

## Implementation of Blue Light Switchable Bacterial Adhesion for Design of Biofilms

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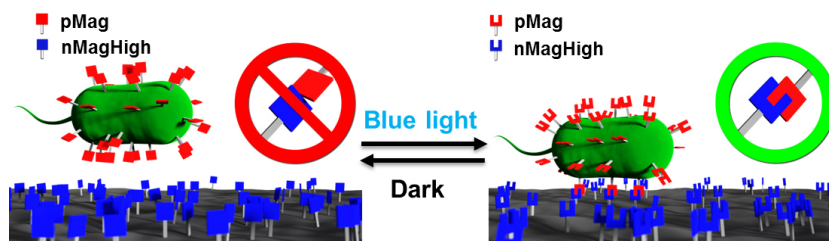
**[Abstract]** Control of bacterial adhesions to a substrate with high precision in space and time is important to form a well-defined biofilm. Here, we present a method to engineer bacteria such that they adhere specifically to substrates under blue light through the photoswitchable proteins nMag and pMag. This provides exquisite spatiotemporal remote control over these interactions. The engineered bacteria express pMag protein on the surface so that they can adhere to substrates with nMag protein immobilization under blue light, and reversibly detach in the dark. This process can be repeatedly turned on and off. In addition, the bacterial adhesion property can be adjusted by expressing different pMag proteins on the bacterial surface and altering light intensity. This protocol provides light switchable, reversible and tunable control of bacteria adhesion with high spatial and temporal resolution, which enables us to pattern bacteria on substrates with great flexibility.

**Keywords:** Bacterial adhesion, Optogenetics, nMag-pMag, Photoswitching, Biofilm

**[Background]** Controlling the biofilm formation is crucial to understand the social interactions between bacteria in naturally occurring biofilm (Flemming *et al.*, 2016). This is also particularly important for the biotechnological application of biofilms in biocatalysis, biosensing and waste treatment (Zhou *et al.*, 2013; Jensen *et al.*, 2016). The biofilm formation always begins with the bacterial adhesion to a substrate, which determines the spatial organization in biofilms (Liu *et al.*, 2016; Nadell *et al.*, 2016). Many strategies have been proposed to control bacterial adhesion such as modifying bacterial surface with bio-orthogonal reactive groups via liposome fusion (Elahipanah *et al.*, 2016), immobilization of adhesive molecules on the substrates (Sankaran *et al.*, 2015; Zhang *et al.*, 2016; Peschke *et al.*, 2017) and conjugating surface tags on bacteria (Poortinga *et al.*, 2000; Rozhok *et al.*, 2005; Lui *et al.*, 2013). Among these, the light responsive approaches provide the highest spatiotemporal control, which is important to precisely control the fine structure of the biofilms. For instance, azobenzene linkers have been used as a photoswitchable tool to reversibly control the bacterial adhesion to substrates by altering the presentation of mannose, which is recognized by the bacterial surface receptor FimH (Voskuhl *et al.*, 2014; Weber *et al.*, 2014; Sankaran *et al.*, 2015). In addition, azobenzene-based molecules have also been used to control bacteria adhesion to mammalian cells (Mockl *et al.*, 2016), bacterial quorum sensing (Van der Berg *et al.*, 2015) and biofilm formation (Hu *et al.*, 2016) with UV-light. One of the major drawbacks of using UV-light is that it is toxic to bacteria. In this protocol, we present a new approach of how to control bacterial adhesion to substrates with blue light based on photoswitchable proteins. Besides being a non-invasive, reversible and tuneable technique to control bacterial adhesion to substrates, it also provides high spatiotemporal control required to form

well-defined biofilms. Photoswitchable proteins are commonly used in the field of optogenetics to regulate gene expression, receptor activation and protein localization in cells with visible light (Müller and Weber, 2013; Tischer and Weiner, 2014). These optogenetic systems are very sensitive to visible light, bioorthogonal and noninvasive. Furthermore, these proteins are genetically encoded so they can be sustainably expressed in the cell. Here, we used the blue light responsive proteins, nMag and pMag, as photoswitches control bacterial adhesion. These proteins heterodimerize under blue light (480 nm) and dissociate from each other in the dark (Kawano *et al.*, 2015). The strength and back conversion kinetics of the nMag and pMag interaction are different for the point mutants. The point mutant pMagHigh (and nMagHigh) has a stronger interaction with its binding partners and slower back conversion, while the opposite is true for the mutant pMagFast1 (and nMagFast1) (Zoltowski *et al.*, 2009).

In our method we display the first interaction partner of the photoswitchable proteins, pMagHigh, pMag or pMagFast1 on the surface of *E. coli* using the circularly permuted OmpX (outer membrane protein X) protein (Daugherty, 2007). The pMag variants are attached through their C-terminal to the OmpX protein. The second interaction partner the photoswitchable protein, nMagHigh, is immobilized through a His6-tag at its C-terminal on a glass substrate with a PEG (polyethylene glycol) coating, which contains a Ni<sup>2+</sup>-NTA group (Schenk *et al.*, 2014). This setup allows bacteria expressing pMag proteins on their surfaces to adhere to nMagHigh functionalized substrates under blue light when the two proteins interact but not in the dark. (Figure 1)



**Figure 1. The engineered *E. coli* that express pMag proteins on their surface adhere to nMagHigh modified substrates under blue light.** In the dark, the pMag-nMag interaction is reversed, which leads to the detachment of the bacteria from the substrate. Reproduced with permission from Chen and Wegner (2017).

## **Materials and Reagents**

1. Pipette tips (STARLAB, catalog number: S1111-6700)
2. 50 ml Falcon tube (Greiner Bio One International, catalog number: 227261)
3. 1.5 ml Eppendorf tube (Eppendorf, catalog number: 0030120086)
4. 0.2 ml PCR tubes (Thermo Fisher Scientific, catalog number: AB0620)
5. 0.45 µm cellulose filter (Carl Roth, catalog number: KH55.1)
6. Ni-NTA column (GE Healthcare, catalog number: 17524801)
7. 50 ml syringe (VWR, catalog number: 53548-010)

*Manufacturer: Air-Tite Products, catalog number: 4850001000.*

8. 20 x 20 mm glass slides (VWR, catalog number: 631-0122)

*Notes: The glass slide is used as the glass surface for the protein functionalization and bacterial experiments.*

9. Parafilm (Sigma-Aldrich, Bemis, catalog number: P7668)
10. Aluminum foil (Carl Roth, catalog number: 1770.1)
11. 35 mm Petri dish (SARSTEDT, catalog number: 82.1135.500)
12. Dialysis tubing (Repligen, Spectrum, catalog number: 132592)
13. Plasmid pB33eCPX (Addgene plasmid) (Addgene, catalog number: 23336)
14. GFP and mCherry pTrc99A plasmids (Prof. Victor Sourjik lab, Chen and Wegner, 2017)
15. nMagHigh pET-21b(+) plasmid (Genescript, Chen and Wegner, 2017)
16. nMagHigh-eCPX plasmid (homemade, Chen and Wegner, 2017)

*Note: The nMagHigh gene is inserted between the KpnI and SacI cutting sites of pB33eCPX.*

17. pMag-eCPX, pMagHigh-eCPX, pMagFast1-eCPX plasmids (homemade, Chen and Wegner, 2017)

*Note: The different pMag variants are generated by point mutagenesis from the nMagHigh-eCPX plasmid using QuikChange II Site-Directed Mutagenesis Kit.*

18. *E. coli* K12 MG1655 (DSMZ, catalog number: 18039)
19. BL21(DE3) competent *E. coli* (homemade, Chen and Wegner, 2017)
20. DH5 $\alpha$  competent *E. coli* (homemade, Chen and Wegner, 2017)
21. PBS Tablets (Thermo Fisher Scientific, Gibco™, catalog number: 18912014)
22. Mowiol (Sigma-Aldrich, catalog number: 81381)
23. LB medium (Carl Roth, catalog number: X968.3)
24. Ampicillin (Carl Roth, catalog number: HP62.2)
25. IPTG (Sigma-Aldrich, catalog number: I6758)
26. PMSF (Sigma-Aldrich, catalog number: P7626)
27. DTT (Sigma-Aldrich, catalog number: D0632-10G)
28. PEG-azide (homemade)
29. Triethylamine (Sigma-Aldrich, catalog number: T0886)
30. Toluene, anhydrous (Alfa Aesar, catalog number: 41464-AK)
31. EDTA (Sigma-Aldrich, catalog number: 798681)
32. NiCl<sub>2</sub> (Sigma-Aldrich, catalog number: 339350)
33. Chloramphenicol (Sigma-Aldrich, catalog number: C0378)
34. L-arabinose (Sigma-Aldrich, catalog number: A3256)
35. Paraformaldehyde, reagent grade (Sigma-Aldrich, catalog number: P6148)
36. Tris Base (Sigma-Aldrich, catalog number: T1503)
37. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
38. Imidazole (Sigma-Aldrich, catalog number: I2399)
39. L-Ascorbic acid (Sigma-Aldrich, catalog number: A5960)

40. NTA-alkyne (homemade, Schenk *et al.*, 2014)
41. PEG azide (homemade, Schenk *et al.*, 2014)
42. Copper sulfate (CuSO<sub>4</sub>) (Sigma-Aldrich, catalog number: 451657)
43. 30% H<sub>2</sub>O<sub>2</sub> (Carl Roth, catalog number: 8070.4)
44. H<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, catalog number: 30743)
45. TEM (Transmission electron microscopy) grid (Ted Pella, catalog number: 1GC150)
46. Centrifuge tubes 500 ml and 50 ml (Thermo Fisher Scientific, catalog numbers: 3141-0500, 3119-0050)
47. Methanol (Fisher Scientific, catalog number: 10224490)
48. N<sub>2</sub> gas (Westfalen)
49. Ethyl acetate (VWR, catalog number: 23882.321)
50. Picodent twinsil 22 (Picodent, catalog number: 13001000)
51. Buffer A (see Recipes)
52. Buffer B (see Recipes)
53. Click reaction solution (see Recipes)
54. Riranha solution (see Recipes)

## **Equipment**

1. Dumont #7 Tweezers (Carl Roth, catalog number: K344.1)  
*Note: Dumont #7 Tweezers is used to pick up the glass slides.*
2. 0.1-2.5 µl, 0.5-10 µl, 10-100 µl, 100-1,000 µl Pipettes (Eppendorf, catalog numbers: 3123000012, 3123000020, 3123000047, 3123000063)
3. Vortexer (neoLab, catalog number: 7-0092)
4. Microcentrifuge (VWR, model: Micro Star 17, catalog number: 521-1646)
5. High-speed centrifuge (Beckman Coulter, model: Avanti® J-26S)
6. Rotors for high-speed centrifuge (Beckman Coulter, models: JA-10, JA-25.50)
7. Incubator (VWR, catalog number: 444-0732)
8. Sonicator (OMNI, model: Sonic Ruptor 400)
9. Invert fluorescence microscope (Leica Microsystems, model: Leica DMi8)
10. Ultrasonic cleaner (BANDELIN electronic, model: Sonorex Super RK 31)
11. Blue LED panel (Albrillo, model: LL-GL003)
12. OD Meter (Biochrom, BioWave, model: WPA CO8000)
13. Nanodrop (Thermo Fisher Scientific, model: NanoDrop™ 8000)

## **Software**

1. ImageJ
2. Originlab

*Note: Oringinlab is used for data analysis.*

## **Procedure**

### **A. nMagHigh Protein Expression and purification**

1. Transform nMagHigh with a C-terminal His6-tag in a pET-21b(+) expression plasmid into BL21(DE3) *E. coli* cells using a standard protocol.
2. Prepare a starter culture from a single colony in 10 ml LB with 50 µg/ml ampicillin. Incubate overnight at 37 °C at 250 rpm.
3. Transfer 10 ml overnight culture into 1 L LB with 50 µg/ml ampicillin and grown at 37 °C at 200 rpm till OD<sub>600</sub> = 0.6-0.8. When the desired OD<sub>600</sub> is reached, add 500 µl IPTG (1 M stock, 500 µM final concentration) to the culture and incubate overnight at 16 °C at 250 rpm.
4. Pellet bacteria at 6,371 x g at 4 °C for 8 min using the high-speed centrifuge with the JA-10 rotor (500 ml centrifuge tube). Discard the supernatant. All the following steps must be done on ice.
5. Resuspend the pellet in 20 ml Buffer A (Recipe 1) containing supplemented with 1 mM PMSF (100 mM stock in methanol) and 1 mM DTT (1 M stock in water). Transfer the suspension into a 50 ml Falcon tube.
6. Lyse bacteria by sonication. Settings for tip sonicator: 50% frequency, 40% power, 10 min. Keep the Falcon on ice during sonication. Transfer lysate into plastic high-speed centrifuge tubes, Centrifuge at 17,418 x g, 4 °C for 30 min using the JA-25.50 rotor (50 ml centrifuge tube).
7. Filter the supernatant through 0.45 µm cellulose filter twice.
8. Equilibrate Ni-NTA column (column volume = 5 ml) with 20-30 ml Buffer A + 1 mM DTT.
9. Load clarified cell lysate on to the Ni-NTA column by passing the lysate twice dropwise using a 50 ml syringe.
10. Wash Ni-NTA column with 10 ml Buffer A + 1 mM DTT and 50 ml (47.5 ml Buffer A + 2.5 ml Buffer B + 1 mM DTT) (Recipe 2).
11. Elute with 10 ml Buffer B + 1 mM DTT. Discard the first 3 ml and collect the last 7 ml which contain the protein.
12. Wash the column once more with Buffer B (50 ml, without DTT) and then Buffer A (50 ml, without DTT). Store Ni-NTA column in the fridge.
13. Put the protein solution in dialysis bags (MW cut-off = 3,500 Da) and dialyze in 2 L Buffer A with stirring at 4 °C for 3 h. Replace the Buffer A with a fresh one and dialyze another 3 h at 4 °C. Collect the protein solution and store in -80 °C. Protein concentration is measured by Nanodrop.

## B. Functionalization of glass surfaces with nMagHigh

1. Label the upper right corner of glass slides (20 x 20 mm) to distinguish the upper side. Clean glass surfaces in freshly prepared Piranha solution for 1 h, rinse three times with Milli-Q water and dry in an N<sub>2</sub> stream.
2. For the PEGylation reaction, immerse surfaces in a solution of PEG-azide (10 mg PEG-azide, MW = 3,500 g/mol) and a drop of triethylamine in 50 ml dry toluene and kept at 79 °C overnight under an N<sub>2</sub> atmosphere in a closed container. Note that surfaces should not be touching each other.
3. Wash the surfaces first with ethyl acetate for 5 min by sonication (Ultrasonic frequency: 35 kHz), then with methanol for 5 min by sonication and dry in an N<sub>2</sub> stream.
4. Prepare the humid chambers for click reaction by putting wet tissue paper in the lid of a Petri dish and cover it with the clean side of the parafilm.
5. Prepare the click reaction solution (for six surfaces) (Recipe 3).
6. Put 100 µl of click reaction solution on the parafilm in the Petri dish.
7. Place one glass slide upside down on each the click reaction solution droplets (marked surface should be in contact with the click reaction solution). Incubate at room temperature for 2 h.
8. After click reaction, wash the glass slides with 1) 50 mM EDTA in Buffer A for 5 min to remove Cu<sup>2+</sup>, and 2) Buffer A twice for 5 min.
9. Incubate the slides with 100 µl 100 mM of NiCl<sub>2</sub> on a parafilm at room temperature for 5 min. Ni<sup>2+</sup> complexes to the NTA groups on the surface for His-tag binding. Wash the slides twice with Buffer A to remove excess Ni<sup>2+</sup>.
10. Place 100 µl nMagHigh protein solution (10 µM, in Buffer A) droplets on a parafilm and put the glass slides upside down on the droplets. Incubate at room temperature for 30 min.  
*Note: The glass surfaces not incubated with nMagHigh protein will be used as negative control in D.*
11. Wash the slides twice with sterile PBS and keep them in the PBS before use.  
*Note: The nMagHigh functionalized glass slides should be freshly prepared for the bacterial experiments.*

## C. Bacteria preparation

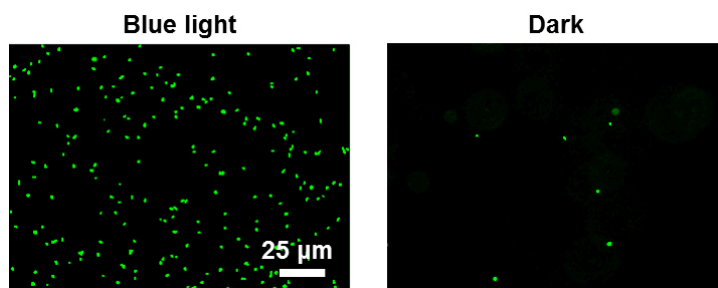
1. Co-transform *E. coli* K12 MG1655 with the pMagHigh-eCPX or pMag-eCPX or pMagFast1-eCPX plasmid (chloramphenicol resistant) and the GFP pTrc99A plasmid (ampicillin resistant) and selected on an LB-agar plate with 35 µg/ml chloramphenicol and 50 µg/ml ampicillin.
2. Inoculate a single colony into 5 ml LB medium containing 35 µg/ml chloramphenicol and 50 µg/ml ampicillin and incubate overnight at 37 °C at 250 rpm. Wrap all the tubes in aluminum foil.
3. Add 500 µl of the overnight cultures into fresh 20 ml LB medium containing 35 µg/ml chloramphenicol and 50 µg/ml ampicillin and culture for 2 h at 37 °C, 250 rpm. Wrap all the tubes in aluminum foil.



4. When the  $OD_{600} = 0.5$ , add 0.04% m/v L-arabinose to induce the expression of the pMag-eCPX proteins and 0.5 mM IPTG to induce the production of the fluorescent protein and incubate the cultures at 25 °C, 250 rpm for 4 h.
5. Spin down the bacteria at 1,500 x g for 10 min and wash with PBS twice. Finally, re-suspend the bacteria in PBS to  $OD_{600} = 1.0$ .

#### D. Bacterial adhesion assays

1. Use glass surfaces functionalized with nMagHigh protein by the method mentioned above and keep them in PBS in the dark. Use a surface that was not incubated with nMagHigh protein as a negative control to assure that the functionalization with the PEG worked.
2. Place each glass surface in a 35 mm Petri dish. Add 3 ml of bacterial solution (the bacteria express pMagHigh or pMag or pMagFast1) with  $OD_{600} = 1.0$  in PBS to each Petri dish.
3. Place one set of surfaces with bacteria under blue light illumination (blue LED panel, 640  $\mu\text{W}/\text{cm}^2$ ) for 1 h at room temperature. Place the second set of surface in the dark for 1 h.
4. Wash the surfaces gently three times with PBS. Fix the bacteria with 4% paraformaldehyde for 20 min and mount them with Mowiol. Acquire nine fluorescent images in the GFP channel for each surface on the inverted fluorescence microscope (DMI8, Leica) through a 20x objective. (Figure 2)
5. Analyze the numbers of bacteria on the surfaces using the particle analyzer tool in ImageJ.



**Figure 2. Fluorescence images of *E. coli* displaying pMagHigh which adhere on nMagHigh functionalized substrates under blue light but not in the dark.** The bacteria are labeled with GFP for detection. Reproduced with permission from Chen and Wegner (2017).

#### E. Bacterial attachment kinetics

1. Functionalize five Glass surfaces with nMagHigh protein by the method mentioned above.
2. Place each glass surface in a 35 mm Petri dish. Add 3 ml of pMagHigh-eCPX bacteria in PBS ( $OD_{600} = 1.0$ ) to each Petri dish.
3. Incubate the surfaces under blue light illumination (blue LED panel, 640  $\mu\text{W}/\text{cm}^2$ ) for different time (10, 30, 60, 120 to 180 min) at room temperature.
4. Wash each surface gently three times with PBS. Fix the bacteria with 4% paraformaldehyde and mount them with Mowiol. Acquire nine fluorescent images in the GFP channel ( $E_x = 488 \text{ nm}$ ,  $E_m$

= 510 nm) for each surface on the inverted fluorescence microscope (DMI8, Leica) through a 20x objective.

5. Analyze the numbers of bacteria on the surfaces using the particle analyzer tool in ImageJ.
6. Quantify the attachment kinetics of the pMag-eCPX and pMagFast1-eCPX bacteria the same way following steps from E1 to E5 with corresponding bacteria.

F. Bacterial attachment adjusted by blue light illumination intensity

1. Use six glass surfaces functionalized with nMagHigh protein prepared as mentioned above.
2. Place each glass surface in a 35 mm Petri dish. Add 3 ml of pMagHigh-eCPX bacteria in PBS ( $OD_{600} = 1.0$ ) to each Petri dish.
3. Incubate the surfaces under blue light illumination with different intensity (0, 3.2, 32, 320, 640 or 3,200  $\mu W/cm^2$ ) for 1 h at room temperature.
4. Wash the surfaces gently three times with PBS. Fix the bacteria with 4% paraformaldehyde and mount them with Mowiol. Acquire nine fluorescent images in the GFP channel for each surface on an inverted fluorescence microscope (DMI8, Leica) through a 20x objective. Analyze the number of bacteria on the surfaces using the particle analyzer tool in ImageJ.

G. Bacterial detachment kinetics

1. Use six glass surfaces functionalized with nMagHigh protein prepared by the method mentioned above.
2. Place each glass surface in a 35 mm Petri dish. Add 3 ml of pMagHigh-eCPX bacteria in PBS ( $OD_{600} = 1.0$ ) to each Petri dish and incubate under blue light illumination (blue LED panel, 640  $\mu W/cm^2$ ) for 1 h at room temperature.
3. Move the Petri dishes to the dark for 0, 10, 30, 60, 120 or 240 min, respectively.
4. Wash each surface gently three times with PBS. Fix the bacteria with 4% paraformaldehyde and mount them with Mowiol. Acquire nine fluorescent images in the GFP channel for each surface on an inverted fluorescence microscope (DMI8, Leica) through a 20x objective. Analyze the number of bacteria on the surfaces using the particle analyzer tool in ImageJ.

H. Multiple attachment and detachment cycles

1. Use five glass surfaces functionalized with nMagHigh protein prepared by the method mentioned above.
2. Place each glass surface in a 35 mm Petri dish. Add 3 ml of pMagHigh-eCPX bacteria in PBS ( $OD_{600} = 1.0$ ) to each Petri dish and incubate under blue light illumination (blue LED panel, 640  $\mu W/cm^2$ ) for 1 h attachment at room temperature.
3. Wash the first surface gently three times with PBS. Fix the bacteria with 4% paraformaldehyde and mount them with Mowiol. Acquire images imaged as described above.
4. Meanwhile, keep the other four surfaces in the dark for 2 h for bacterial detachment.



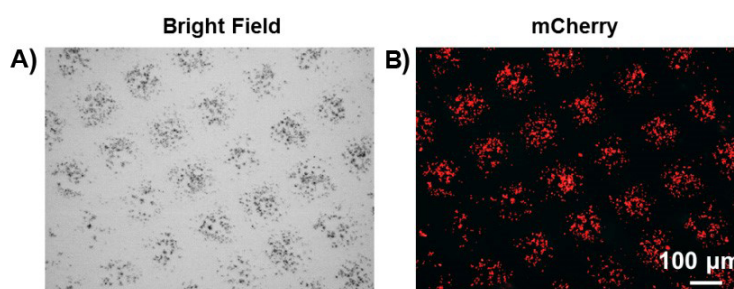
5. Wash the second surface gently three times with PBS and fix the bacteria with 4% paraformaldehyde.
6. Meanwhile, place the remaining three surfaces under blue light (blue LED panel, 640  $\mu\text{W}/\text{cm}^2$ ) for 1 h for attachment.
7. Repeat the Steps H3 to H6 for another cycle. For each surface, acquire nine fluorescent images in the GFP channel on an inverted fluorescence microscope (DMI8, Leica) through a 20x objective. Analyze the number of bacteria on the surfaces using the particle analyzer tool in ImageJ.

#### I. Bacterial patterning

1. Co-transform *E. coli* K12 MG1655 with pMagHigh-eCPX (chloramphenicol resistant) and mCherry pTrc99A plasmid (ampicillin resistant) plasmids. Prepare bacteria as described above.
2. The glass surface with nMagHigh protein immobilization is stuck to the bottom of a 35 mm Petri dish (with a round hole in 15 mm diameter). 3 ml pMagHigh-eCPX bacteria solution with  $\text{OD}_{600} = 1.0$  is added into the Petri dish and kept in the dark.

*Note: Picodent twinsil 22 is used as a glue to stick the glass surface to the Petri dish bottom.*

3. The glass surface is locally illuminated with blue light through photomask (TEM grid without support film) for 1 h on an inverted fluorescence microscope (DMI8, Leica) through a 10x objective.
4. The photomask is removed and the glass surface is gently washed by removing 2 ml solution and adding 2 ml PBS into the Petri dish. Repeat the washing steps for three times. Don't let the surface dry.
5. The surface is imaged by the inverted fluorescence microscope (DMI8, Leica) through a 10x objective. (Figure 3)



**Figure 3. Photopatterning of pMagHigh displaying bacteria to nMagHigh functionalized glass surfaces.** A) Bright field and B) fluorescent images of bacteria patterns. The bacteria are labeled with mCherry for detection. Reproduced with permission from Chen and Wegner (2017).

#### Data analysis

Data information can be access via <https://pubs.acs.org/doi/abs/10.1021/acssynbio.7b00197>.

## **Notes**

1. All the experiments are performed in the dark room.
2. Bacteria are cultured in the dark to avoid the activation of the photosensitive protein.

## **Recipes**

1. Buffer A (1x)  
50 mM Tris-HCl, pH 7.4  
300 mM NaCl (17.5 g for 1 L buffer)
2. Buffer B (1x)  
Buffer A  
250 mM Imidazole (17 g for 1 L buffer)
3. Click reaction solution (for six surfaces)  
473  $\mu$ l MilliQ water  
60  $\mu$ l 1 M Tris-HCl at pH 8.5  
60  $\mu$ l 1 M L-Ascorbic acid (prepare fresh: 102 mg L-Ascorbic acid in 741  $\mu$ l MilliQ water)  
0.9  $\mu$ l 25 mM NTA-alkyne  
6  $\mu$ l 100 mM CuSO<sub>4</sub> (should be added last; when added, locally a brownish color appears. After mixing, the solution becomes colorless)  
Mix the solution by vortexing
4. Riranha solution  
3:1 (v/v) conc. H<sub>2</sub>SO<sub>4</sub>:H<sub>2</sub>O<sub>2</sub> (30%)  
20 ml H<sub>2</sub>O<sub>2</sub> (30%)  
60 ml H<sub>2</sub>SO<sub>4</sub>

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## **Competing interests**

There are no conflicts of interest.

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