivery has just started to be explored.[7–9] Living bio-interfaces consisting of biofilm-forming probiotic *L. lactis* engineered to surface display cell-adhesive ligands have been used as biofunctional coatings to mediate contact between cells and the host material and direct cell fate.[10–12] In a previous report, we showed how such interactions can be remotely controlled by light in a temporally regulated manner using an endotoxin-free, optogenetically engineered *E. coli* strain as living component of the interface.[13] Furthermore, exploiting a recently identified fluorescent protein-mediated secretion mechanism,[14] immobilized bacteria contacting an eukaryotic cell were able to

deliver a fluorescently labelled protein in a specific manner. Though the actual mechanism of fluorescent protein delivery is not fully understood, it seems to happen through diffusion of the fluorescent protein across the membrane.[14] In this manuscript, we hypothesized that this mechanism can be extended to transport other proteins genetically fused to the fluorescent protein into the external medium. Such *in situ* production of proteins, coupled to localized delivery and external regulation, represents an attractive property combination and opens interesting strategies for new therapeutic approaches, beyond current drug-delivery systems mostly based on protein-loaded nanoparticles. Issues like long-term release and cost-effective production can be improved with this approach.[17,19,20] A few recent examples of living materials producing biochemicals provide preliminary evidence to this approach. An antibiotic-releasing living material was constructed by encapsulating penicillin-producing fungi in a hydrogel matrix sandwiched between an elastomer and a porous membrane.[15] The material released the antibiotic for several days and killed gram-positive bacteria *in vitro*. In a different report, 3D-printed living tattoos for chemical detection on skin were made using *E. coli* trapped within a pluronic matrix on a skin-adhesive elastomer.[16] These tattoos turned fluorescent in the presence of specific chemicals due the responsive production of a green fluorescent protein by the bacteria.

As a step forward, in this manuscript a living material that enables *in situ* production of a protein, and regulation of protein release by means of optogenetically engineered living components is presented. While most bacterial protein expression systems are regulated through chemicals, here we use light as external stimulus for regulating protein synthesis and subsequent delivery.[21–23] The optogenetics approach allows non-invasive and localized activation of protein synthesis, easy regulation of pharmacokinetic profiles by tuning exposure dose, and can be eventually multiplexed using different optogenetic constructs and wavelengths. While these attributes have been impressively demonstrated in different biomedical contexts,[21,22] this work applies it for the first time of the biomaterials field. From this perspective, a bacterial protein factory is directly incorporated within a simple hydrogel, and the release is externally controlled by external light exposure. Bacterial protein secretion,[14] optogenetics[21] and hydrogel encapsulation[15] are all combined to engineer these living hydrogels **(Figure 1a)**. Due to its robustness, protein overexpression capability and adaptability to be extended to an enormous range of genetic modules and strains, *E. coli* was chosen as the living component in this study. We employed an entirely genetically encoded optogenetic protein expression plasmid, pDawn,[21] with which we were able to express and secrete, in response to low doses of blue light, a streptavidin binding peptide tag containing red fluorescent protein (RFP). The bacteria were effectively and functionally immobilized within agarose gels, and the light-driven local triggering of protein expression and release of different dosages of the protein is demonstrated.

2. Results and Discussions

2.1. Optoregulated living material design and construction

To engineer the light-responsive protein-releasing bacteria, the RFP gene was inserted into an optogenetic protein-expression plasmid and produced in *E. coli*. Based on reported data demonstrating *E. coli* to be able to secrete β-barrel shaped fluorescent proteins with a net negative charge,[14] and our own previous experience with *E. coli* delivering a red fluorescent protein to cells,[13] we decided to use RFP with an N-terminal streptavidin-binding tag (Strep-tag® II) (Figure 1a) for monitoring protein release. Light-regulation was realized by cloning RFP into the well-established optogenetic protein expression plasmid, pDawn.[21] This *E. coli*-based plasmid activates expression of a protein cloned into it when irradiated with blue light. Since most commonly used *E. coli* lab strains are incompatible for medical purposes due to

their endotoxic outer membranes, a commercially available endotoxin-free strain of *E. coli* (ClearColi®)[24] was used in this study. Upon exposure to white light, RFP expression within the bacteria in culture was clearly observed using epifluorescence microscopy, while negligible leaky expression occurred in the absence of light (Supporting information, Figure S1).

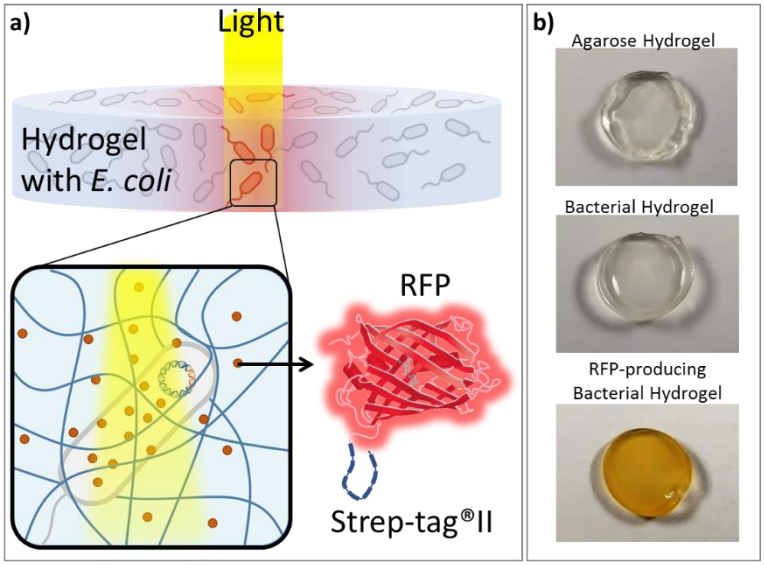

Figure 1. a) Graphical representation of light-regulated protein secreting living material concept with *E. coli* encapsulated in hydrogel and secreting RFP. b) Images of hydrogels that are encapsulating nothing, bacteria and bacteria that were allowed to produce RFP for 7 days. Diameter of gels is 12 mm.

To engineer the living material, bacteria were encapsulated within agarose hydrogel by simple mixing bacterial cultures with low-melt agarose solutions, maintained at 37ºC. The gels were formed by pouring the suspension into well plates and cooling to room temperature. Bacteria were loaded at high density (ca. 2 x $10^{\wedge 9}$ cells/ mL). After a week of protein expression, the gel visibly changed color (Figure 1b), indicating the production and accumulation of RFP within the gel. Even though bacterial viability was retained during hydrogel encapsulation **(Figure 2a)**, RFP production upon exposure to 470 nm light occurred poorly when the gels were completely submerged in medium (Supporting information Figure S2). This was most likely due to the fact that RFP requires molecular oxygen for proper folding,[25,26] which might have not been sufficient when the hydrogels were submerged in medium. To overcome this issue, thin layers of bacterial hydrogels (<500µm) were used with one surface exposed to air and resulted in robust RFP expression (Supporting information Figure S2).

2.2. Localized and tuned protein expression

Localized activation of protein expression was attempted by exposing the gel to light through a photomask. However, this resulted in RFP expression over the entire hydrogel, most likely due to scattering effects. Using pulsed exposure, as suggested by Ohlendrof et al.,[21] localized expression of RFP was achieved within a circular region of diameter 1 mm. Pulse cycles of 470 nm light (2 mW) irradiated for 100 ms every 10 mins ensured minimal light scattering, while maintaining continuous activation of protein expression. Tuning of protein expression level was possible by varying the number of pulse cycles, thereby varying the amount of irradiation time (Figure 2b). Fluorescence within the activated areas was detected after 1 hour of pulsed irradiation (Figure 2c) and 5 hours after commencement of irradiation. Fluorescence

intensities correlated almost linearly with the number of pulse cycles. It should be noted that in the absence of light exposure, the 0-pulse cycle region displays a non-zero fluorescence intensity at the 5-hour timepoint. This is due to leaky protein expression, inherent to such systems, and was approximately 36 times lower than that of the highest intensity spot. These results suggest that protein expression can be finely regulated in time, space and dosage through light irradiation.

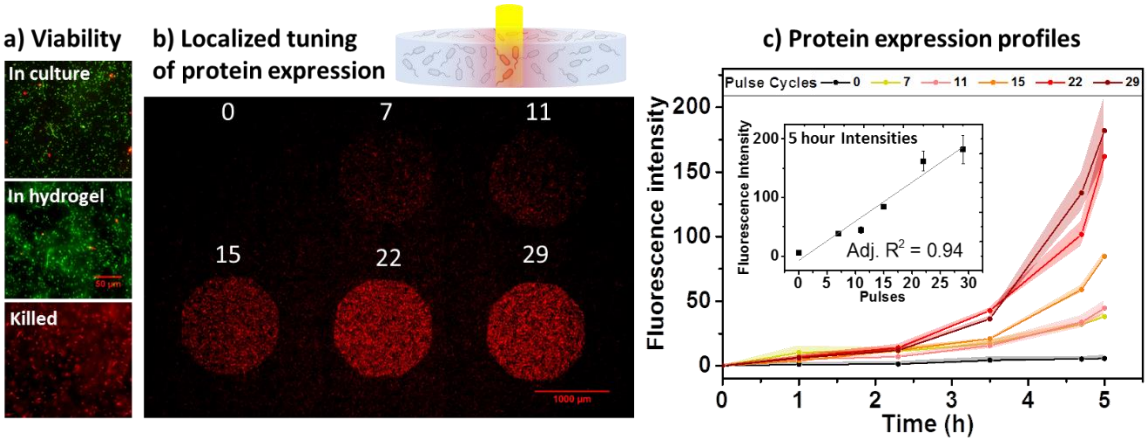

Figure 2. a) Epifluorescence images showing bacterial viability analyzed using BacLight bacterial viability kit. Hydrogel immobilized bacteria show similar levels of viability to the bacteria in culture (~98%). As a negative control, bacteria within the hydrogel were killed with 70% ethanol. Green = Live bacteria, Red = Dead bacteria. b) Epifluorescence image of 5-hour timepoint of bacterial hydrogel film expressing RFP with spots irradiated

2.3. Protein secretion and diffusion through hydrogel

Next, protein secretion and diffusion through the hydrogel was tested. Protein secretion was confirmed in bacterial cultures using Strep Tactin® affinity beads, to which secreted RFP is expected to bind through its N-terminal streptavidin-binding peptide tag (Supporting information, Figure S1). Bacteria and affinity beads were mixed with agarose separately and filled into wells adjacent to one another **(Figure 3a)**. 4x4 mm^2 areas within the bacterial gel were exposed to 470 nm light for 29 pulse cycles lasting 5 hours. After 2 hours of light exposure the affinity beads within the gel located closest to the bacterial gel gained faint red fluorescence (Figure 3b), ca. 1 hour after RFP expression was observed in the bacteria gel. This result indicates that RFP was secreted and diffused across the gel to become accumulated at the affinity beads. The fluorescence intensity of the affinity beads increased with time and was observable beyond 1 mm distance from the bacterial layer. 5 hours after the end of the light pulse cycles (10-hour timepoint), a clear fluorescence gradient was observed across the gel with the beads, where the fluorescence intensity inversely correlated to the distance from the bacterial layer (Figure 3d).

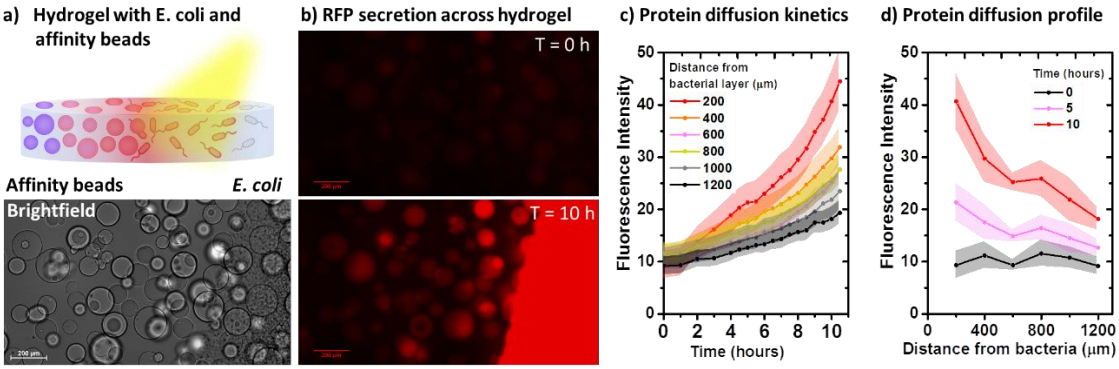

Figure 3. a) Graphical representation of the hydrogel containing spatially segregated bacteria and affinity beads. The brightfield image shows the *E. coli* to the right as a speckled region and the affinity beads on the left. b) Epifluorescence images at 0-hour and 10-hour time points showing red fluorescence of the bacterial and affinity bead layers. c) Fluorescence intensities of the affinity beads at different distances from the bacterial layer plotted as a function of time after light pulse irradiation was started. d) Fluorescence intensities of affinity beads plotted as a function of distance from the bacterial layer at 0, 5 and 10-hour timepoints. Semi-transparent bands in both plots represent standard error values obtained from a total of 49 beads from 3 individual samples.

2.4. Light-regulated dosed release of protein

Encouraged by these results, we proceeded to test the ability of the bacterial hydrogels to secrete proteins into the surrounding medium. For the experimental design, 2 issues observed in the previous experiments had to be addressed: (i) one side of the hydrogel should be exposed to air for oxygen availability, and (ii) bacteria immobilized at the surface of the gel were able to grow out of the gel and contaminate the medium. Both these issues were solved by forming a bacterial hydrogel in a transwell insert using a porous membrane with pores smaller than bacteria **(Figure 4a)**. *E coli* have dimensions of 1 µm diameter and 2 - 3 µm length[27] and therefore membranes with pore size 0.4 µm were tested. This strategy prevented contamination of the medium by *E. coli*. One side of the gel was exposed to air and the other was in contact with the medium through the porous membrane. Within the medium, affinity beads were added to detect and quantify the secreted RFP. A 4x4 mm^2 area of the gels was irradiated with increasing number of pulse cycles and the fluorescence intensity of the affinity beads was measured after 24 hours. A clear correlation between fluorescence intensity and irradiation dose was observed (Figure 4b). Using a calibration curve, the mass of RFP secreted by the bacterial hydrogels into the medium was calculated (Figure 4c). The amount of secreted RFP showed a non-linear correlation with the irradiation dose, indicating that RFP secretion is non-linearly dependent on cytoplasmic concentration of RFP and is considerably improved by its overexpression. At the highest dose applied, approximately 50 ng of protein was accumulated in the medium from nearly 10 µL of bacterial hydrogel in 24 hours (Details of calculations provided in the Supporting Information). Most importantly, it is evident that the amount of secreted protein can be easily regulated by light.

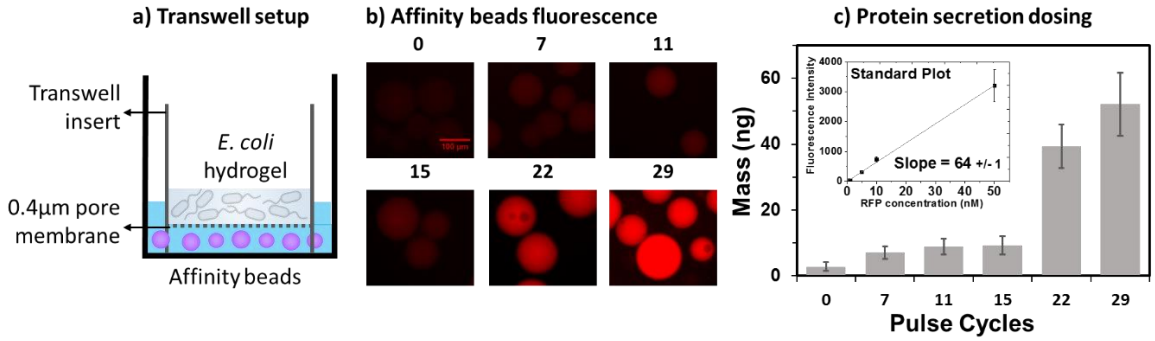

Figure 4. a) Experimental design with bacterial hydrogel within the transwell, separated by a porous membrane from the medium containing affinity beads. b) Epifluorescence images of affinity beads after 24 hours of protein secretion from bacterial hydrogels. Numbers on top of the images correspond to number of pulse cycles. c) Mass of protein secreted into the medium depending on the number of light pulses cycles delivered. Inset represents the standard plot of affinity beads fluorescence intensity as a function of known RFP concentrations. Error bars indicate standard deviation values (n = 2).

3. Conclusion and outlook

Using relatively simple material and optogenetic engineering we demonstrate the fabrication of a living hydrogel capable of producing and releasing proteins in light-dependent doses. The modularity of the system allows for a wide range of modifications, adaptations and extensions of this approach. Most obviously, several medically relevant proteins can be genetically fused to RFP for controlled release.[14] Protein secretion can be achieved using other genetically encodable transporters,[28] thereby eliminating the need for oxygen that posed as a limitation in this study. The optogenetic toolbox contains other constructs that allow activation of protein expression with green, red and even near infrared light.[22,23] Peptide tags that promote degradation of the expressed protein[29] can be included to rapidly eliminate excess protein within the cytoplasm after light activation is stopped, thereby establishing a sharp cut-off of secretion and reducing leaky expression in the dark that was observed in this study. Bacterial metabolism can be controlled through genomic modifications to prevent outgrowth[30] and a kill-switch in the form of a lysis protein can be incorporated to eliminate the bacteria when required.[31] Furthermore all these genetic modules can be mixed and combined within a single bacterium thereby establishing genetic circuits with multiple functions. Alternatively, bacterial strains with different functions can be incorporated in a material. From the materials perspective, various different types of encapsulating matrices such as polyacrylamide,[32] alginate,[33] PEG diacrylate,[34] pluronic,[16] etc. can be achieved and processed into various forms such as microbeads,[33,34] bioprinted scaffolds[16] and even electrospun fibers.[35] Such modifications will be explored in future studies to identify the true potential and limitations of these living materials for biomedical applications.

4. Experimental Section

Materials: Clearcoli BL21(DE3) electrocompetent cells were purchased from BioCat, NEBuilder® Hifi DNA assembly cloning kit and Q5® Site-directed mutagenesis kit from New England Biolabs (NEB), primers from Integrated DNA Technologies (IDT), Low-melting point agarose (molecular biology grade) from Fisher Scientific, MilliCell® CM standing cell culture inserts (24 well, Biopore PTFE hydrophilic membrane) from Merck Millipore Sigma Aldrich, Strep-Tactin® XT affinity beads from IBA life sciences and pDawn plasmid was purchased from Addgene (plasmid # 43796).

For electroporation transformation Bio-Rad Micropulser™ Electroporator was used in combination with Bio-Rad 0.1 cm electroporation cuvettes (1652083). Microscopy analysis

was performed using two different Nikon Ti-Ecllipse (Nikon Instruments Europe B.V., Germany) microscopes. One used an Intensilight (Nikon Instruments Europe B.V, Germany) pre-centered mercury lamp for illumination and an Andor Clara (Andor Technology Ltd, Belfast, United Kingdom) CCD camera for detection. The second microscope used a Sola SE 365 II (Lumencor Inc., Beaverton, USA) solid state illumination device and an Andor Clara CCD camera for detection. For fluorescence imaging, both devices used the appropriate Semrock (Semrock INC., Rochester, USA) filter set mounted on a filter-cubes. On both microscopes, temperature was maintained with help of an Okolab (Okolab SRL, Pozzuoli, Italy) incubation cage. Blue light irradiation and Image acquisition were performed using either a 10x Plan-Apo objective, with a numerical aperture of 0,45 (Nikon) or a 20x S Plan Fluor phase contrast objective with a numerical aperture of 0,45 and an extra-long working distance of 8mm (glass coverslips).

Construction of plasmids and bacterial strains: An N-terminal hexa-histidine tag in RFP from our previously reported plasmid, pET-Duet1-TagRFP, was mutated to a Strep-tagII® (WSHPQFEK) using Q5® site directed mutagenesis kit. This gene was then amplified and cloned into pDawn using NEbuilder® Hifi DNA assembly cloning kit with the following primers – pDawn-Rev 5'-GCTGCTGCCCATGGTATATC-3', pDawn-Fwd 5'-GATCCGGCTGCTAACAAAG-3', RFP-Fwd 5'-gatataccatgggcagcagcTGGAGCCATCCGCAGTTTG-3', RFP-Rev 5'-gctttgttagcagccggatcTCACCAGACTACGAGTTCCTTG-3'. This yielded pDawn-RFP plasmid which was then transformed into Clearcoli BL21(DE3) electrocompetent cells exactly as specified by the provider. A glycerol stock was made from a bacterial culture grown overnight at 37^{o}C, 250 rpm in the dark from a single colony. A final glycerol concentration of 15% was used.
Image processing and analyses were performed using Fiji edition of ImageJ.[1]
Gene sequence:
ATGGGCAGCAGCTGGAGCCATCCGCAGTTTGAAAAAGATCTGTACGACGATGAC
GATAAGGATCCaATGAGCGAGCTGATTAAGGAGAACATGCACATGAAGCTGTAC
ATGGAGGGCACCGTGAACAACCACCACTTCAAGTGCACATCCGAGGGCGAAGGC
AAGCCCTACGAGGGCACCCAGACCATGAGAATCAAGGTGGTCGAGGGCGGCCCT
CTCCCCTTCGCCTTCGACATCCTGGCTACCAGCTTCATGTACGGCAGCAGAACCTT
CATCAACCACACCCAGGGCATCCCCGACTTCTTTAAGCAGTCCTTCCCTGAGGGC
TTCACATGGGAGAGAGTCACCACATACGAAGACGGGGGCGTGCTGACCGCTACC
CAGGACACCAGCCTCCAGGACGGCTGCCTCATCTACAACGTCAAGATCAGAGGG
GTGAACTTCCCATCCAACGGCCCTGTGATGCAGAAGAAAACACTCGGCTGGGAG
GCCAACACCGAGATGCTGTACCCCGCTGACGGCGGCCTGGAAGGCAGAAGCGAC
ATGGCCCTGAAGCTCGTGGGCGGGGGCCACCTGATCTGCAACTTCAAGACCACAT
ACAGATCCAAGAAACCCGCTAAGAACCTCAAGATGCCCGGCGTCTACTATGTGG
ACCACAGACTGGAAAGAATCAAGGAGGCCGACAAAGAGACCTACGTCGAGCAGC
ACGAGGTGGCTGTGGCCAGATACTGCGACCTCCCTAGCAAACTGGGGCACAAGG
AActcgTAGTCTGGTGA
Blue = Strep-tagII®; Green = Xpress Epitope; Red = TagRFP

Bacterial culture conditions: Bacterial cultures were grown for 16h at 34^{o}C, 180 rpm in LB Miller medium supplemented with 50 µg/mL of Kanamycin to an optical density at 600 nm wavelength (OD_{600}) value between 0.4 – 0.8. The culture was then spun down at 3300 g for 10 min at 22^{o}C (Hettich Rotanta 460 RS). The supernatant was discarded and replaced with fresh medium at a volume to ensure a final bacterial density with OD_{600} around 4.0. All procedures were performed either in the dark or under a laminar hood with an orange film that cut out blue light.

Bacterial hydrogel preparation: Low melting point agarose was mixed with medium at a concentration of 2% w/v, dissolved at 70^{o}C and cooled to 37^{o}C. The high-density bacterial cultures were brought to 37^{o}C and mixed at a 1:1 volume ratio to have a final bacterial OD_{600} of 2.0 and an agarose concentration of 1% w/v. These gel solutions were pipetted in the required form and allowed to form a gel at room temperature in the dark.
Localized tuning of protein expression:
100 µL of bacterial gels were pipetted into 24 well cell culture plate wells, allowed to spread evenly over the whole surface and allowed to form a gel at room temperature. This resulted in an extremely thin gel at the central region with a width of approximately 20 µm that allowed imaging of the fluorescent patterns with minimal background fluorescence. Individual spots were irradiated with the microscope bearing the Intensilight mercury lamp illuminator through a 10x objective with a partially closed field diaphragm resulting in a 1 mm diameter circular irradiation zone of power 2 mW at 470 nm wavelength. NIS Elements JOBS module was used to program the microscope to irradiate each spot with the specified number of pulses. Each pulse occurred once in 10 mins and irradiation lasted for 100 ms. Large area images were also captured at regular intervals of time through the red fluorescence channel.

Protein diffusion through gel: Strep-Tactin® XT affinity beads were spun down at 1000 g for 30s and washed twice with 5 column volumes of medium and finally resuspended in 2 column volumes. This was mixed in a 1:1 volume ratio with 2% Agarose solution at 37^{o}C. 75 µL of the bacterial gels were first added to one side of 24 well plate wells and allowed to form gels. The gels were thick at the edges and formed a fine film near the mid-point of the well. 75 µL of the affinity beads gel was then added on the other edge and this too formed a fine film at the mid-point of the well, while also partially overlapping over the bacterial gel. Activation of protein expression in the bacterial layer was then performed using the microscope bearing the Sola solid-state illuminator through a 10x objective, shining blue light with an intensity of 2 mW at 470 nm. The irradiation was scanned over an area of 4 mm X 4 mm, with an irradiation time of 100 ms for each spot. The irradiation was done over 4 hours with an interval of 10 mins between each pulse scan. Large area images of the affinity beads gel directly adjacent to the irradiated area was imaged at regular intervals using the red fluorescence channel. The irradiation and imaging were performed to automatically occur for the whole time period using the ND Sequential Acquisition tool in NIS-Elements.

Dosed protein secretion: 50 µL bacterial gels were formed in MilliCell CM inserts within 24 well plate wells. Strep-Tactin® XT affinity beads were spun down at 1000 g for 30s and washed twice with 5 column volumes of medium and finally resuspended in 15 column volumes of medium. 400 µL of this suspension was then pipetted into the well area below and around the MilliCell insert. Activation of protein expression in the bacterial layer was then performed using the microscope bearing the Sola solid-state illuminator through a 10x objective, shining blue light with an intensity of 2 mW at 470 nm. The irradiation was scanned over an area of 4 mm X 4 mm, with an irradiation time of 100 ms for each spot. Each MilliCell CM insert containing a bacterial gel was irradiated for the number of pulse cycles mentioned in the main text. Pulses were done the same way as mentioned in the previous paragraph. The affinity beads were imaged 24 hours after the start of the irradiation and only after removing the inserts from the wells. To obtain the standard curve, purified RFP of known concentrations were mixed with affinity beads prepared the same way as explained above and imaged under the same microscopy conditions.
The standard curve provided as slope of 64 ± 1 intensity units/nM of RFP. RFP has a molecular weight of 29.3 kDa. Using these values and the affinity bead intensity values obtained from the above-mentioned experiment, the mass of protein secreted into the 400 µL of medium was calculated. The MilliCell inserts have an inner diameter of 1 cm with an

effective membrane area of 0.6 cm^2 within which 50 µL of bacterial gel was formed, which would result in a 0.083 cm thick uniform gel. The irradiated area was 0.16 cm^2 around the center, therefore the volume of uniform gel irradiated would have been approximately 13 µL. Since the gel is actually not uniform but thicker at the edges and thinner near the center, the total volume of irradiated gel was approximated as 10 µL.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Supporting Information

Optoregulated Protein Release from an Engineered Living Material

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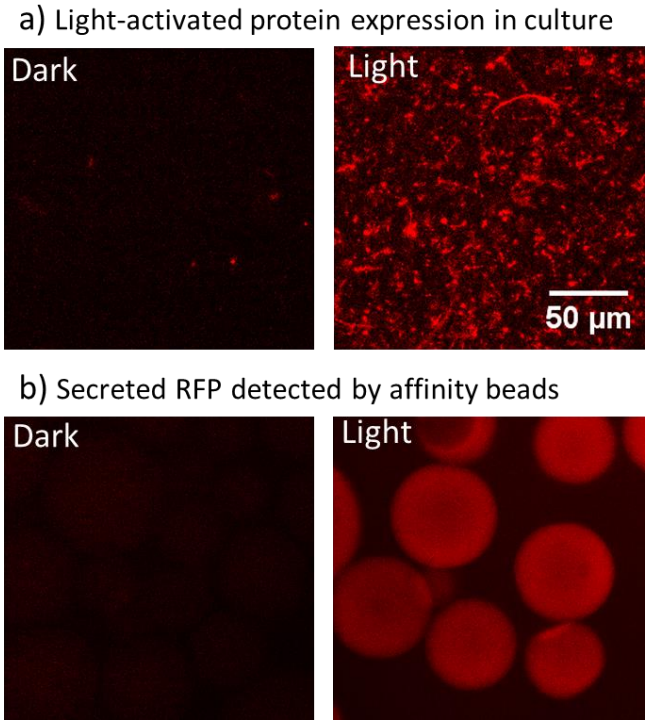

Figure S1. a) Epifluorescence red channel images of *E. coli* in culture expressing RFP in the absence and presence of light after 5 hour of white light irradiation having a blue component of power 20 µW. b) Strep Tactin® affinity beads exposed to *E. coli* culture supernatants with or without 7 hours of white light exposure.

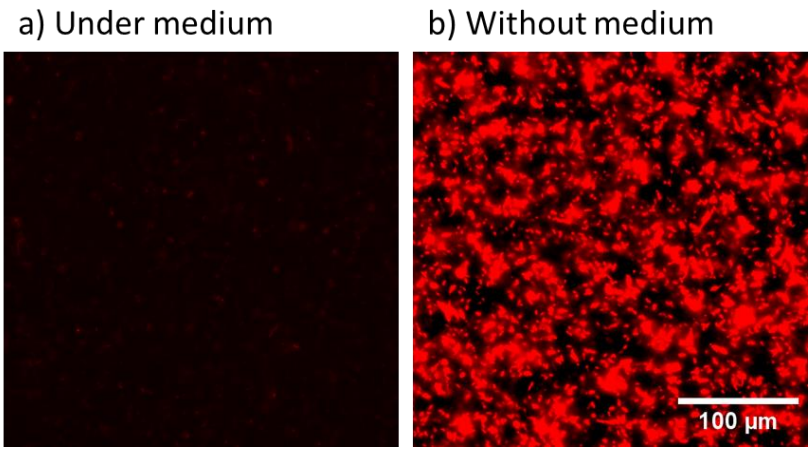

Figure S2. RFP expression in light-responsive E. coli cells immobilized in agarose hydrogel matrices with 4 hours of white light activation a) with medium on top of the gel and b) without any medium on the gel