



# In Vivo Light-Driven DNA Binding and Cellular Uptake of Nucleic Acid Stains

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## Accepted Manuscript

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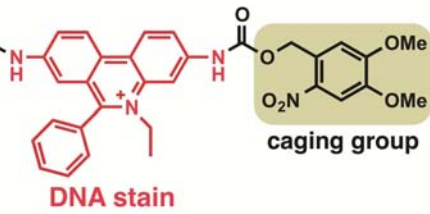
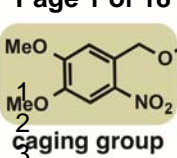
## How to cite:

Sánchez, M., Martínez-Costas, J., Gonzalez, F., Bermudez, M., Vázquez, M., Mascareñas, J. (2012). In Vivo Light-Driven DNA Binding and Cellular Uptake of Nucleic Acid Stains. *ACS Chem. Biol.*, 7 (7), 1276–1280. DOI: 10.1021/cb300100r

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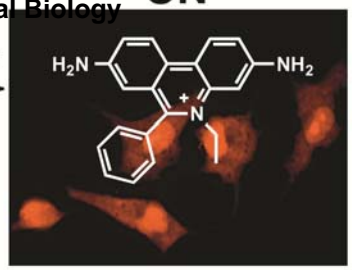
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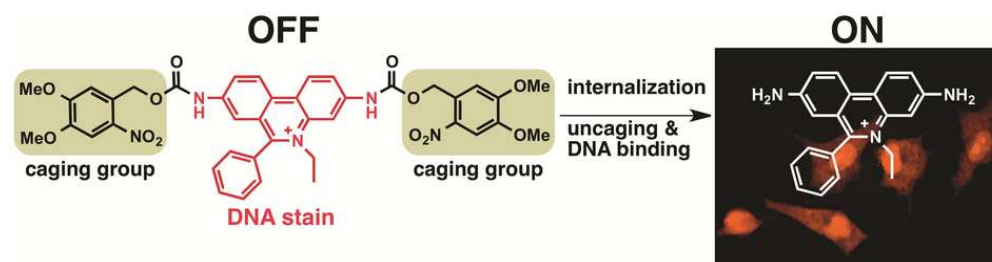


ACS Chemical Biology

**ON**

internalization  
uncaging &  
DNA binding





40x10mm (600 x 600 DPI)

## ***In vivo* light-driven DNA binding and cellular uptake of DNA stains**

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**Abstract:** Chemical derivatization of DNA stains such as ethidium bromide or DAPI with tailored, photoresponsive caging groups, allows for “on demand” spatiotemporal control of their “*in vivo*” DNA binding, as well as for improving their cellular uptake. This effect was particularly noteworthy for a Nvoc-caged derivative of ethidium bromide that, in contrast with the parent stain, is effectively internalized into living cells. The activation strategy works in light-accessible, therapeutically relevant settings, such as human retinas, and can be even applied for the release of active compounds in the eyes of living mice.

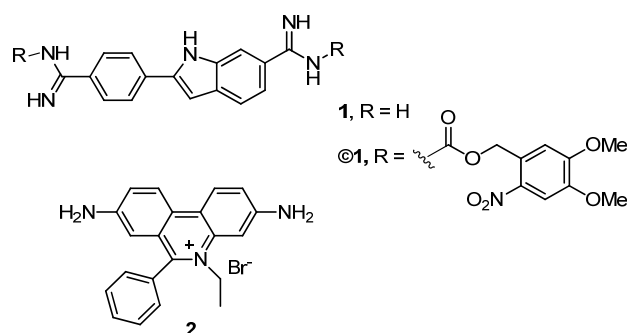
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6 The development of nucleic acid-targeted drugs continues to be a major research endeavor at  
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8 the interface between chemistry and biomedicine. A large variety of DNA-binding agents, have  
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10 been developed, and many of them have found application as drugs or as DNA stains.<sup>1,2,3,4,5</sup>  
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12 Unfortunately, many of these molecules present selectivity and toxicity problems that seriously  
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14 restrict their therapeutic potential. In this context, derivatizing these molecules with photolabile  
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16 appendages that confer advantageous physicochemical features while providing for external  
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18 control of their activity might open important therapeutic avenues.<sup>6,7,8</sup> Needless to say that the  
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20 use of light-activated compounds in photodynamic therapy might greatly benefit from current  
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22 technical advances that make possible to irradiate almost any tissue in the human body.<sup>9</sup>  
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26 DAPI (4',6-diamidino-2-phenylindole, **1**) and ethidium bromide (EtBr, 3,8-diamino-5-ethyl-6-  
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28 phenyl-phenanthridinium bromide, **2**) are among the best known DNA stains,<sup>10,11,12,13,14</sup> and have  
29  
30 found wide and important applications in molecular and cell biology. Ethidium bromide binds  
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32 DNA by intercalation, and has been extensively used for detecting double stranded DNA, mainly  
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34 in gel electrophoresis assays. However, it cannot be used for direct staining of cellular nucleic  
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36 acids, as it is poorly internalized. In contrast, the blue-fluorescent DAPI can be used to stain cell  
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38 nuclei and, unlike the non-specific EtBr, it prefers to bind A/T-rich DNA sequences, inserting into  
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40 their narrow minor groove.  
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43 We have recently demonstrated that caging of the amidinium moieties of DAPI and other  
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45 bisbenzamidine DNA binders with suitable photoresponsive protecting groups suppresses  
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47 their DNA binding.<sup>15</sup> Importantly, irradiation with UV light removes the caging groups and  
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49 regenerates the parent, active compounds, thus allowing the control of the *in vitro* DNA binding  
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51 activity of these molecules by external stimuli (UV light). We were then challenged to  
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53 demonstrate that such conditional activation strategy could also be used in living cells and  
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55 tissues, a process that should be easily monitored by the increased fluorescence of these  
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6 molecules upon staining of the cell nuclei. In addition, we were also intrigued by the possibility of  
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8 extending the caging strategy to other DNA binders lacking amidinium groups, such as ethidium  
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10 bromide (EtBr).

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13 Herein we demonstrate that both, DAPI (1) and EtBr (2), can be efficiently caged using  
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15 photolabile groups, and that their DNA binding can be restored in living cells and in  
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17 therapeutically-relevant tissues such as retinas, in an *spatially* and *temporarily*-controlled  
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19 manner. We also show that it is even possible to release the DNA stains in the eyes of living  
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21 mice by using an external irradiation source.  
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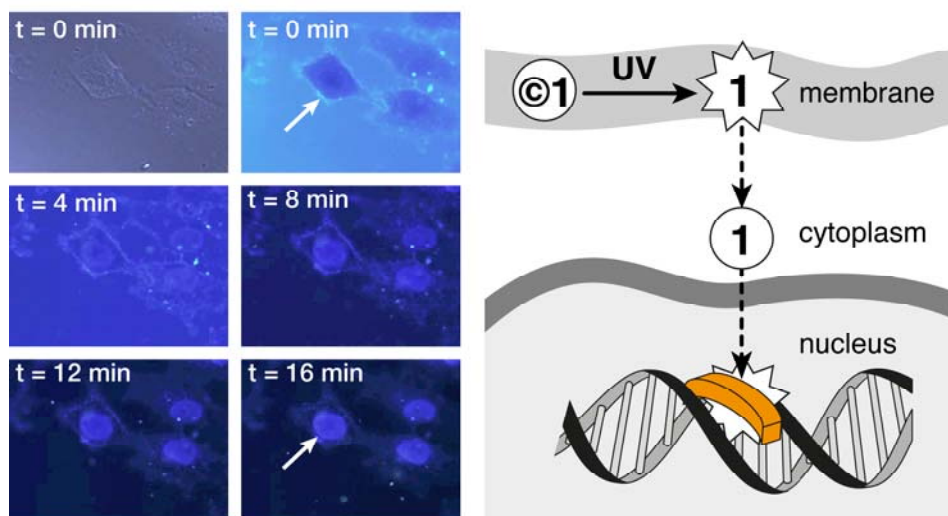


36  
37 **Figure 1.** Structures of DAPI (1), its caged derivative (©1) and ethidium bromide (2).

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40 As expected from the relatively low pKa of the amidines and their propensity to act as leaving  
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42 groups, the caging groups of DAPI derivative ©1<sup>15</sup> can be readily removed in presence of ds-  
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44 DNAs, in just 1.5-2 min, using a standard gel transilluminator lamp as irradiation source ( $\lambda = 300$   
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46 to 375 nm, see the supporting information, Figure S1). For the in vivo assays, we incubated  
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48 independent samples of Vero cells,<sup>16</sup> with 5  $\mu$ M ©1 for 30 minutes, and monitored their evolution  
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50 by fluorescence microscopy. Initial irradiation experiments were carried out using the  
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52 transilluminator, but the UV light source from the microscope could also effectively photolyze ©1.  
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54 This allowed an easy monitoring of the changes in fluorescence emission of the dye inside the  
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56 cells in real time. Remarkably, the fluorescence emission is initially relatively weak and diffuse,  
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6 but apparently more intense on the perimeter (membrane) of the cells. Over time it becomes  
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8 progressively concentrated in the cell nuclei, while growing fainter at the cell edges. Indeed,  
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10 after 20 minutes under the microscope, the edges become barely distinguishable and only the  
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12 nuclei are observed (see time-lapse video S1 in the supporting information). Control experiments  
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14 with liposomes confirm that, as expected, ©1 is basically non-emissive, however addition of  
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16 DAPI to a liposome solution results in a marked increase in its emission intensity (see  
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18 Supporting Information, page S11). Therefore it can be safely stated that the fluorescence  
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20 observed in the membrane is due to uncaged DAPI.  
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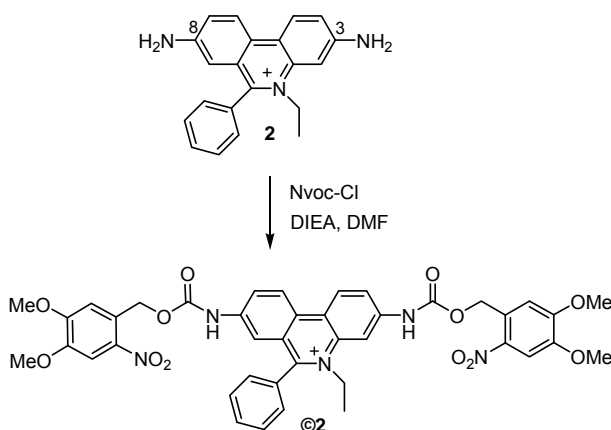
23  
24 In order to separate the effects of the uncaging and cellular redistribution, Vero cells were  
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26 incubated with ©1 in PBS for 30 min, and irradiated for 2 min in the transilluminator (enough to  
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28 uncage most of the stain). Comparison of photographs obtained immediately after the  
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30 irradiation, and after five minutes, confirms that the uncaged dye requires some time to  
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32 accumulate and stain the cell nuclei (see supporting information, page S16). All these results are  
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34 consistent with the presence of the hydrophobic caged DAPI (©1) in the cytoplasmic membrane  
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36 and probably also in other cell locations. Upon irradiation, photolysis of ©1 releases the  
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38 fluorogenic DAPI, which is highly emissive in the hydrophobic phospholipid matrix. However, the  
39  
40 uncaged DAPI is very hydrophilic (ACD/Labs LogD (pH 7.4) = -1.03),<sup>17,18</sup> and is therefore driven  
41  
42 out of the membrane and freely diffuse towards the cell nuclei, finally forming a stable,  
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44 fluorescent DNA complex.  
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**Figure 2.** Left: Fluorescence microscopy of Vero cells incubated with 5.0  $\mu\text{M}$  ©1 showing the fluorescence changes inside the cells ( $t = 0$ : brightfield and fluorescence;  $t = 4$  min to  $t = 16$  min: fluorescence); arrows highlight the brighter areas. Right: Schematic representation of the proposed activation dynamics of caged DAPI, which is, at least in part, stored in the cell membrane. Initial background fluorescence at  $t = 0$ , most probably results from non-specific interaction between uncaged DAPI and the hydrophobic polymeric Mowiol® matrix used as coverslip mounting solution.

Once demonstrated the possibility of *in vivo* UV-controlled DNA interaction with a minor groove binder, we sought to extend this strategy to EtBr (**2**), which binds DNA by intercalation and lacks the amidinium groups of DAPI. Despite its wide use as an *in vitro* DNA stain, EtBr has very limited value for *in vivo* experiments due to its poor cell membrane permeability. We envisioned that masking the amino groups of EtBr with the non-polar Nvoc caging group might not only interfere with its DNA binding, but also improve the internalization properties of the stain by increasing its hydrophobic character. Therefore the bis-Nvocphenantridyl derivative ©2 was prepared by treatment of the commercial EtBr with nitroveratryl (Nvoc) chloride under basic conditions.

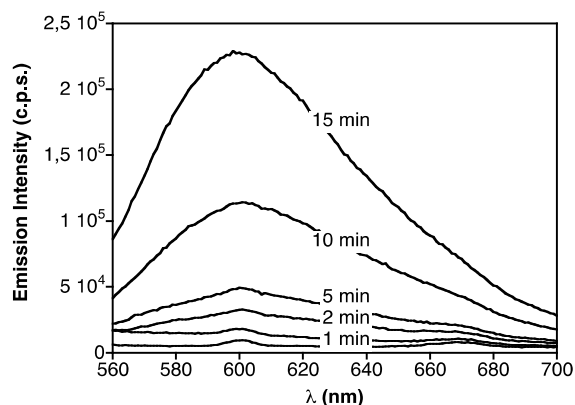




**Scheme 1.** Synthesis of Nvoc-caged ethidium bromide ©2.

As expected, ©2 did not show any fluorescence in the presence of a short double stranded DNA oligonucleotide, and competition experiments with uncaged EtBr confirmed that ©2 is unable to bind DNA. Irradiation of a solution of ©2 in Tris-HCl buffer; 100 mM NaCl; pH 7.5, with UV light (standard transilluminator lamp,  $\lambda = 300$  to 375 nm), promoted the cleavage of the photolabile groups, thus regenerating the active EtBr intercalator (2). Importantly, this can be achieved in the presence of DNA, leading to a large increase (60-fold) in the emission intensity at 600 nm, which is consistent with the intercalation of the released EtBr into the DNA (Figure 3).

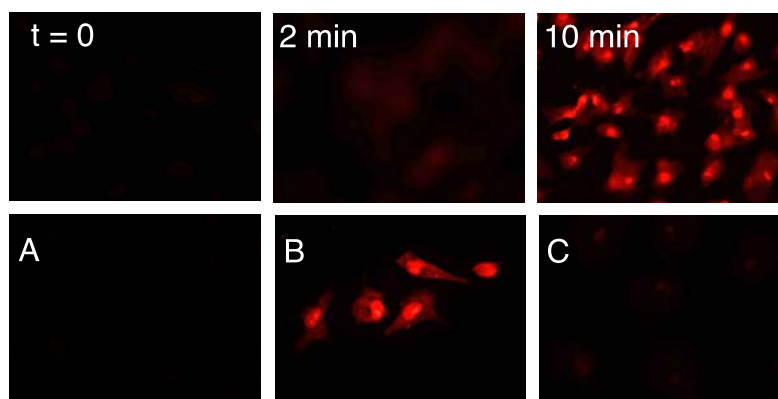
HPLC monitoring of the UV uncaging showed that the process generates temporary Nvoc-monoprotected intermediates. Control experiments with a mixture of specifically synthesized C3 and C8 Nvoc-monoprotected EtBr showed that these compounds are not fluorescent in presence of DNA, despite retaining some DNA binding affinity (see the supporting information, pages S9-S10). Therefore, uncaging of both groups is required for good DNA binding, and for DNA-dependent fluorescent staining.



**Figure 3.** Fluorescence emission spectra of a 0.8  $\mu\text{M}$  solution of ©2 in Tris-HCl buffer at increasing irradiation times in the presence of a hairpin ds-oligonucleotide. No further increase in fluorescence is observed after 15 min. irradiation; therefore shining light for 10-15 min is enough for complete uncaging.

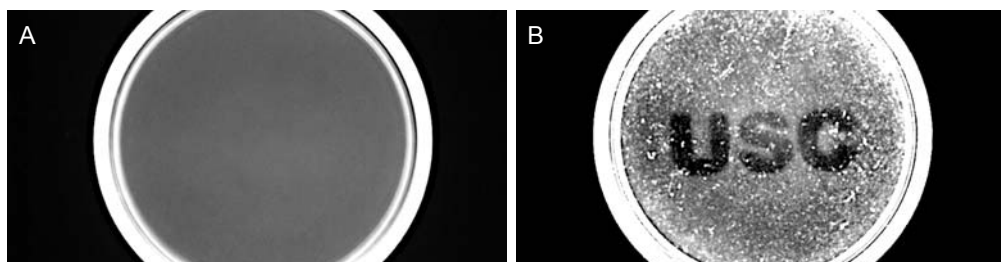
Once the photocontrolled DNA binding of ethidium was confirmed, we studied whether the presence of the hydrophobic appendage could improve the cell transport properties of the dye. Therefore, independent samples of Vero cells were treated in parallel with 12.5  $\mu\text{M}$  caged ©2 and 20.0  $\mu\text{M}$  of EtBr (2), and examined by fluorescent microscopy after 30 min (excitation filter 530-550 nm/emission filter 590 nm). As shown in Figure 4A/C, in both cases the cells were essentially non-fluorescent, and only a very faint emission could be detected in the case of EtBr. Remarkably, UV irradiation of the cells incubated with ©2 (10 min) resulted in a very significant increase in the emission intensity in the cell nuclei (Figure 4B).<sup>19</sup> This result must be correlated with photorelease of ethidium bromide from the inactive derivative ©2. Therefore, in contrast to the parent phenantridinium 2, the designed photoresponsive derivative ©2 seems to efficiently cross cell membranes, accumulating inside the cell in a latent form, until irradiation with UV light releases the active DNA binder. A control experiment at different irradiation times revealed a progressive increase in the fluorescence emission from the cells, reaching a maximum after approximately 10 min of irradiation (Figure 4, top). Interestingly, colocalization experiments with

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6 DAPI and mitotracker dyes show that the uncaged ethidium is mainly located in the nucleoli,  
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8 therefore cannot be really considered as a true nuclear stain (See the supporting information,  
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10 pages S15 and S16).  
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27  
28 **Figure 4.** Top: Fluorescence microscopy of Vero cells ( $12.5 \mu\text{M}$  ©2) at increasing irradiation  
29 times. Bottom:  $12.5 \mu\text{M}$  ©2, after 30 min incubation. A) before irradiation and uncaging; B) after  
30 uncaging by UV irradiation for 15 min; C) control experiment with  $20.0 \mu\text{M}$  of ethidium bromide 2  
31 after 30 min incubation. Pictures were taken with a Olympus DP-50 digital camera mounted on  
32 an Olympus BX51 microscope, equipped with U-MNG2 fluorescence mirror unit.  
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39 In addition to temporal activation, the caging strategy should also allow for the spatial control of  
40 the interaction. To demonstrate that, we prepared a cell monolayer on a 100 mm tissue culture  
41 plate (Figure 5A), and irradiated it through a cardboard cutout of our university acronym, USC.  
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43 After 15 minutes of irradiation through the mask, we looked at the culture through a gel imaging  
44 system, observing the expected cellular photopatterning only for the cell populations that were  
45 irradiated (Figure 5B). Therefore, derivative ©2 is a light responsive, transport-efficient version of  
46 ethidium bromide, and should represent a significant addition to the yet short arsenal of nucleic  
47 acid stains that are effective *in vivo*.  
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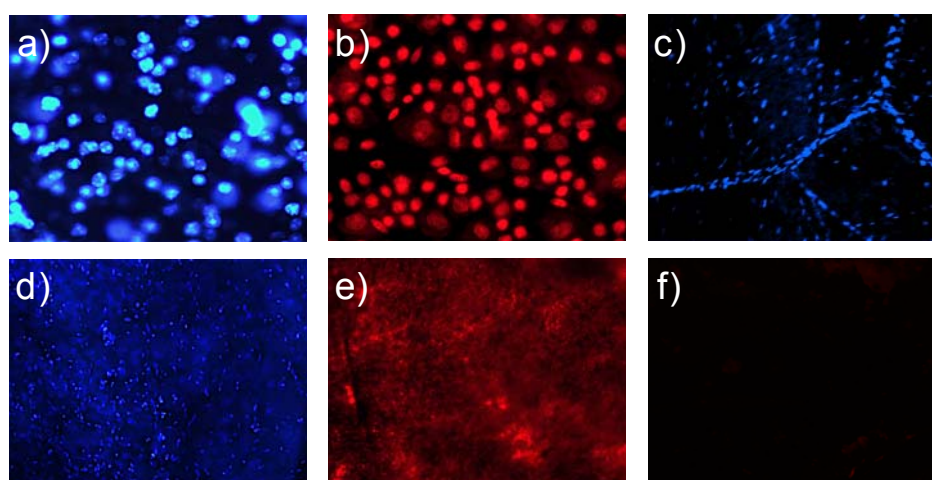


**Figure 5.** A) cell monolayer in the culture plate before irradiation; B) same culture after irradiation through a cardboard cutout.

The above results encouraged us to evaluate the performance of both caged DAPI (©1) and caged ethidium (©2) in a more stringent, therapeutically-relevant setting. Therefore we incubated mice eye retinas with 5.0  $\mu\text{M}$  ©1 and 12.5  $\mu\text{M}$  of ©2 (PBS buffer) and analyzed the resulting cellular staining before and after irradiation. Before irradiation, no staining was visible, whereas after irradiation both ©1 and ©2 were effectively uncaged in the retina, and the cell nuclei became fluorescent (Figure 6a, 6b, see also time-lapse video S2 in the supporting information). Importantly, the same results were reproduced with human retinas (Figure 6d, 6e), confirming that these compounds can be activated in the complex cellular environment of human tissues.

In a step forward, we also explored the possibility of *in vivo* activation of these molecules by direct external irradiation of the eyes in living mice. Therefore, live mice were treated with caged DAPI (©1) using a femoral vein injection, and a transpupillar irradiation of one of their eyes was made. The mice were sacrificed and the retinas extracted and flat mounted for study. As expected, only the retina of the irradiated eyes showed a clear fluorescence emission, which was mostly concentrated in the vascular walls (Figure 6c), possibly because the blood-retinal barrier prevented most of the molecules to enter the retinal cells.<sup>20</sup> These results confirm that the designed compounds can be selectively activated in the posterior segment of eyes of living

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6 animals using an external irradiation source, and suggest the eye as a suitable initial target  
7 organ for future therapeutic intervention using this type of light-activatable compounds. Delivery  
8 of drugs in a minimally invasive, safe and effective manner in the eye is still a major  
9 therapeutical challenge.<sup>21</sup> Given the well-established use of lasers in ophthalmology, the  
10 uncaging tactic should open new opportunities to treat specific eye diseases that require the  
11 selective release of active drugs in specific areas of the eye.<sup>22,23</sup>  
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37 **Figure 6.** Fluorescence microscopy of mouse and human retinas. a) Mouse retina incubated with  
38 5.0  $\mu\text{M}$  of **1**, after  $\sim 5$  min irradiation. b) Mouse retina incubated with 12.5  $\mu\text{M}$  **2**, after  $\sim 10$  min  
39 irradiation. c) Retina extracted from a mouse previously injected with **1** in the right femoral vein  
40 (100  $\mu\text{L}$ , 5.0  $\mu\text{M}$ ), and transpupillary irradiated for  $\sim 10$  min before sacrificing the animal; the  
41 nuclei of aligned cells forming a vessel image can be clearly observed. d) Human retina  
42 incubated with 5.0  $\mu\text{M}$  **1**, after  $\sim 5$  min irradiation. e) Human retina incubated with 12.5  $\mu\text{M}$  of  
43 **2**, after  $\sim 10$  min irradiation. f) Human retina incubated with 12.5  $\mu\text{M}$  of **2** before irradiation.  
44 Control, non-irradiation experiments were carried in all cases and none of them showed traces of  
45 fluorescence. Images a and b were acquired with 1 s exposure time, images c-f with 400 ms. All  
46 images with ISO 400 sensitivity.  
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56 In conclusion, we have demonstrated that the Nvoc caging strategy can be applied to classic  
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6 DNA intercalators like EtBr. Since these molecules are nucleic acid-dependent fluorescent  
7 probes, they provide an excellent opportunity for real-time monitoring of the activation process in  
8 living cells. For the Nvoc-DAPI (©1), we could observe the uncaging process and cell  
9 redistribution of the released stain in real time. Moreover, the caging strategy can be exploited  
10 not only for the “*on demand*” spatiotemporal control of the DNA-binding, but also for fine-tuning  
11 the physicochemical properties of the substrates. In the case of the Nvoc-caged derivative ©2,  
12 this allows for a rapid cell internalization, overcoming the poor cell transport ability of the parent  
13 ethidium bromide. Importantly, we have also demonstrated that this strategy can be successfully  
14 implemented in therapeutic-relevant tissues such as the retina, being even possible to activate  
15 them with external irradiation in the eyes in living mice. To the best of our knowledge, our results  
16 provide the first evidence on the use of caged compounds in eyes, and demonstrate that  
17 focalized release of drugs in the eye can be made with a minimally invasive technique, opening  
18 a new door for therapeutic and perhaps diagnostic applications.  
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### 37 **Materials/methods**

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39 General: All reagents were acquired from commercial sources. Reactions were followed by  
40 analytical RP-HPLC with an *Agilent 1100* series LC/MS using an *Eclipse XDB-C18*(4.6 x 150  
41 mm, 5 µm) analytical column. Standard conditions for analytical RP-HPLC consisted on a linear  
42 gradient from 5% to 95% of solvent B for 30 min at a flow rate of 1 mL/min (solvent A: water with  
43 0.1% TFA, solvent B: acetonitrile with 0.1% TFA). Compounds were detected by UV absorption  
44 at 220, 270, 304 and 330 nm. Final products were purified on a *Büchi Sepacore* preparative  
45 system consisting on a pump manager *C-615* with two pump modules *C-605* for binary solvent  
46 gradients, a fraction collector *C-660*, and UV Photometer *C-635*. Purification was made using  
47 reverse phase of water/acetonitrile 0.1% TFA, using a pre-packed preparative cartridge (150 x  
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6 40 mm) with reverse phase RP<sub>18</sub> silica gel (Büchi order #54863). The fractions containing the  
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8 products were freeze-dried, and their identity was confirmed by ESI-MS(+). NMR spectra were  
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10 recorded using *Varian Mercury 300* or *Brucker DPX 250* spectrophotometers, and processed  
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12 using the *MestReNova v6.1.1-6384* suite (*Mestrelab Research*). <sup>1</sup>H NMR spectra were  
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14 processed applying Global Spectrum Deconvolution (GSD).  
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## 17 18 **Synthetic procedures**

### 19 20 **3,8-bis(4,5-dimethoxy-2-nitrobenzyl)oxy]carbonyl]amino)-5-methyl-6-**

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22 **phenylphenanthridinium** (Nvoc<sub>2</sub>-ethidium, ©2). To a solution of commercially available  
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24 ethidium bromide (2), (100 mg, 0.253 mmol) in 25 mL of DIEA/DMF (0.195 M) was added  
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26 Nitroveratryl chloride (209 mg, 0.759 mmol). The resulting mixture was stirred for 16 h under Ar  
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28 and in the dark. After concentration, the residue was purified by preparative reverse-phase  
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30 chromatography (*Büchi Sepacore*) (5 min isocratic 15% B, followed by linear gradient from 15%  
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32 to 95 % B 30 during min, and 10 min isocratic at 95% B). The combined fractions were  
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34 concentrated and freeze-dried to obtain the desired product (©2) as a trifluoroacetic salt (77 mg,  
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36 0.086 mmol, 34%).  
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40 **In vitro fluorescence experiments with ©2:** A fluorescence cuvette containing a 0.82 μM  
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42 solution of ©2 in Tris-HCl buffer 20 mM; 100 mM NaCl; pH 7.5, and 0.136 μM hairpin DNA  
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44 oligonucleotide AACGTT was irradiated with UV light, and fluorescence emission spectra were  
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46 recorded after different irradiation times. Hairpin oligonucleotide: **AACGTT**: GGC AAGCTT CGC  
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48 TTTTT GCG AAGCTT GCC.  
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51 **Cell uptake experiments.** Vero cells were maintained in DMEM (Dulbecco Modified Eagle  
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53 Medium) containing 10% of FBS (Fetal Bovine Serum). The day before the cellular uptake  
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55 experiments, cells were seeded in twelve well plates containing glass coverslips (15 mm). Cells  
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57 were then washed 3 times in PBS and overlaid with 1 mL of fresh PBS with no serum added.  
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6 Compound ©1 was added in order to obtain a final concentration of 5.0  $\mu\text{M}$ , and in the case of  
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8 ©2 12.5  $\mu\text{M}$ . Samples were incubated for 30 min at room temperature in the absence of light.  
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10 For video recording and snapshots of Figure 2, after incubation the coverslips were mounted on  
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12 glass slides with *Mowiol*4-88® [100 mg/mL in 100 mM Tris-HCl pH 8.5, 25% glycerol and 0.1%  
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14 DABCO (as an antifading agent)]. Irradiation of selected samples was performed with a standard  
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16 gel UV transilluminator (8 watt,  $\lambda_{\text{exc}}$  300-370 nm). Images were obtained with an *Olympus DP-*  
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18 *50* (Olympus Corp., Tokyo, Japan) digital camera mounted on an Olympus BX51 fluorescence  
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20 microscope (Olympus BX51, Olympus Corp., Tokyo, Japan) equipped with a 360-370 nm  
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22 excitation filter, 420 nm emission filter for the cells treated with ©1 and were further processed  
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24 (cropping, resizing and contrast global contrast and brightness adjustment) with *Adobe*  
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26 *Photoshop* (Adobe Systems). In the case of ©2 we used a 530-550 nm excitation filter, and a  
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28 590 nm emission filter.  
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32 **Experiments with retinas and with animals.** Animal retinas were obtained from 3 month-old  
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34 wild type SV129 mice originally acquired from Charles River Laboratories (Boston,  
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36 Massachusetts, USA). The animals were terminally anesthetized, the eyes enucleated, the  
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38 retinas dissected from the eyecup, and flat mounts prepared with the ganglion cell layer  
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40 uppermost. Then they were incubated with a solution of caged ethidium (©2, 12.5  $\mu\text{M}$  in PBS  
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42 buffer), or caged DAPI (©1, 5.0  $\mu\text{M}$ , PBS buffer). The uncaging was performed with a standard  
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44 gel UV transilluminator for ©2 ( $\lambda = 300$  to 375 nm). In the case of ©1, it was found that the light  
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46 of the microscope (*Olympus BX51*) was intense enough to cleave the photolabile groups. The  
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48 irradiated samples were observed in a fluorescence microscope (*Olympus BX51*, Olympus  
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50 Corp., Tokyo, Japan) equipped with a 530-550 nm excitation filter, 590 nm emission filter for the  
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52 cells treated with ©2, and a 360-370 nm excitation filter, 420 nm emission filter for the cells  
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54 treated with ©1. Pictures were taken with an *Olympus DP-50* camera (*Olympus Corp.*, Tokyo,  
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56 Japan) mounted on the microscope. The same procedure was followed in the case a human  
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6 retina obtained from a patient who underwent ocular evisceration because of a painful terminal  
7  
8 glaucoma. Once the retina was removed from the eye, it was placed in DMEM (*Dulbecco*  
9  
10 Modified Eagle Medium) containing 10% of FBS (Fetal Bovine Serum), 1%  
11  
12 penicillin/streptomycin, and 1% glutamate, and cut into several pieces. Each piece was placed  
13  
14 on a microscope slide and the incubation procedure described above was followed. Consent  
15  
16 permission was obtained from the patient and the procedure was authorized by the Ethical  
17  
18 Committee for Clinical Research of our local government (Xunta de Galicia).  
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20  
21 For in vivo experiments the animals were anesthetized by intraperitoneal injection of 4% chloral  
22  
23 hydrate (400 mg/kg body weight) and a drop of tropicamide solution (10 mg/ml) was instilled on  
24  
25 the eyes to produce mydriasis. The right femoral vein was cannulated, and 0.1 ml of a solution of  
26  
27 caged DAPI (©1, 5.0  $\mu$ M in PBS) was injected. Then, the right eye was transpupillary irradiated  
28  
29 ( $\lambda$  = 300 to 375 nm). Finally, the animals were sacrificed by cervical dislocation, the eyes were  
30  
31 enucleated, and the retinas mechanically dissected out were mounted and viewed in the  
32  
33 fluorescent microscope. All experiments involving animals were conducted according to the  
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35 Bioethical Committee of our institution and adhered to the ARVO statement for the Use of  
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37 Animals in Ophthalmic and Vision Research.  
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#### 44 **Acknowledgments**

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47 *We thank the support given by the Spanish grants SAF2007-61015, SAF2010-20822-C02,*  
48  
49 *CTQ2009-14431/BQU, ConsoliderIngenio 2010 CSD2007-00006, and the Xunta de Galicia*  
50  
51 *INCITE09 209 084PR, GRC2010/12, PGIDIT08CSA-047209PR. M.I.S. thanks the Spanish*  
52  
53 *Ministry of Education for FPU PhD fellowships.*  
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