

## PAPER

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## Photo-controllable cytotoxicity in cell culture using a diarylethene photoswitch†

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For diarylethene (DAE) derivatives, whose dark cytotoxicity varies greatly depending on their isomerization state, we observe that the non-toxic open-ring isomer taken up by cells is converted to a toxic closed-ring isomer after only 10 seconds of UV light irradiation. This enables these derivatives to cause pronounced cell death after 1 day. Furthermore, when the closed-ring isomer is detoxified by opening the ring with green light irradiation after several intervals, cell damage increases with the period that DAE remains in the closed-ring state. The timescale at which the closed-ring isomer triggers the onset of cytotoxicity is considered in conjunction with the intercalation properties of DAE into DNA, and we also discuss the application of photonic molecular machines as a new technology for the control of biosystems.

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## Introduction

The ability to act on an object locally, remotely, and instantaneously makes light particularly suited for on-demand sterile control of microscopic biosystems. In practice, this property has led to the commercialization of automated systems for rapid processing of cultured cellular systems by combining light-responsive culture substrates and lasers.<sup>1</sup> In addition to cell killing, photo-responsive control of cell differentiation or detachment has been implemented using photo-functional substrates.<sup>2,3</sup> On the other hand, several attempts have been made to incorporate photoresponsive molecules into cells to control them by light from the outside. This technology kills cells by leveraging molecules that produce reactive oxygen species as sensitizers when exposed to light inside the cells, and it has already been widely applied clinically as photodynamic therapy (PDT).<sup>4,5</sup> Recently, combined with antibody targeting, cancer photoimmunotherapy has attracted attention as the latest therapeutic technology.<sup>6,7</sup> In addition, research is also underway to control biosystems by inducing photochromic molecules that change their structure under light to work

inside cells based on a different mechanism from sensitization.<sup>8–10</sup> In the first example of such an attempt, Branda *et al.* demonstrated that the movement of *C. elegans* that have taken up diarylethene (DAE) can be remotely controlled by externally inducing isomerization of DAE with light.<sup>11</sup> And some diarylethenes have been applied in photopharmacology.<sup>12–14</sup> Subsequently, in cell culture systems to which DAE derivatives were added at very low concentrations (<0.1 ppm), we found that the intercalation of DAE can be triggered by irradiation with UV light at 365 nm, which closes the open-ring form, and that irradiation of the molecules in this state with blue light at 436 nm causes pronounced phototoxicity, leading to rapid apoptosis of the cells.<sup>9</sup> The cytotoxicity was found to be increasingly dependent on the concentration of DAE incorporated as a closed-ring isomer and the dose of blue light irradiation; moreover, it was observed that light irradiation on a timescale of 100 s at light intensities on the order of 100 mW cm<sup>−2</sup> is required to induce cell death.<sup>10</sup>

In this study, we investigate the photo-switching of cytotoxicity of a different type newly found for the DAE derivative **1**,<sup>15</sup> based on the dark toxicity which varies greatly depending on the isomerization state. We discuss the timescale required for the transient ring closure to doom the cells to death after 1 day, along with the intercalation properties of DAE into DNA.

## Results and discussion

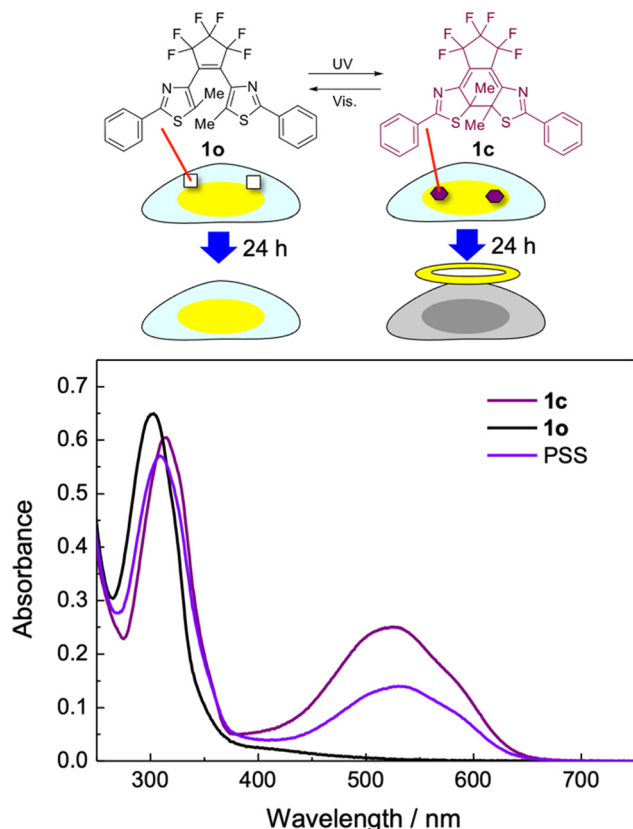
The structures and cellular effects of the two photoisomers of DAE used in this study are shown in Fig. 1(a). Prior to this experiment, we found that **1c**, the closed-ring isomer, exhibi-

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**Fig. 1** Chemical structures and the effect on cell culture of **1o** and **1c** (a) and UV-vis absorption spectra of **1o**, **1c** in a solution (b, D-PBS : DMSO = 95 : 5 and DAE concentration = 25  $\mu$ M) and the photostationary state (PSS, **1o** : **1c** = 44.5 : 55.5) under UV irradiation at 365 nm (b).

ted dark toxicity at concentrations of 5–10 ppm, whereas the corresponding open-ring isomer, **1o**, had little effect on cell viability at these concentrations. In addition, we observed that the dark toxicity effects exhibited by **1c** were not observed until about half a day after addition. This slow response is in contrast to the rapid (<1 h) cellular damage that occurs upon photoirradiation of dye molecules incorporated into cells, including our previous report on DAE (see the ESI†).<sup>9,10</sup> UV-vis absorption spectra of **1o**, **1c** and the photostationary state under UV irradiation at 365 nm are shown in Fig. 1(b). Here, irradiation of **1o** with UV light resulted in a 55.5% conversion to **1c** and irradiation with a sufficient amount of green light at 500–550 nm resulted in a complete conversion to **1o**.

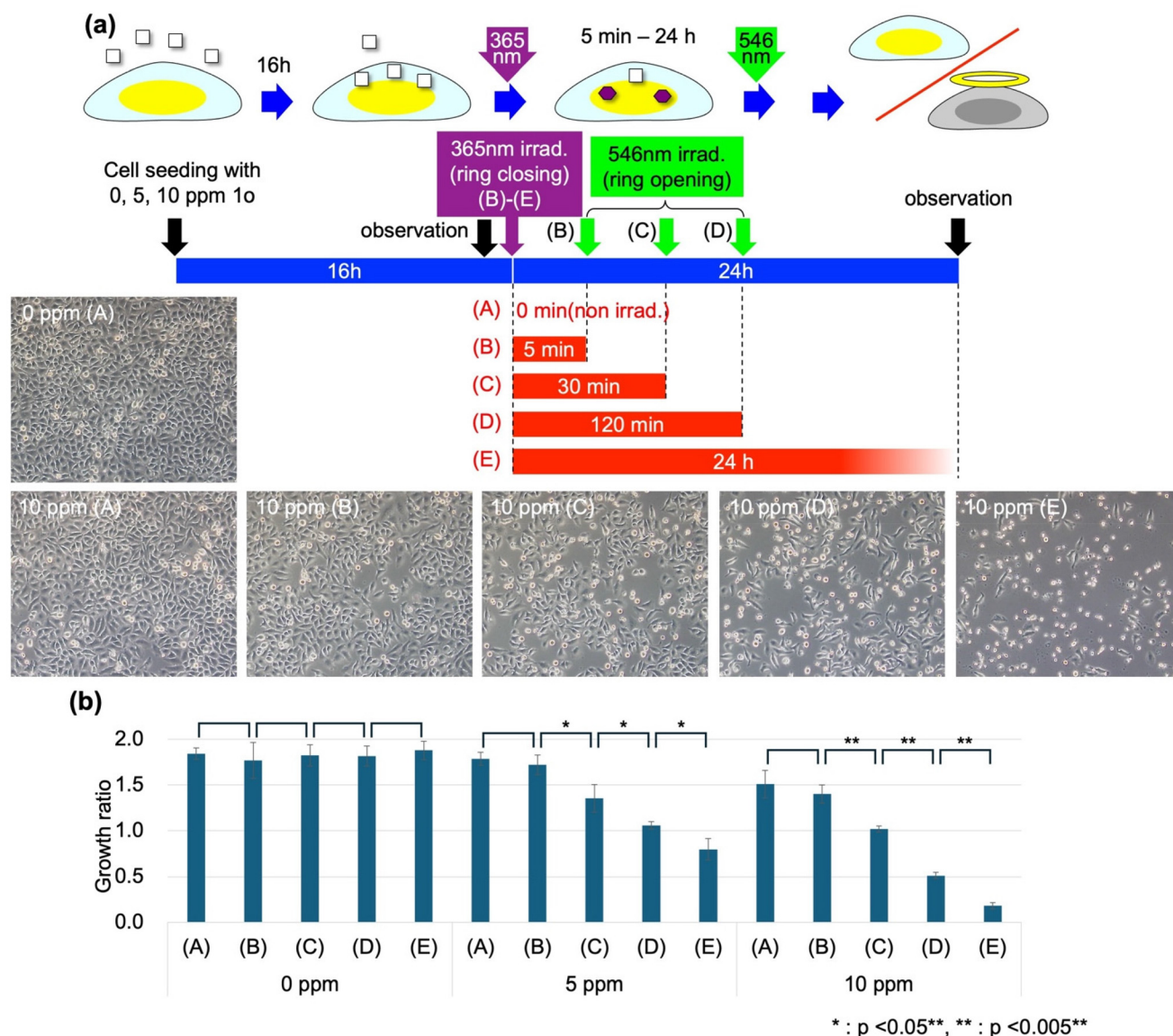
Fig. 2(a) shows an illustration of the overall experimental flow used to investigate the effects of photoswitching on the dark toxicity of DAE on cells. Experiments were conducted under three conditions (0, 5, and 10 ppm) for DAE coexistence concentration and five conditions ((A)–(E)) for the light irradiation schedule, as shown in Table 1, with a total of 15 conditions. HeLa cells were seeded on culture dishes in a culture medium containing 0, 5, or 10 ppm **1o**. After 16 h of incubation, we observed the cells under a microscope, replaced the culture medium with a DAE-free one, and irradiated the

samples with UV light at 365 nm for 10 s for conditions (B)–(E). The cells were then incubated for the prescribed time ( $t_i$ , (B): 5 min, (C): 30 min, and (D): 120 min) and then irradiated with green light at 546 nm for 30 s. Twenty-four hours after the first observation, the cells were gently flushed with the culture medium to remove the cells detached due to damage, and microscopic observation was performed again. Fig. 2(a) shows the microscopy images of the cells in the second observation.

Under conditions without light irradiation, the coexistence of 10 ppm **1o** had only a slight effect on cell density, but when UV light irradiation activated the toxicity by converting **1o** to **1c**, most cells were dead in 24 h (10 ppm (E)). Since no DAE was present in the culture medium at the time of UV irradiation due to the preceding medium change, this effect was thought to be due to the action of DAE already incorporated into the cells. **1o** is nearly insoluble in water, and when added to an aqueous solution system after dissolving in ethanol, it was observed to gradually precipitate out. On the other hand, when added to cell culture systems, it was taken up efficiently by cells that are rich in lipids. The effects of UV light irradiation did not appear immediately and the irradiation dose was necessary and sufficient to convert more than half of the **1o** to **1c**; these observations clearly indicate that this cytotoxicity was due to the dark toxicity of **1c**, which was totally different from the dose-dependent one reported in our previous papers.<sup>8,10</sup> We have summarized the characteristics of the photo-switching of cytotoxicity studied in this work and that reported previously in Fig. S3 and Table S3 in the ESI.† Fig. 2(b) shows the cell growth rate (number of cells in the second observation/number of cells in the first observation) in 24 hours in the presence of three coexisting concentrations of DAE under each irradiation condition. It was confirmed that none of the light irradiation conditions alone without DAE (0 ppm) had any effect on cell viability.

On the other hand, it was confirmed that the toxicity activated by the conversion of **1o** to **1c** could be nearly cancelled by irradiation with green light after 5 min (B). Even under the condition of 10 ppm DAE concentration, which killed most of the cells within 24 h after UV light irradiation, irradiation with green light after 5 min maintained the same viability as under the condition with no light irradiation at all (A). Fig. 3 shows the results of micropatterned irradiation performed to clearly demonstrate this situation: HeLa cells cultured in a **1o**-containing medium and incorporating **1o** were irradiated with UV light of 365 nm wavelength (Fig. 3(a), rectangular area), and immediately after that, a micropattern (Fig. 3(b), “546” area) was irradiated with green light of 546 nm wavelength. Then, many cells that experienced only UV light irradiation were damaged and detached from the substrate surface in 24 h, while the cells that received overlapping green light irradiation escaped damage (Fig. 3(c)). The ON/OFF (activation/cancellation) of cell damage was localized to the irradiated area for each corresponding condition, clearly indicating that the toxicity of the DAE molecule can be controlled on-demand at this spatio-temporal scale, and the effects on the cells appeared in 24 h.





**Fig. 2** Illustration of the overall experimental flow conducted to investigate the effects of photoswitching on the dark toxicity of DAE on cells and the microscopy images of the cells after all processes under the conditions of 0 ppm (A) and 10 ppm (A)–(E) (a) and the cell growth rate in 24 hours in the presence of three coexisting concentrations of DAE under each irradiation condition (b).

**Table 1** Schedules of light irradiation

| Condition | UV irradiation | $t_i$ (min) | Green irradiation |
|-----------|----------------|-------------|-------------------|
| (A)       | No             | —           | No                |
| (B)       | Yes            | 5           | Yes               |
| (C)       | Yes            | 30          | Yes               |
| (D)       | Yes            | 120         | Yes               |
| (E)       | Yes            | —           | No                |

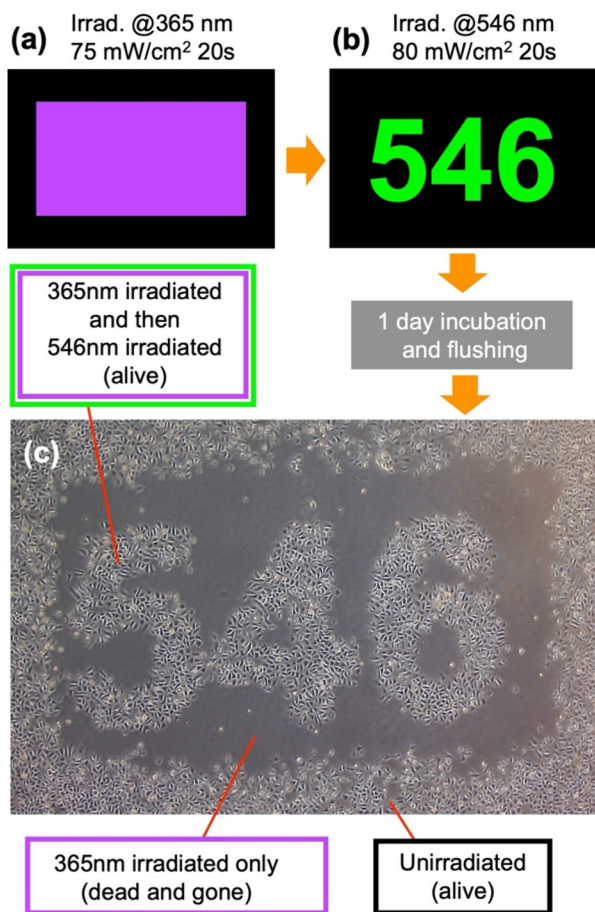
Irradiation with UV and green light carried out at 16 and 40 h, respectively, after cell seeding.

However, it was also found that the cancellation of this toxicity by green light irradiation decreased as  $t_i$  (time elapsed since UV irradiation) increased (Fig. 2, 10 ppm, (C) and (D)). In

connection with this finding, we investigated the intercalation of DAE added as **1o** into DNA. We discuss below the results of experiments in which **1o** DMSO solution was added to an aqueous DNA solution and light irradiated under conditions corresponding to the above cell experiments ((A)–(E)). Fig. 4(a) shows the absorbance spectra of the solutions at each stage of light irradiation and after the elapse of time for the irradiation conditions (A), (B), (D), and (E). Under condition (A) without UV irradiation, the absorbance spectrum attributed to **1o** gradually changed its profile over time, and the absorbance decreased significantly 40 h after the addition of **1o**. This change was consistent with the change corresponding to the progression of aggregation and precipitation (decrease in concentration in solution) seen when **1o** DMSO solution is added

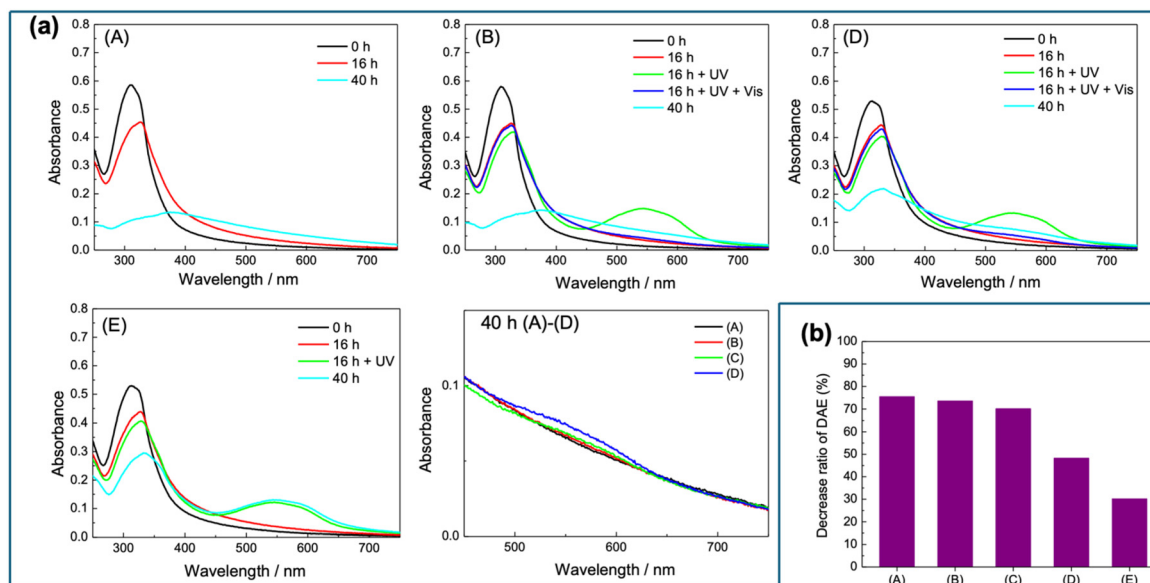






**Fig. 3** Demonstration of micropatterned irradiation of cell culture. UV irradiation area (a), green light irradiation area (b), and experimental result (c).

to water. Fig. 4(b) shows the decrease ratio (**1** (residual ratio)) of DAE in the solution estimated from the absorbance decrease. Even under condition (B), in which the samples underwent UV irradiation followed by green light irradiation 5 min later, the decrease ratio 40 h after the addition of **1o** was similar to that under condition (A), in which no irradiation was performed. However, we observed a decrease as the interval to green irradiation ( $t_i$ : time that most DAEs remain in the **1c** state) increases. In our previous study, we observed that **1c** intercalates with DNA when added to aqueous DNA solution, thereby stabilizing the dispersion and preventing precipitation, whereas **1o** hardly intercalates at all. From this finding, it was inferred that the experimental results shown in Fig. 4(b) were due to the gradual intercalation of DAE into DNA during ring closure, which improved dispersion stability in an aqueous system. Under condition (D), where  $t_i = 120$  min, the rate of decrease of DAE from the solution during the 22 hours following green light irradiation was predominantly smaller than that under conditions (A) and (B). In previous studies, we have observed that **1c**-incorporated cells show sensitivity (dose-dependent damage) to blue light at a wavelength of 436 nm and that green light irradiation,<sup>10</sup> which should cause **1c** ring opening, does not reduce this sensitivity. All of these results suggest that once DAE is intercalated into DNA, DAE is not expelled from inside the DNA by green light irradiation but remains there. Furthermore, the timescale for the decrease of **1o** from the DNA solution was on the order of 1 h, consistent with that of the  $t_i$  for DAE to reduce cell viability. As  $t_i$  lengthened, the slight absorbance bulge around the wavelength of 550 nm, which is attributed to **1c**, gradually increased even after green light irradiation (Fig. 4(a) “40 h (A)–(D)”). This suggests that a small fraction of DAE intercalated into DNA in



**Fig. 4** UV-vis absorption spectra of DNA–DAE solutions (D-PBS/DMSO = 95 : 5 (v/v), DNA: 75.8  $\mu$ Mbp, and DAE: 25  $\mu$ M) at various stages of light irradiation and time lapse schedules of conditions (A)–(E) (a) and the decrease ratio of DAE (loss from the solution due to precipitation) under different irradiation conditions (b).



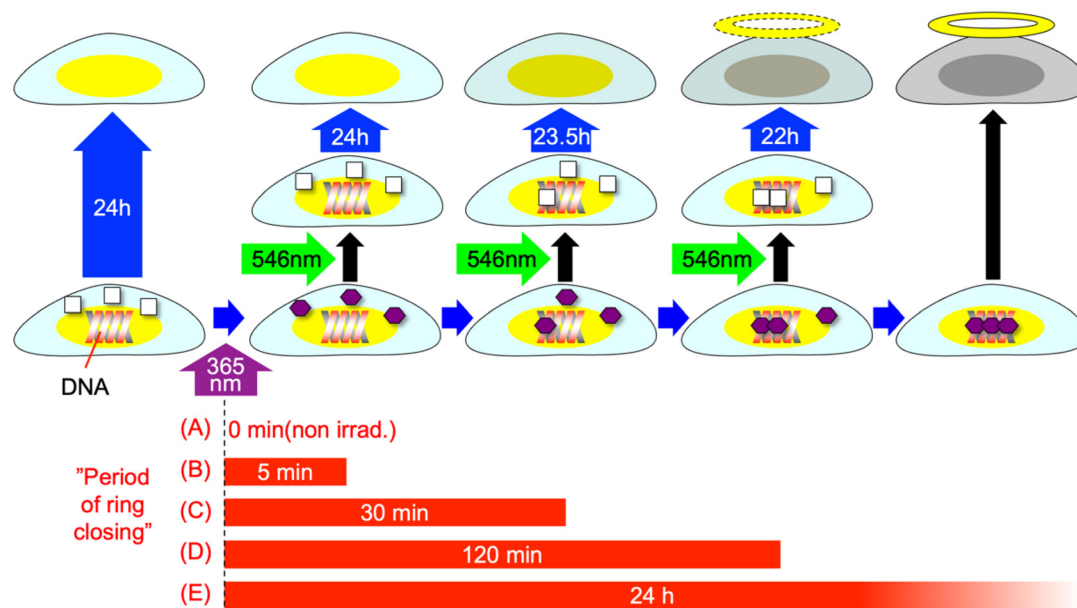


Fig. 5 Schematic illustration of the mechanism of decreased viability of cells after UV irradiation.

the **1c** state was strongly stabilized inside the DNA, making it less likely to become ring-open.

Based on the above results, we have considered the following mechanism for the decreased viability of the cells after 10 s of UV irradiation (Fig. 5): DAE, which is taken up by cells as **1o** and converted to **1c** by light, is gradually transferred into the DNA on a timescale of 1 h in the cells due to its planar structure (ESI<sup>†</sup>). This, like other DNA intercalators, causes dark toxicity and dooms the cells to a decreased viability after about 1 day. The mechanisms of cell death due to doxorubicin, one of the DNA intercalators used for anticancer drugs, are complex and are not fully understood but topoisomerase II poisoning is considered to be one of the mechanisms.<sup>16–18</sup> If a similar mechanism works for **1c**, the slow response of the dark toxicity of **1c** may be explained. However, even **1c** that is converted from **1o** in the cells by UV irradiation, if done prior to transfer to DNA, will return to the **1o** state almost stoichiometrically by green light irradiation and will not damage the cells.

## Conclusions

We introduced DAE, a molecular machine that exhibits photoisomerization, into cultured cells and demonstrated that its intercalation ability can be switched on by external light irradiation for as little as 10 s, thus dooming the cells to remarkable cell death after 1 day. This new scheme of photo-induced cell death stands in contrast to the dose-dependent phototoxic systems reported for PDT-related technologies as well as our previous work. In particular, dissolved oxygen plays an important role in the photosensitization-based systems used in PDT,<sup>5</sup> but in many cases cancer tissue is placed in an

anaerobic environment, which has been identified as a problem that reduces the efficiency of PDT.<sup>19</sup> However, the scheme presented in this study, in which light is used to switch dark toxicity, can avoid such problems. Although major issues, such as the applicable concentration and wavelength of irradiating light, still need to be overcome for clinical application, our approach is expected to provide a new tool for controlling biosystems *in vitro* with light.

## Experimental

### Materials and apparatus

The DAE used in this study was synthesized and purified by methods given in the existing literature.<sup>12</sup> The HeLa cell line derived from human cervical adenocarcinoma was purchased from the Riken Cell Bank (no. RCB0007). Tissue culture polystyrene dishes (IWAKI no. 3000-035, AGC Techno glass Co., Ltd), minimum essential medium (MEM, no. 051-07615, Wako Pure Chemical Industries, Ltd) and FBS were used for cell culture. Light irradiation of the cell culture system was carried out using a PC-controlled micro-projection system (DESM-01, Engineering System Co.) installed in an inverted research microscope (IX70, Olympus Co.).<sup>20,21</sup> DNA was obtained from salmon testes-derived DNA (sodium salts of DNA from salmon testes, average molecular weight:  $1.3 \times 10^6$  ca. 2000 bp, SIGMA). For UV irradiation, CL-1503 and CL-H1-365-9-1-B from Asahi Spectra Co. Ltd. were used. For green light irradiation, an AC-powered LED hand light, LED-EXHD/RFP (center wavelength: 540 nm) from Optocode Corporation, was used. A Hitachi spectrophotometer, UH-4150, was used to record absorption spectra in solution.



## Experimental methods

**Effects of light irradiation conditions on the expression of cytotoxicity.** Experiments were performed under three conditions (0, 5, and 10 ppm) for DAE coexistence concentration and five conditions ((A)–(E)) for the light irradiation schedule shown in Table 1, resulting in a total of 15 conditions (Fig. 2(a)). Here,  $2 \times 10^5$  HeLa cells were dispersed in a 2 mL culture medium containing a predetermined concentration of **1o** and seeded into 35 mm diameter culture dishes with a  $10 \text{ cm}^2$  culture area. The cells were incubated for 16 hours, and then micrographs of the cells were taken. After the culture medium was replaced with a DAE-free one for conditions (B)–(E), a particular rectangular area of  $1.4 \text{ mm} \times 1 \text{ mm}$  was irradiated with UV light of 365 nm wavelength and  $410 \text{ mW cm}^{-2}$  intensity for 10 seconds to convert **1o** to **1c**. For conditions (B)–(D), after incubation for a predetermined time ( $t_i = 5 \text{ min}$  (B), 30 min (C), and 120 min (D)), the previously UV-irradiated area was irradiated with green light of 546 nm wavelength and  $470 \text{ mW cm}^{-2}$  intensity for 30 s to convert **1c** to **1o**. Twenty-four hours after the first cell observation, the cells were lightly flushed with culture medium to remove detached cells and microscopic observation of the cells was performed again. Next, the number of live cells in a given area ( $0.6 \text{ mm} \times 0.75 \text{ mm}$ ) was counted in the first and second observation images ( $N = 4$ , see the ESI†).

**Demonstration of cell patterning by photoswitching of toxicity.** HeLa cells were dispersed in a culture medium containing 10 ppm **1o** at a density that would make them confluent the next day, and then they were seeded in 35 mm diameter culture dishes. After 18 hours of incubation, the culture medium was replaced with a DAE-free one, and for a certain rectangular area of  $1.7 \text{ mm} \times 3.2 \text{ mm}$ , UV light irradiation of 365 nm wavelength and  $75 \text{ mW cm}^{-2}$  intensity was conducted for 20 seconds to convert **1o** to **1c**. Immediately thereafter, green light irradiation of 546 nm wavelength and  $80 \text{ mW cm}^{-2}$  intensity was carried out for 20 s along the character pattern “546” ( $1.3 \text{ mm} \times 2.8 \text{ mm}$ ) inside the previously irradiated area to convert **1c** to **1o**. Twenty-four hours later, the detached cells were removed by gently flushing with culture medium, and the cells were observed under a microscope.

**Intercalation of DAE into DNA.** In this process, 57 mL of D-PBS buffer solution, 3.0 mg of DNA (sodium salts of DNA from salmon testes), and 1.5 mL of DMSO were mixed in a 100 mL sample tube and stirred at room temperature for 24 hours. This solution was added in 3.9 mL portions to each 9 mL sample tube, to which 100  $\mu\text{L}$  of 1 mM **1o** or **1c** DMSO solution was added to prepare the DNA–DAE solution (D-PBS/DMSO = 95 : 5 (v/v), DNA: 75.8  $\mu\text{M}$  (bp), DAE concentration: 25  $\mu\text{M}$ ). Light irradiation of the **1c**-added system was performed immediately after the addition of **1c**, and for conditions (B)–(D), to convert **1o** to **1c**, light irradiation of 365 nm wavelength and  $200 \text{ mW cm}^{-2}$  intensity was performed for 10 s to convert **1o** to **1c**. The DNA solution was kept in the dark for a predetermined time ( $t_i = 5 \text{ min}$  (B), 30 min (C), and 120 min (D)) and then irradiated with green light of 540 nm

wavelength and  $10 \text{ mW cm}^{-2}$  intensity for 2 minutes to convert **1c** to **1o**. In both cases, absorbance spectra were recorded 40 h after the introduction of **1o** into the DNA solution.

## Author contributions

KS, SY and KU designed and directed the project. NM performed DNA experiments. MK performed cell experiments. KS wrote the original draft.

## Data availability

The data that support the findings of this study are available from the corresponding authors (KS and KU) upon reasonable request.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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