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Modification-Free Photocontrol of β-Lactam Conversion with Spatiotemporal Resolution

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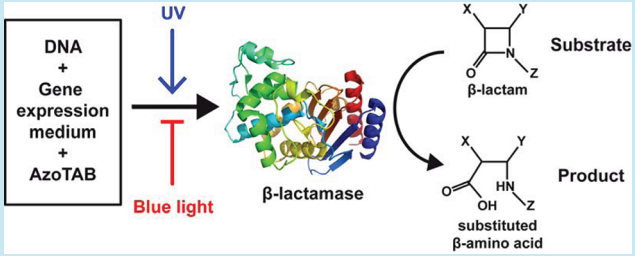
Supporting Information

ABSTRACT: β-Lactams can be converted into β-amino acids by β-lactamase, a bacterial enzyme, leading to significant change in the biological function of the substrate molecules. Here we describe a method for photocontrol of β-lactam conversion without gene nor enzyme modification. This is achieved by the addition of a cationic photosensitive surfactant, AzoTAB, to a gene expression medium containing DNA coding for β-lactamase, the enzyme capable of the desired conversion. In the absence of UV (365 nm) or after illumination by blue light (480 nm) for 4 min, conversion of β-lactam is strongly reduced while the application of UV for 4 min results in a strong enhancement of substrate conversion. Several cycles of activation/inhibition are obtained upon successive UV/blue light illuminations. When both reconstituted photoresponsive gene expression medium and β-lactamase substrate are encapsulated in independent microfluidic chambers, selective UV illumination results in spatially resolved activation of substrate conversion.



KEYWORDS: *enzyme, photocontrol, cell-free expression, β-lactamase, AzoTAB*

Triggering chemical reactions through a light stimulus is an important challenge with promises for chemical and biological applications. Light is a well-adapted stimulus to achieve a finely resolved spatiotemporal control down to micrometer and microsecond scales, and many strategies are now available to set up a light-actuated chemical[1,2] or biological[3,4] system. A classical approach consists in designing photosensitive molecules to elicit a change in the function of a target molecule upon illumination. This is typically accomplished by grafting photocleavable moieties, as is the case for photocaged compounds,[5,6] or by adding photoisomerizable groups through covalent modification or, in the case of proteins, incorporation of non-natural amino acids.[3,4,7−10] The chemical reaction we wish to control using light is the conversion of a non-photosensitive substrate by implementing a photocontrol of the enzyme capable of the desired conversion. Chemically modifying the enzyme or its environment to endow them with photosensitive properties is, as previously mentioned, a useful strategy.[11−13] Its success, however, is based on a protein-specific approach and requires developing *ad hoc* protocols for each new system of interest. In contrast, we describe herein a strategy where photocontrol is introduced at the level of the enzyme synthesis, which in turn allows us to attain a control over substrate conversion. To achieve this photocontrol without any chemical modification, we added a photosensitive nucleic acid binder to a reconstituted gene expression medium containing DNA coding for the desired enzyme. Based on light-induced conformational change of DNA, this approach has the advantage to offer sequence-independent and reversible photocontrol of gene expression. It was previously demonstrated using a DNA coding for a model green fluorescent protein (GFP),[14] but it has never been applied to control the synthesis of an enzyme and the resulting substrate conversion. We applied this method to control for the first time the conversion of a β-lactam to a β-amino acid using light, through the photocontrolled synthesis of TEM-1 β-lactamase enzyme. The β-lactam hydrolysis is a particularly interesting reaction as it can considerably affect the biological function of the substrate molecule. For instance, once ampicillin, a β-lactam antibiotic, undergoes this conversion, it loses most of its toxicity. Interestingly, such conversion can be exploited for medical applications. In particular, non-toxic β-lactam prodrugs (*e.g.*, protax, c-dox) can be converted by β-lactamase into highly cytotoxic anticancer drugs (paclitaxel, doxorobucin, respectively).[15,16] To our knowledge, our approach thus constitutes the first demonstration of photocontrol of the synthesis of an enzyme capable of prodrug−drug conversion. We show that it provides photocontrol over substrate conversion with both spatial and temporal resolution.

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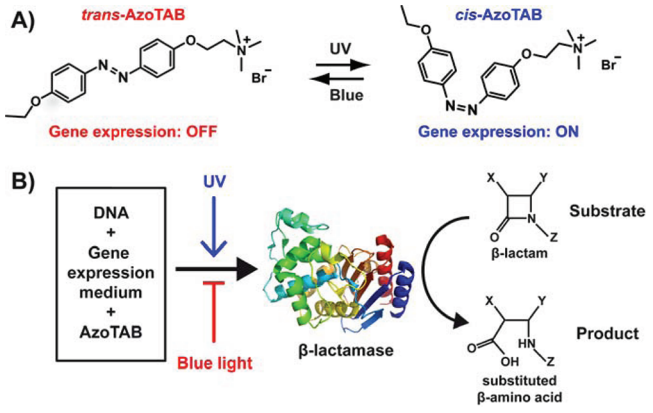

Figure 1. Concept of photocontrol of β-lactam conversion without gene nor protein modification. (A) Under dark conditions, *trans*-AzoTAB strongly binds to nucleic acids[17−19] and inhibits gene expression.[14,23,24] Upon UV illumination (365 nm), it isomerizes into *cis*-AzoTAB, a weaker nucleic acid binder[17−19] allowing gene expression.[14,23,24] *cis*-AzoTAB is stable under dark conditions. Upon blue illumination (480 nm), it isomerizes back to *trans*-configuration and inhibits gene expression again.[14] (B) A photosensitive gene expression medium is prepared by assembling DNA coding for β-lactamase, a cell-free gene expression medium (PURExpress) and AzoTAB. The system is inactive under dark conditions. UV illumination triggers the synthesis of β-lactamase, which converts a substrate containing a β-lactam moiety into a product with a substituted β-amino acid. The system is reversible, and β-lactamase expression can be stopped by blue illumination. The protein figure was created using Pymol and TEM-1 β-lactamase structure, PDB code 1ZG4.

Figure 1 shows our concept and experimental system. We used an azobenzene trimethylammonium bromide surfactant (AzoTAB)[17−22] to reversibly control gene expression as a function of light illumination through photodependent binding of AzoTAB to DNA (Figure 1A).[14,23,24] A photoresponsive gene expression medium was prepared by assembling PURExpress cell-free expression kit, a plasmid containing a sequence coding for TEM-1 β-lactamase (full sequence in Supporting Information) and AzoTAB. We characterized the ability of this reconstituted gene expression medium to express β-lactamase and consequently convert β-lactam substrates as a function of AzoTAB concentration for different illumination conditions (Figure 1B).

First, the photoresponsive gene expression medium was submitted to illumination at 365 nm for 4 min (+UV) or kept in the dark for 4 min (−UV) prior to incubation at 37 °C for $t_{incubation}$ = 75 min. Gene expression was characterized by Western blot analysis for different AzoTAB concentrations and illumination conditions (Figure 2 top). It shows that, for [AzoTAB] ≤ 1.5 mM, the characteristic band of β-lactamase is not significantly affected by the presence of AzoTAB or by the application of the UV stimulus. In contrast, for [AzoTAB] ≥ 2 mM in the absence of UV, the band intensity significantly decreases with an increase in AzoTAB concentration down to levels below the detection threshold ([AzoTAB] = 4 mM). In this concentration range, the application of the UV stimulus results in partial recovery of the band intensity. In the absence of DNA, no band was observed, which confirms the absence of β-lactamase in the initial expression medium. When the same solutions were analyzed in a Coomassie gel, we did not observe any modification of the bands due to proteins from the PURExpress kit regardless of AzoTAB concentration and

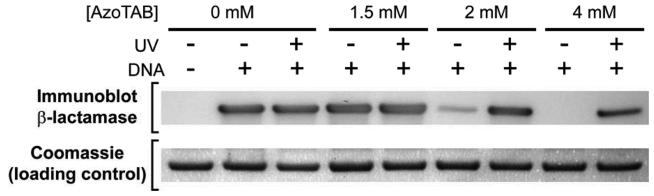

Figure 2. Photocontrol of *in vitro* β-lactamase synthesis. A characteristic Western blot showing TEM-1 β-lactamase (top) and the corresponding Coomassie gel showing a characteristic band of the cell-free expression medium (bottom) after 75 min of gene expression at 37 °C for various concentrations of AzoTAB, with or without UV illumination, in the absence or in the presence of DNA. [DNA] = 10 ng μL^{-1}. +UV/−UV: 365 nm/dark for 4 min at RT before incubation.

illumination conditions (Figure 2, bottom). This shows that the variations in band intensities on the Western blot are due to different levels of β-lactamase expression and not to variations in the initial composition of the gene expression medium. All of these results show that AzoTAB strongly reduces the amount of β-lactamase produced by the reconstituted gene expression medium and that the application of a short UV stimulus before incubation allows partial yet significant recovery of β-lactamase synthesis.

We then looked at the conversion of a specific β-lactam known as nitrocefin. It is a chromogenic substrate of β-lactamase,[25] routinely used to characterize β-lactam conversion,[26] especially in the context of prodrug−drug conversion.[27] Nitrocefin (N) strongly absorbs in the visible range (yellow, $\lambda_{max} \approx 390$ nm) and is converted into a β-amino acid product (nitrocefoic acid, NA) with a neat bathochromic effect (red, $\lambda_{max} \approx 490$ nm). As previously, the photoresponsive gene expression medium was submitted to different illumination conditions and incubated at 37 °C for $t_{incubation}$ = 75 min. Nitrocefin was then added to a concentration of $[N]_0 = 70$ μM. The concentration of nitrocefoic acid [NA] was then followed by measurements of optical density at 490 nm as a function of conversion time (Figure 3A). In the absence of AzoTAB and UV illumination, [NA] strongly increases to reach a plateau after approximately 15 min (−AzoTAB, −UV), which indicates that the synthesized β-lactamase is active and able to convert nitrocefin into nitrocefoic acid. In contrast, with [AzoTAB] = 2 mM, this increase is much smaller, and on the time scale of the experiment no plateau is observed, which corresponds to a much reduced nitrocefin conversion (+AzoTAB, −UV). Interestingly, when UV was applied, an intermediate behavior was observed, which corresponds to a partial recovery of nitrocefin conversion (+AzoTAB, +UV). A simple control experiment, with $t_{incubation}$ = 0 min, showed only a very slight increase in [NA] (Supporting Information Figure S3), which shows that, under our experimental conditions, the components of PURExpress do not convert nitrocefin to nitrocefoic acid. With $t_{incubation}$ = 75 min, when AzoTAB was removed from initial solutions but added at the end of incubation, absorption curves were very similar regardless of AzoTAB concentration and UV illumination (Supporting Information Table S1). This demonstrates that both AzoTAB and UV have only minor effects on the already synthesized β-lactamase. All of these results show that the presence of AzoTAB in the gene expression medium induces a strong effect of UV illumination on nitrocefin conversion and that this likely originates in the photodependent binding of AzoTAB to DNA coding for β-lactamase.

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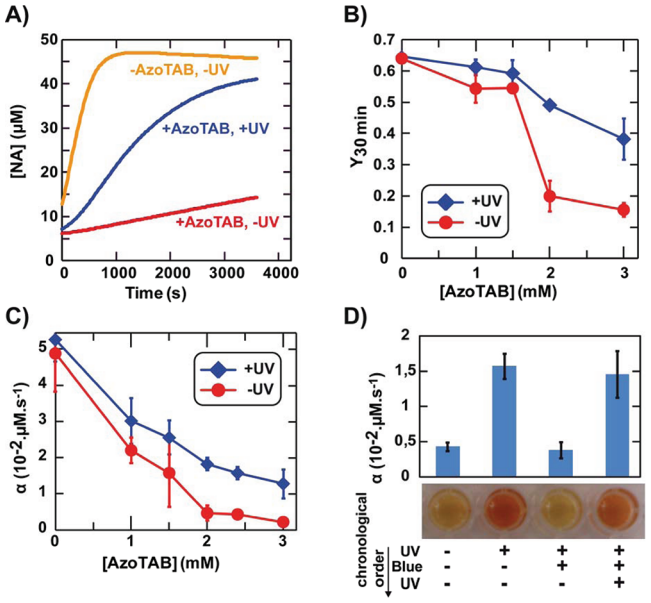

Figure 3. Reversible photocontrol of the conversion of a chromogenic β-lactam, nitrocefin (N, $\lambda_{max} \approx 390$ nm), into nitrocefoic acid (NA, $\lambda_{max} \approx 490$ nm). (A) Nitrocefoic acid concentration ([NA]), determined by optical density measurement at 490 nm ($OD_{490\ nm}$), as a function of time in the absence or presence of AzoTAB (2 mM), with or without UV. (B) Conversion rate after 30 min of nitrocefin conversion ($Y_{30\ min} = [NA]_{30\ min}/[N]_0$) as a function of AzoTAB concentration, with or without UV. Symbols are data points showing mean ± SD for at least three replicates. (C) Initial slope α of [NA] *vs* time as a function of AzoTAB concentration, with or without UV. Symbols are data points showing mean ± SD for at least three replicates. (D) Initial slope α of [NA] *vs* time (top) and real color photograph of solutions after 60 min of nitrocefin conversion (bottom) for [AzoTAB] = 2.4 mM and different illumination conditions. All experiments were performed in this order: assembly of photosensitive gene expression medium ([DNA] = 10 ng μL^{-1}, PURExpress, AzoTAB) at 4 °C; illumination at RT (−UV/−Blue: dark for 4 min, UV: 365 nm for 4 min, Blue: 480 nm for 4 min); incubation for 75 min at 37 °C; addition of nitrocefin (70 μM); measurement of $OD_{490\ nm}$ at 37 °C as a function of time.

To quantify the effects of AzoTAB and UV on nitrocefin conversion, we measured NA concentration 30 min after nitrocefin introduction ($[NA]_{30\ min}$) and calculated the conversion rate $Y_{30\ min} = [NA]_{30\ min}/[N]_0$ as a function of AzoTAB concentration, with or without UV illumination (365 nm for 4 min) (Figure 3B). For $0 \leq [AzoTAB] \leq 1.5$ mM, $Y_{30\ min}$ is almost constant regardless of AzoTAB concentration and illumination. In contrast, for $[AzoTAB] \geq 2$ mM, $Y_{30\ min}$ shows a sharp drop without UV and a marked recovery upon UV illumination. For [AzoTAB] = 2 and 3 mM, UV illumination results in a 2.5-fold increase in $Y_{30\ min}$ from 0.20 ± 0.05 to 0.49 ± 0.01 and from 0.16 ± 0.02 to 0.38 ± 0.06, respectively. Another crucial parameter is the initial rate of nitrocefin conversion. To study the effects of AzoTAB and UV on this parameter, we measured α, the slope at the beginning of conversion (see Supporting Information Figure S2 for slope measurement), as a function of AzoTAB concentration and illumination conditions (Figure 3C). Without UV, α undergoes a 20-fold decrease from 4.9 ± 1.1 to 0.22 ± 0.09 × 10^{-2} μM s^{-1} when [AzoTAB] increases from 0 to 3 mM, which shows that AzoTAB strongly reduces initial conversion rate. With UV, α decreases with an increase in AzoTAB but is higher than the value in the absence of UV, regardless of AzoTAB concentration. Interestingly, in the range of strong inhibition ($2 \leq [AzoTAB] \leq 3$ mM), UV results in a 3- to 5-fold increase in α, *e.g.*, from 0.43 ± 0.06 to 1.6 ± 0.2 × 10^{-2} μM s^{-1} for [AzoTAB] = 2.4 mM. All of these results (Figure 3A−C) show that AzoTAB allows for strong decrease in substrate conversion, while a short UV illumination before expression induces a marked increase in both initial conversion rate and extent of conversion after a given time. These results correlate well with the photocontrol of β-lactamase expression as demonstrated by Western blot analyses (Figure 2). When similar experiments were carried out for a shorter UV illumination time (1.5 min), a marked enhancement of nitrocefin conversion was obtained for [AzoTAB] = 2 mM, but a lower increase was observed at higher AzoTAB concentrations (Supporting Information Figure S4). All of these results demonstrate a modification-free photocontrol of a substrate conversion, which originates from a control at the gene expression level of the corresponding enzyme.

Since AzoTAB enables both switching on and off of gene expression upon UV and blue light illumination, respectively (Figure 1), we measured α on solutions containing 2.4 mM AzoTAB that had been successively exposed to UV and blue light before gene expression. Figure 3D shows that α remarkably follows the illumination pattern. α increases from 0.43 ± 0.06 to 1.6 ± 0.2 × 10^{-2} μM s^{-1} upon UV, drops to 0.38 ± 0.1 × 10^{-2} μM s^{-1} when blue light is applied after UV, and increases back to 1.5 ± 0.3 × 10^{-2} μM s^{-1} upon a second UV exposure following the blue light illumination.

In previous experiments, the enzyme substrate was added once β-lactamase was expressed. It is thus important to check whether substrate conversion can be triggered using light when the substrate is initially present in the reconstituted gene expression medium. To this end we assembled our photosensitive gene expression medium ([DNA] = 10 ng μL^{-1}, PURExpress, [AzoTAB] = 1 mM) with fluorocillin, a fluorogenic β-lactam whose fluorescence strongly increases upon conversion by β-lactamase. Note that fluorocillin slightly inhibits gene expression, so that 1 mM AzoTAB was enough to strongly reduce gene expression without UV while allowing significant enhancement upon UV illumination. The assembled solution was distributed in an array of independent microfluidic chambers (Figure 4A,B), using the so-called oil microsealing technique.[28] We applied UV illumination (365 nm for 10 min) through a photomask right below the chambers and incubated the device at 37 °C. Figure 4C shows a typical fluorescence microscopy image obtained after 90 min of incubation. While all chambers contain the same solution, chambers that have been exposed to UV exhibit higher fluorescence than those that have been shielded from UV by the photomask (Figure 4C−E). Slight pixel intensity variations are observed within individual chambers, but these fluctuations are smaller than the difference between +UV and −UV chambers (Figure 4D). Similarly, some variations can be observed from one chamber to the other with the same illumination conditions, but chambers exposed to UV are reproducibly and significantly more fluorescent than those without UV (Figure 4E). A similar experiment was carried out under the same conditions except for UV illumination that was applied after incubation. In this case, fluorescence was similar from one chamber to another and did not correlate with the UV illumination pattern, which shows that changes of fluorescence observed in Figure 4C−E are not due to the effect of UV on the fluorogenic substrate itself. All of these

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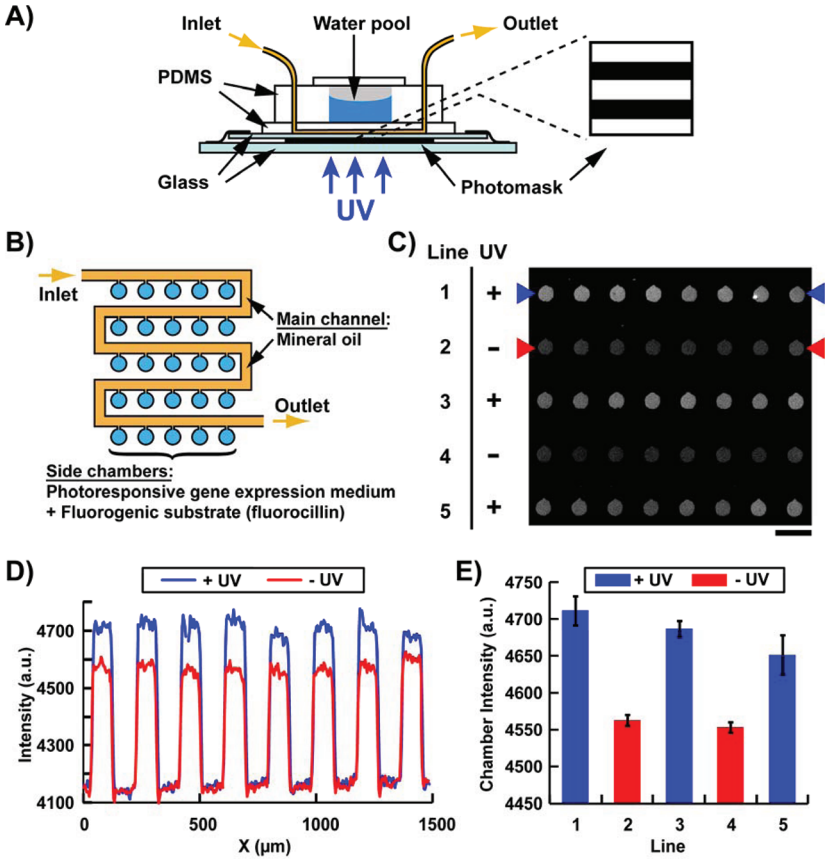

Figure 4. Spatially resolved photoactivation of a fluorogenic β-lactam substrate in an array of independent microfluidic chambers. (A) Side-view of the experimental setup. UV illumination was applied through a photomask clamped between a microscopy glass slide and the substrate of a two-layer PDMS microfluidic device. The upper layer consists of a water pool to avoid evaporation. (B) Schematic representation (top view) of the microfluidic channel (50 μm high) in the bottom layer (500 μm to 1 mm thick). Side chambers (100 μm diameter) are connected to a main channel (50 μm wide) through a narrow neck (30 μm long, 15 μm wide). Side chambers are filled with a mixture of the photoresponsive gene expression medium ([DNA] = 10 ng μL^{-1}, PURExpress, [AzoTAB] = 1 mM) and fluorocillin (2.5 $\mu g\ mL^{-1}$), a weakly fluorescent substrate that is converted into a highly fluorescent product by β-lactamase, prior to sealing by a gentle flow of mineral oil in the main channel.[28] (C) Fluorescence microscopy image of chambers illuminated by UV (365 nm) for 10 min through a photomask hiding a line out of two prior to incubation at 37 °C for 90 min. Scale bar is 200 μm. (D) Intensity profile along a line between the blue (+UV) or the red (−UV) arrows shown in panel C. (E) Mean pixel intensity per chamber averaged on 5 consecutive chambers in a line. Error bars show ± SD.

results show that the *in situ* conversion of fluorocillin into its highly fluorescent product is enhanced by local UV exposure and that our photocontrol method provides a micrometer-scale spatial resolution.

We have described a method for *in vitro* photocontrol of enzyme synthesis resulting in light-controllable substrate conversion, without gene or protein modification. It consisted in adding a photosensitive nucleic acid binder to a reconstituted gene expression medium where the only requirement is a gene coding for the desired enzyme. This was demonstrated using a commercially available gene expression system (PURExpress) and β-lactams as target enzyme substrates. We showed that the conversion ability of the system is strongly reduced in the absence of light while a short UV illumination triggers β-lactamase synthesis and the subsequent conversion of different kinds of β-lactam substrates. Reversible control was demonstrated by achieving several cycles of activation/inhibition using successive UV/blue light stimuli. Finally, our method allowed for *in situ*, spatially resolved photoactivation of substrate conversion in an array of microfluidic chambers. Because our approach is sequence-independent and does not require any modification of the gene expression machinery or of the enzyme itself, it can be easily applied to potentially all kinds of enzymes that can be synthesized *in vitro* in a functional form. Moreover, since it has been shown that AzoTAB-based nonspecific photocontrol can be combined with sequence-specific regulation using small RNAs,[24] such an approach could also be applied for selective photoactivation of a target enzyme among others. By controlling the enzyme at its expression step, unlike standard strategies based on covalent modification of the protein itself, our method offers the advantage of preserving the activity of the native protein and does not rely on extensive knowledge of the protein structure. Finally, photocontrol of β-lactam hydrolysis by β-lactamase, which to our knowledge has been demonstrated here for the first time, is particularly interesting from the perspective of improved prodrug-based therapies for anticancer treatments. Compared to endogeneous enzymes, exogenous enzymes such as β-lactamase offer marked selectivity in prodrug conversion. Tumor-targeting is usually obtained by conjugating the enzyme to a monoclonal antibody specific to antigens expressed on tumor cells.[29,30] However, the paucity of tumor-selective antigens and the adverse immune effects are severe limitations in such strategies.[31] In contrast, our method, which provides a mean to synthesize the active enzyme only when and where it is necessary using a noninvasive light stimulus, is a first step toward the develop-

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ment of gene-carrying autonomous nanomachines that can be triggered using light to deliver anticancer drugs with improved efficiency while minimizing immunogenicity and side effects.

METHODS

For gene expression experiments, RNase-free water, PURExpress solution A (5 μL), DNA (10 ng μL^{-1}), the desired amount of AzoTAB (from a 15 mM solution), and PURExpress solution B (2 μL) were assembled at 4 °C in this order to give a total volume of 10 μL. The solutions were then either kept in the dark or illuminated with UV (365 nm) for 4 min at RT before being incubated at 37 °C, usually for 75 min. Western blots were carried out with 2.5 μL of the previous solution, along with a reducing agent and a blue stain in SDS. The gels were run for 30 min at 200 V, and the proteins were then transferred onto the membrane for 1 h at 30 V. The anti-β-lactamase in mouse antibody was used at 10 μg mL^{-1} and the anti-mouse HRP linked antibody at 1:1000 dilution. For nitrocefin assays, the photosensitive gene expression medium was assembled at 4 °C and then submitted to illumination (UV and/or blue light) or kept in the dark at RT prior to incubation at 37 °C for 75 min. Then 145 μL of nitrocefin (final concentration 70 μM) in phosphate buffer (100 mM, pH 7) was added to the gene expression medium in a microplate kept at 37 °C. The optical density at 490 nm ($OD_{490\ nm}$) was then followed, typically with a measure every 20 s for 1 h. For fluorocillin experiments in the microfluidic device, RNase-free water, PURExpress solution A, DNA, PURExpress solution B, and fluorocillin were assembled at 4 °C and introduced in an array of microchambers that were then sealed with oil. A photomask was adjusted, and 10 min of UV illumination was carried out through the mask. The device was then incubated for 90 min at 37 °C. The resulting fluorescence was monitored. Full methods are in Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Materials and methods; plasmid (Figure S1); slope measurement (Figure S2); effect of gene expression incubation time (Figure S3); effect of AzoTAB concentration for a ±UV illumination for 1.5 min before incubation (Figure S4); influence of AzoTAB and UV illumination on already-synthesized β-lactamase (Table S1); and supplementary references. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

D.B. supervised the project and designed research; A.V.-M. performed *in vitro* gene expression, Western blot analyzes, and nitrocefin assays; Y.-J.L. performed microfluidics experiments; A.D. prepared the DNA template; T.dM. contributed to microfluidics experiments; A.V.-M., Y.-J.L., A.G., and D.B. analyzed the data; A.V.-M. and D.B. wrote the paper with contributions from all authors.

Notes

The authors declare no competing financial interest.

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