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Dual-color control of nucleotide polymerization sensed by fluorescence actuator

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Abstract

Spatial and temporal control of molecular mechanisms can be achieved using photolabile bonds that connect biomolecules to protective caging groups, which can be cleaved upon irradiation of a specific wavelength, releasing the biomolecule ready-to-use. Here we apply and improve a previously reported strategy to tightly control *in vitro* transcription reactions. The strategy involves two caging molecules that block both ATP and GTP nucleotides. Additionally, we designed a molecular beacon complementary to the synthesized mRNA to infer its presence through a light signal. Upon release of both nucleotides through a specific monochromatic light (390 and 325 nm) we attain a light signal indicative of a successful *in vitro* transcription reaction. Similarly, in absence of irradiation, no intense fluorescence signal was obtained. We believe this strategy could further be applied to DNA synthesis or the development of logic gates.

1. Introduction

Caged molecules are photosensitive molecules that have shown great potential for the study and control of molecular mechanisms involved in cellular and physiological processes. Caging can be achieved by linking a biomolecule of interest through a photolabile bond to a protecting group, thus rendering it temporarily inactive.¹ Upon specific wavelength irradiation, release of the bioactive molecule occurs, allowing for time-controlled burst in concentration with tight spatial control of the biological process under study, without subsequent addition of mass to the system. Many biological applications for caging molecules have been proposed, such as caged calcium and caged ATP to control physiological processes, caged neurotransmitters like glutamate and caged nucleic acids to regulate gene expression.² This way it is possible to switch biological processes on/off via a light/irradiation input.^{3,4} However, the choice of the photoremovable protecting group must fulfill several requirements mainly related to the molecule concentration and velocity of photodeprotection.^{1,5} Coumarin derivatives stand out, presenting relatively high extinction coefficients and photochemical quantum yields. They also absorb in the visible region (<420 nm) and have the advantage of fast photocleavage kinetics.^{6,7} We have previously reported the use of caged nucleotides for controlled polymerization of nucleic acids.⁵ The system is based on ATP caged with [7-(diethylamino)coumarin-4-yl]methyl (DEACM), which can be released by light irradiation and, thus, allowing for the controlled triggering of *in vitro* RNA polymerization. The earlier developments of coumarin derivatives to cage ATP were achieved by Geibler and co-workers in 2003.⁸ Here, we take one step further and introduce GTP caged with (7-methoxycoumarin-4-yl)methyl (MCM) for the development of a dual color setup that allows for the control of integration of either nucleotide: caging renders both nucleotides biologically inactive

and release of either ATP or GTP (or both simultaneously) is attained via irradiation using a monochromatic light pulse of the respective wavelength (Fig. 1). Once the free nucleotides are present in the reaction mixture, RNA polymerization can proceed. Additionally, we have introduced a Molecular Beacon (MB) to detect the formation of the RNA product.⁹ MBs have been widely used as nucleic acid reporters.^{10,11} The MB consists of a single-stranded oligonucleotide probe labeled with a fluorophore (5'-FAM) in one extremity and a quencher molecule (3'-DABCYL) at the other end. In the absence of a complementary sequence, the MB assumes a stem-loop conformation, leading to an energy transfer from the excited fluorophore to the quencher, and no fluorescence signal is observed. However, upon hybridization to the complementary RNA target, the stem-loop conformation is disrupted, which in turn causes the fluorophore and the quencher to be moved apart from each other, increasing the fluorescence signal.^{12,13} As such, transcription is translated into a fluorescent light signal. This work represents one more step towards controlling nucleic acid synthesis.

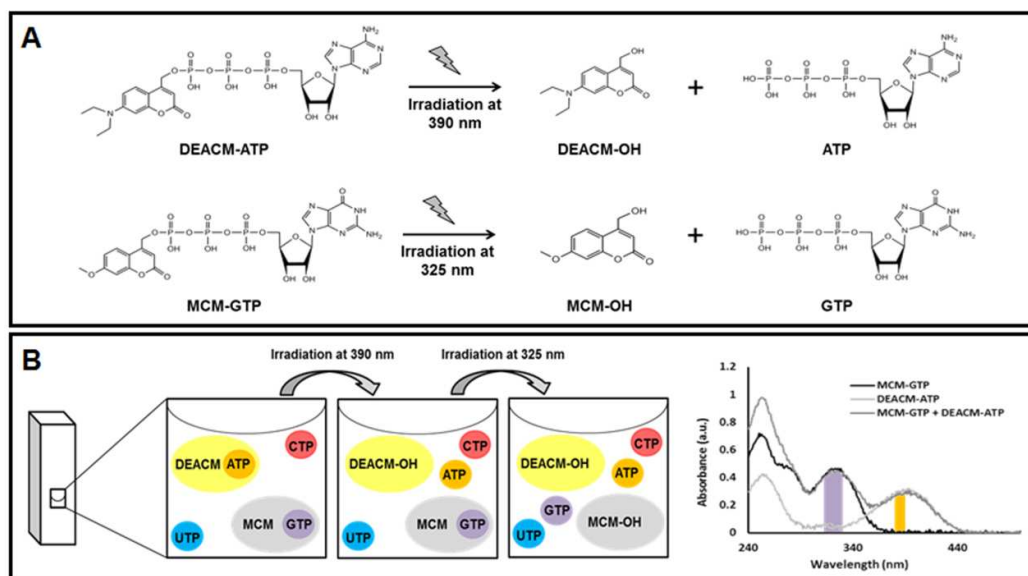


Fig. 1 Light induced release of ATP and GTP from its precursors DEACM-ATP and MCM-GTP. (A) DEACM-ATP and MCM-GTP structure photolysis. Upon irradiation at specific wavelengths (390 nm for DEACM-ATP and 325 nm for MCM-GTP), the coumarin-nucleotide ester bond is cleaved and free ribonucleotides are produced. (B) Representation of dual color ribonucleotide activation prior to *in vitro* transcription. A mixture of two caging molecules is subjected to light irradiation at 390 (yellow) and 325 nm (purple), with subsequent release of nucleotides, available for downstream applications. The absorption spectra of both coumarins, in the experiment conditions, are represented on the right (yellow and purple areas highlight irradiation bandwidth).

2. Results

2.1 Photochemical characterization

Both caging molecules selected for this study were fully characterized prior to use. The photochemical properties of DEACM-ATP have been described elsewhere.^{5,14} The absorption spectrum of MCM-GTP is characterized by three bands, with maxima at 250 nm, 268 nm and 325 nm (Fig. 1B). The band with maximum at 268 nm corresponds to the guanine ring absorption, while the other two correspond to MCM

ring absorption. The DEACM-ATP ($\lambda_{\max}=390$ nm, $\epsilon_{\max}=15000$ M⁻¹cm⁻¹, $\Phi_{\text{chem}}=0.0125$) and MCM-GTP ($\lambda_{\max}=325$ nm, $\epsilon_{\max}=13500$ M⁻¹cm⁻¹, $\Phi_{\text{chem}}=0.0100$) solutions in 10 mM phosphate buffer (pH 8.0) present absorption maxima in different regions (Fig. 1B), therefore 390 nm light was used to excite the DEACM caging group in DEACM-ATP molecule to release specifically the nucleotide functionalized to it. Likewise, for MCM-GTP, 325 nm light was chosen, leading to the formation of GTP and MCM-OH (Fig. 1A).

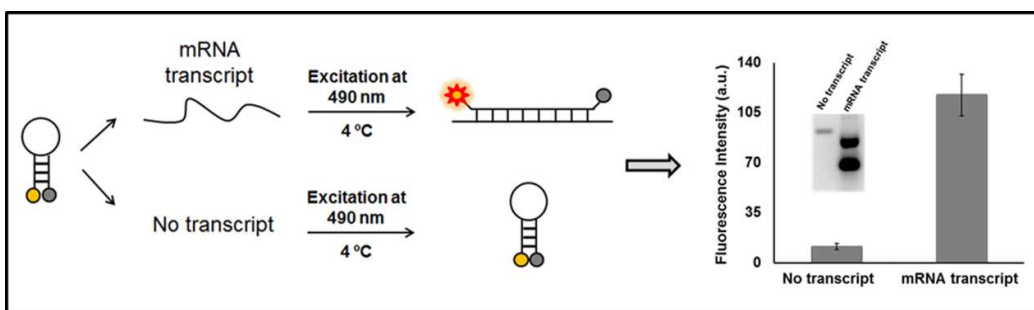


Fig. 2 Representation of a molecular beacon target detection. Upon hybridization to the complementary target (mRNA transcript), the molecular beacon hairpin structure opens and binds to the target, resulting in an intense fluorescence signal. In absence of target (no transcript), the molecular beacon maintains its hairpin conformation bringing the fluorophore (left) and quencher (right) in close proximity resulting in a residual emission signal.

2.2 Molecular Beacon Detection of RNA transcript

To ensure maximum efficiency of the caging and photorelease, different reactions containing solely one of the caging agents, either DEACM-ATP or MCM-GTP, and the other three remaining free nucleotides, were irradiated at the selected wavelength (390 and 325 nm, respectively). The release of ATP and GTP upon irradiation (in each case), and concomitant activation of transcription, was detected by measuring the fluorescence intensity resulting from the hybridization of the MB with the

complementary target (see Fig. 2). The MB performance and behavior were fully characterized and calibrated for optimal sequence identification and signal output (Fig. S1 and S2) using negative and positive controls of transcription. We also set up a fluorescence intensity threshold (approximately 25 a.u.) limited by the non-irradiated control above which we consider that the reaction provided a full length mRNA. Results show the detection of a clear fluorescence signal in both cases where ATP and GTP were substituted respectively with irradiated DEACM-ATP and MCM-GTP, indicating the production of a full length RNA transcript (Fig. 3). However, there is residual fluorescence signal of the MB even when there is no irradiation of the coumarin. Moreover, the fluorescence signal from the transcription reaction where the nucleotides were activated by light was lower than the positive control where all nucleotides were free in solution. The obtained fluorescence results are supported by agarose gel electrophoresis.

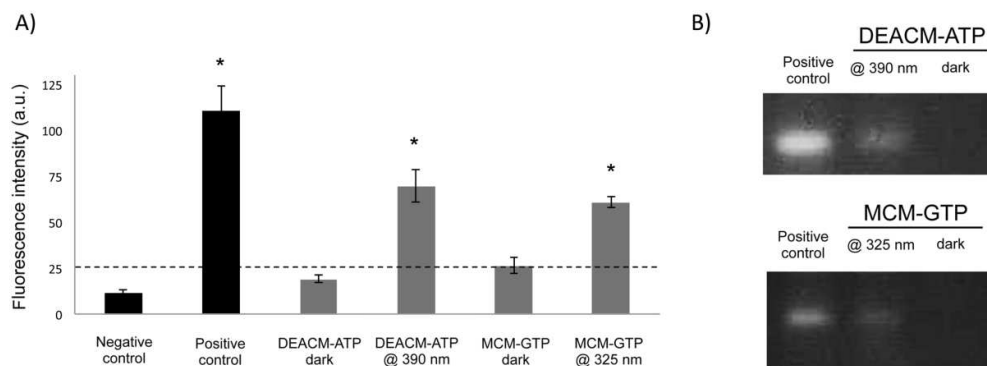


Fig. 3 Fluorescence actuator for sensing light-controlled transcription. (A) Irradiation of transcription reactions at specific wavelengths containing either DEACM-ATP or MCM-GTP and the three remaining free NTPs, followed by addition of the MB, results in a clear increase of fluorescence signal. Dashed line represents the established threshold for absence of transcript. (B) The fluorescence signal correlates to the generation of a full length RNA product.

2.3 Dual color activation of transcription

Following demonstration that both coumarins, when used independently, can breakdown and release ready-to-use nucleotides we tested their performance when put together in the same mixture. First we observed if the small spectral absorption overlap between DEACM and MCM at 325 nm would produce significant ATP leakage (Fig. 4). We fixed MCM-GTP concentration at 75 μM and varied DEACM-ATP. Data depicted in Figure 2 shows that 50 and 37.5 μM of DEACM-ATP are sufficient to produce mRNA visible by agarose gel electrophoresis, thus confirming the existence of a small leakage effect at 325 nm. Considering these results, we performed the dual color activation of transcription where a mixture of both coumarins was irradiated at the selected wavelengths. We denote that, in general, whenever a mixture containing irradiated DEACM-ATP and MCM-GTP is used for

the transcription reaction (Fig. 5), the resulting fluorescence signal is diminished, regarding the use of only one coumarin (data in Fig. 3). The established threshold made this comparison possible between experiments. Taking a closer look at Figure 5, we can see an increase in MB fluorescence intensity when the coumarin mixture was irradiated at both wavelengths, versus single wavelength irradiation at either 325 or 390 nm. Conversely it still presents a higher signal than the fluorescence threshold, establishing the formation of mRNA during *in vitro* transcription. Once more we can correlate the fluorescence results with the ones obtained in agarose gel electrophoresis.

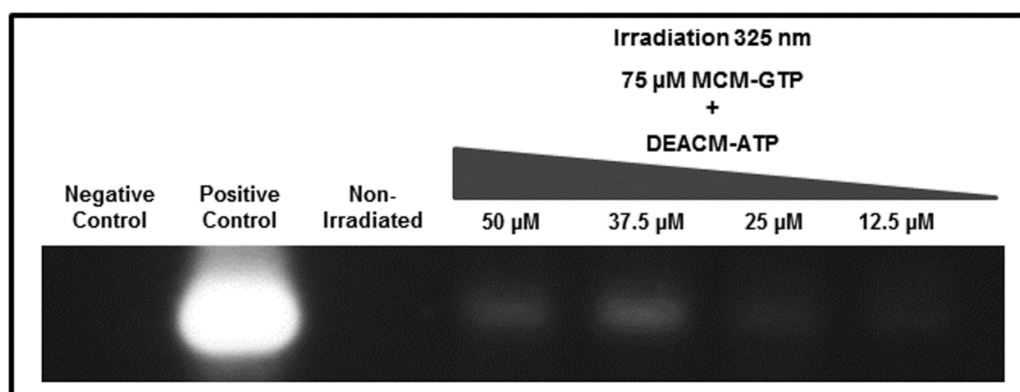


Fig. 4 Effect of DEACM-ATP leakage at 325 nm. Agarose gel electrophoresis of T7 RNA polymerase *in vitro* transcription reaction with DEACM-ATP and MCM-GTP molecules. Solutions were irradiated at 325 nm. Extra time of exposure for image acquisition was used to improve visualization of the leakage effect.

3. Discussion

The possibility of controlling RNA polymerization by means of direct light irradiation of caged-ATP for the release of the functional nucleotide moiety was previously demonstrated by our group.⁵ Here, we have taken one step further in what the control

of RNA polymerization by light is concerned – in addition to DEACM-ATP we simultaneously used MGM-GTP, thus having the possibility to control the release of two substrates required by the enzyme (see Fig. 1A). The choice of caging moieties (DEACM and MCM) was based on minimizing spectral overlap while maintaining similar chemical structure and photorelease behavior, so as to reduce bias between the two nucleotides being released. In this way, by irradiating a RNA polymerization mixture without enzyme at 390 nm, one is capable of releasing functional ATP into the mixture, whereas irradiation at 325 nm will release GTP (Fig. 1B).

To visualize RNA production directly in solution and without the need for further sample manipulation, we designed a MB that in presence of its complementary target (mRNA) adopts an open conformation and fluorescence is detected; in the absence of transcription, the MB retains the native closed hairpin conformation, keeping the fluorophore in close proximity of the quencher and no fluorescence is observed – Fig. 2 (see also Supplementary Information Fig. S2).

When DEACM-ATP and MCM-GTP are used as caging agents there is an increase in MB fluorescence that can be correlated with a successful transcription reaction (Fig. 3). Despite the efficiency of this approach, fluorescence is lower than that attained when the four free nucleotides are directly added to the reaction. The inhibitory effect of coumarin-alcohol derivatives may be responsible for hampering polymerization, as previously described.^{5,15} The DEACM-OH photolysis by-product formed during DEACM-ATP irradiation has been shown to interfere with transcription, due to its poor solubility in water and consequent partition into T7 RNA polymerase, inhibiting its activity.⁵ To assess whether *in vitro* transcription reaction was inhibited by the MCM-GTP photolysis by-product, increasing MCM-OH concentrations were used in samples containing a mixture of the four natural nucleotides. MCM-OH

concentrations above 100 μM eventually lead to decreasing transcription yields (Fig. S3). The inhibition of transcription by the photolysis by-product limits the concentration range of MCM-caged nucleotide that can be used for light-controlled *in vitro* transcription reactions. However one should also consider that not all of the irradiated molecules were able to generate free triphosphate nucleotides ready to be incorporated by RNA polymerase, which results in a lesser amount of mRNA synthesis for the irradiated sample and therefore a lower MB fluorescence signal. By establishing a threshold from which we consider that there is an effective transcription product we can compare results from independent experiments.

Following an experimental design where ATP and GTP are replaced respectively by DEACM-ATP and MCM-GTP within a regular NTP pool, both 390 nm and 325 nm light irradiation are needed for the release of both missing ribonucleotides and for RNA transcription to resume – dual-color system. However, when 325 nm light alone is used as excitation, due to the residual photochemistry of DEACM-ATP derivative (circa 15% of the maximum absorption), there is an ATP leakage effect (Fig. 4). Therefore, MCM-GTP relative concentration was increased in order for excitation light competition to occur and DEACM-caged nucleotide cleavage to be reduced upon 325 nm irradiation, thus minimizing transcription leakage in the absence of the second input. For the non-irradiated sample, no RNA product was observed, indicating efficient DEACM and MCM caging of ATP and GTP, respectively, rendering the ribonucleotides biologically inert and impairing transcription (Fig. 5). When only 390 nm light irradiation is applied, only ATP, but no GTP, is released, hence no transcription product is synthesized. If both wavelengths are used to irradiate the sample, both ATP and GTP are released and transcription resumes. When compared to the usage of a single caging molecule, a lower increase in fluorescence is

denoted, which may be due to joint inhibitory effect of both coumarin-alcohol derivatives formed after irradiation.

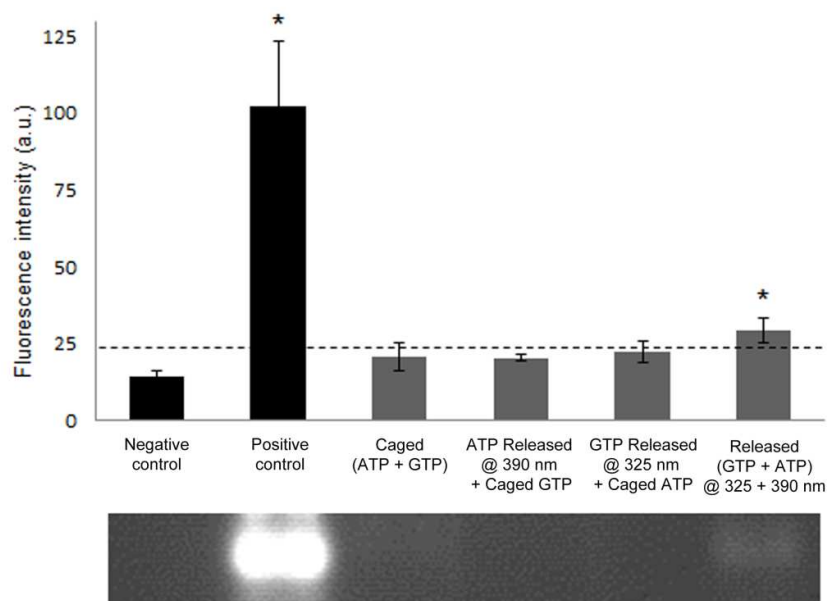


Fig. 5 Control of transcription via light-input/RNA-output “AND” logic gate. DEACM-ATP is used as source of ATP and MCM-GTP as source of GTP. Only in presence of both wavelength light inputs (390 and 325 nm) formation of mRNA product occurs.

4. Experimental Section

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) of the highest purity available and used without further purification. T7 RNA Polymerase was purchased from Fermentas (Vilnius, Lithuania). All oligonucleotides were purchased from STAB Vida (Caparica, Portugal).

4.1 MCM-GTP synthesis and characterization

DEACM-ATP synthesis and purification is described elsewhere.⁵ [7-methoxycoumarin-4-yl]methyl guanine 5'-triphosphate (MCM-GTP) was synthesized following the same procedure, but using [7-methoxycoumarin-4-yl] phosphate

(MCM-P) and GDP as starting materials. A Hitachi-Merck HPLC L6200A Pump with an L-4500 Diode Array Detector using a Polystyrene-Divinylbenzene (PLRP-S, Polymer Labs) semi-preparative column (7.4 mm x 15 mm, 8 μ m, 300 \AA) was employed for separation and purification of MCM-GTP. Eluent A was triethylammonium acetate buffer in water, 20 mM, pH 6.9; eluent B was methanol. The gradient started with 25% of B in A and isocratic separation after 4min; with an increase to 100% B after 5 min, and finished after 16 min at 100% of B. Separations were run at a flow rate of 0.9 mL/min and the column temperature was 35°C. A final MCM-GTP yield of circa 20% was estimated. After peak separation and collection, samples were lyophilized, resuspended in water and stored in the dark at -20°C. A purity of >95% was determined by HPLC. All solutions were protected from light and manipulations were made under red-light illumination.

4.2 Irradiation assays

All spectroscopic measurements and irradiations were performed in a 60 μ L quartz fluorescence cuvette (10.00 mm optical path) at 21 °C. The DEACM-ATP absorption spectrum was acquired with 1.5 nm slit bandwidth for excitation and emission, with correction files. MCM-GTP spectrum was collected with 2.5 nm slit bandwidth for excitation and 5.0 nm bandwidth for emission, with correction files. Coumarin derivatives irradiations were carried out in a Spex Fluorolog 0.22 m Spectrometer with a 150W xenon arc lamp, monochromated for the desired excitation wavelength (35 min of irradiation at 390 nm, 4.5 nm slit bandwidth for DEACM-ATP; 35 min of irradiation at 325 nm, 9 nm bandwidth for MCM-GTP). The actinometry of the irradiation setup was performed with the concentrated potassium ferrioxalate actinometer¹⁶ and intensities of 9.5×10^{-8} and 4.2×10^{-8} Einstein/min were measured, for

325 nm and 390 nm monochromatic irradiation, respectively. Photochemical quantum yields for the formation of alcohol were calculated both for MCM-GTP and DEACM-ATP as described in Pinheiro et al., 2008, which in turn translate to quantum yields of nucleotide release. For irradiations with 325 nm light, 10 mM phosphate buffer: MCM-GTP=0.0100, DEACM-ATP=0.0025. For irradiations with 390 nm light, 10 mM phosphate buffer: DEACM-ATP=0.0125.

4.3 Dual-color *in vitro* transcription

Standard *in vitro* transcription using 250 ng of template was performed with 20 U of T7 RNA Polymerase according to the manufacturer's protocol. Reactions were incubated 1 h at 37°C, followed by heat inactivation of enzyme for 15 min at 75°C. In assays involving caged-ATP and caged-GTP, ATP was substituted by 50 μ M of DEACM-ATP and GTP was substituted by 75 μ M of MCM-GTP. Coumarin-derivatives were irradiated prior to addition to the reaction mixture. For irradiation experiments, the remaining free nucleotides triphosphate were present at concentrations of 100 μ M each. Products were analyzed on 3% agarose gel electrophoresis in 1xTBE buffer. Results were visualized in a Gel Doc XR+ Molecular Imager system (Bio-Rad, USA) following staining with GelRed (Biotium, USA). Transcription template cloning and purification is described elsewhere.⁵

4.4 DEACM-ATP leakage at 325 nm and MCM-OH inhibition

DEACM-ATP leakage assay was performed using standard *in vitro* transcription conditions, 100 μ M of free CTP and UTP, 75 μ M of MCM-GTP and increasing concentrations of DEACM-ATP (12.5, 25, 37.5, 50 μ M). Prior to the addition of T7 RNA Polymerase the solutions were irradiated at 325 nm for 1 hour. Products were

analyzed on 3% agarose gel electrophoresis in 1xTBE buffer. For MCM-OH inhibition assay, the super-saturated 868 μM MCM-OH water solution was prepared by dissolving pure MCM-OH powder (1.79 mg) in 10 mL acetone, and evaporated under vacuum forming a thin film on the volumetric flask walls. Hot sterile water (10 mL) was added to the flask and the solution was put in an ultra-sound bath for 10 minutes. No precipitation was observed after cooling to room temperature.

4.5 Molecular beacon for detection of transcription

In order to rapidly evaluate the efficiency of the reaction, a 25 nt DNA molecular beacon (FAM-5'-TCGATAACAGTTCCTGCATGATCGA-3'-D) complementary to the RNA transcript of the Exon 7 of the human p53 gene was used. It is labelled with a 6-carboxyfluorescein (FAM) at the 5' end and with [4 - [(4 - (dimethylamino)phenyl)azo)benzoic acid] (DABCYL) at the 3' end. For hybridization assays, 1.5 μM of molecular beacon was added to the reaction mix before 1 hour incubation at 37 °C. Then, the reaction mixture was brought up to a total volume of 50 μL with DEPC treated water. Fluorescence measurements were performed at 4 °C, to ensure a closed conformation in the hairpin region by default, using 490 nm as the excitation wavelength.

5. Conclusions

We developed a universally applicable light controlled binary system as proof-of-concept for multi-color selective release of nucleotides. Light allows activation of a biologically active molecule with defined temporal accuracy without the need for further substrate addition into the system. Ultimately, having each of the four nucleotides functionalized with a suitable caging group with different non-

overlapping absorption profiles would allow for the construction of a four letter code instead of a binary one. By coupling the output to a light signal through the use of a MB, we are able to immediately detect and quantify the target molecule without the need for separation of the hybridized MB from the unbound probe in excess. By using light as a trigger, we demonstrate a new universally applicable means to surpass the necessity of continuously adding mass to the system. Furthermore, this approach could be extendable to light-controlled DNA synthesis, another attractive biomolecule for the devise of computational networks and logic gates involved in functioning and regulatory mechanisms in living cells.

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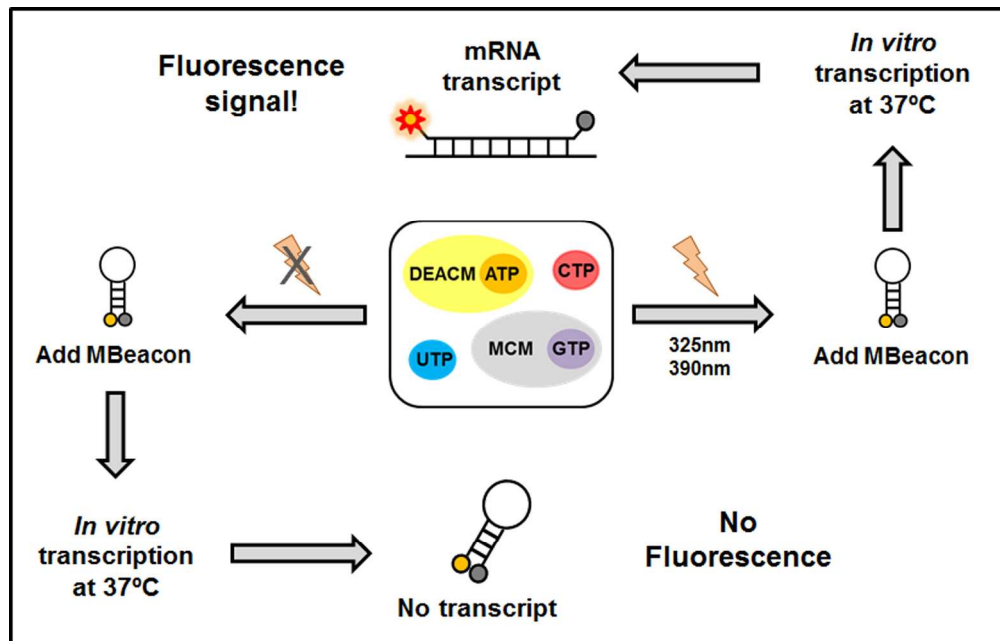
Keywords

Cage compounds, *In vitro* transcription, RNA, Dual-color system, Molecular Beacon

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