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A Photochromic ATP Analogue Driving a Motor Protein with Reversible Light-Controlled Motility: Controlling Velocity and Binding Manner of a Kinesin/Microtubule System in an *In Vitro* Motility Assay

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ABSTRACT

In this study we synthesized two photochromic ATP analogues (ATP-Azos) featuring azobenzene derivatives tethered to the 2' position of the ribose ring of ATP. In the presence of the ATP-Azo featuring a *p-tert*-butyl group on the azobenzene moiety, we observed reversible photo-control of the motility, velocity, and binding manner of a kinesin/microtubule system upon irradiation with UV or visible light in an *in vitro* motility assay.

TEXT

Adenosine 5'-triphosphate (ATP) is a biogenic molecule that functions as an energy donor and interacts with various proteins, ATPases, within an organism. To investigate and artificially control the functions of ATPases, several ATP analogues that respond to stimulation with light, including fluorescently labeled ATPs^[1] and caged-ATPs,^[2] have been developed. The developed caged-ATP system is unique in that the hydrolysis of ATP mediated by an ATPase is initiated by irradiating with UV light to cleave the *o*-nitrophenyl protecting group of the γ -phosphate unit of the ATP derivative. Because this photo-cleavage process is irreversible, it is impossible to modulate the rate of the hydrolysis with desired timing once the protecting group has been released. An ATP analogue having the potential to reversibly photo-control the functions of ATPases would be a promising molecule to control various biochemical phenomena. In this regard, an ATP analogue conjugated to a photochromic unit that could undergo reversible conformational changes would be a candidate molecule for the reversible control of ATPase activity under irradiation with light. Azobenzene, a well-known

photochromic molecule that exhibits reversible conformational changes upon irradiation with light of various wavelengths, is often applied as a photo-controllable mediator of the functions of proteins^[3] and synthetic molecules, including molecular machines,^[4] host molecules,^[5] metal complexes,^[6] liquid crystals,^[7] gels,^[8] and polymers.^[9] The photo-control of protein functions has been studied using azobenzene derivatives conjugated to peptides or proteins; there have been few studies, however, of the development of small-ligand nucleotides connected to azobenzene derivatives.^[10]

In this present study, we synthesized azobenzene derivatives of ATP—so-called “ATP-Azos”—as a novel class of ATP analogues. Herein, we demonstrate the reversible and repeatable photo-control of a motile property of kinesin, a kind of ATPase motor protein, through an *in vitro* motility assay performed in the presence of an ATP-Azo.

We designed our ATP analogues such that a single azobenzene moiety would be appended to the ribose ring of the ATP unit, in consideration of the fact that various ATP analogues modified at these positions can still interact with ATPases.^[1,11] We synthesized ATP analogues tethering an azobenzene or *p-tert*-butylazobenzene moiety at the 2' position of the ribose ring (Scheme 1) using the approach outlined in Scheme 2 (for details, see “Syntheses of Compounds” in the Supporting Information).^[12] This synthetic pathway allowed us to modify ATP only at the 2' position of the ribose ring, thereby avoiding any potential confusion, due to the presence of 2' and 3' isomers, when interpreting the experimental results^[13] and eliminating any possible contamination of ATP, in contrast to other syntheses starting from ATP. We obtained the resultant ATP-Azos, **1a** and **1b**, as their sodium salts after sequential anion and cation ion-exchange chromatographies.^[14]

We examined the photoisomerization behavior of these ATP-Azos through alternating irradiation with UV light ($\lambda = 365$ nm), inducing *trans*-to-*cis* isomerizations of the azobenzene moieties of the ATP-Azos, and visible light ($\lambda = 436$ nm), inducing their reverse *cis*-to-*trans* isomerizations [Scheme 1, Figure 1 (presenting the example for **1b**)]. The ratios of *trans*-**1b** to *cis*-**1b** in the photostationary states upon irradiation with UV and visible light were 8:92 and 86:14, respectively, estimated from analyses of both NMR (Figure S1, Supporting Information) and UV–Vis absorption (Figure 1) spectra.

We used an *in vitro* motility assay of kinesin^[15] to assess the properties of the ATP-Azos in their different isomeric states as substrates of ATPase, thereby allowing the ATPase activity to be visualized in terms of dynamic movements through imaging techniques. In the *in vitro* motility assay, we confirmed that fluorescent-labeled microtubules (MTs)^[16, 17] were driven by recombinant kinesin (kinesin-1) molecules coated on the glass surface in the presence of ATP-Azos (added instead of ATP); this approach allowed us to evaluate the motility behaviors of the MTs (see “Observation of Motility of Kinesin-Microtubule System,” Supporting Information).

Table 1 and Figure 2 display the sliding velocities of the MTs driven by the kinesin molecules at various concentrations of the ATP-Azos in their *trans* and *cis* forms. The average velocities of the MTs at 1 mM of *trans*-**1a** and *cis*-**1a** were 372 ± 7 and 383 ± 7 nm/s, respectively—approximately 60% of the value (ca. 660 nm/s) obtained for ATP at 1 mM, which was the sufficient concentration of ATP for saturating the sliding velocity of the MTs (Table 1). At 100 μ M of **1a**, a concentration close to the value of K_m^{ATP} (85 μ M for ATP, obtained through our motility assay; see Figure S2, Supporting Information), we recorded velocities of 179 ± 4 and 175 ± 3 nm/s for *trans*-**1a** and *cis*-**1a**, respectively—approximately 45% of the value for ATP (ca. 390 nm/s; Table 1). Thus, *trans*–*cis* isomerization of **1a** did not significantly affect the sliding velocity of the MTs. In contrast, for **1b**, which features a *tert*-butyl group at the azobenzene terminus, we observed a significant difference in the sliding velocities of the MTs before and after irradiation with UV light. The average velocity of the MTs at 100 μ M *trans*-**1b** was 75 nm/s [ca. 20% of the velocity at 100 μ M ATP (ca. 390 nm/s); Table 1, Figure 2a]. After converting *trans*-**1b** to *cis*-**1b** through irradiation with UV light ($\lambda = 365$ nm) of the chamber for the *in vitro* motility assay, the velocity reached 117 nm/s (an increase of 56%; Table 1, Figure 2a). Subsequent irradiation with visible light ($\lambda = 436$ nm) induced the reverse *cis*-to-*trans* isomerization of **1b**, resulting in the velocity decreasing to 76 nm/s—almost the same value as that obtained initially prior to UV irradiation (Figure 2a). We also observed this tendency—an increase in velocity after irradiation with UV light and a decrease after irradiation with visible light—at other concentrations of **1b**. When the concentration of *trans*-**1b** was too high (e.g., Figure 2a, without irradiation, 1 mM; with Vis irradiation, 650 μ M or 1 mM), we could not

measure the velocities of the MTs because they were not bound to the kinesin-coated glass surface and drifted in the assay buffer (see below for further details of this phenomenon).

To confirm the reproducibility and repeatability of the changes in the sliding velocities of the MTs upon irradiation with light, we inverted the order of irradiation sequence. We observed the same tendency in the velocity changes as mentioned above when subjecting *cis*-**1b** (“UV 1st”, Figure 2b), itself obtained after irradiation at 365 nm, to irradiation at 436 nm, transforming it into *trans*-**1b** (“Vis”, Figure 2b) with an accompanying decrease in the MT velocity, followed by irradiation at 365 nm to reform *cis*-**1b** (“UV 2nd”, Figure 2b), accompanied by an increase in MT velocity. We could not measure the velocity of the MTs at 1 mM *trans*-**1b** (Figure 2b, Vis) for the same reason mentioned above.

Irradiation with UV light had no effect on the kinesin-induced motilities of the MTs in the presence of ATP (i.e., not the ATP-Azos), as revealed in a control experiment (see Figure S2, Supporting Information). Therefore, our observation of a different sliding velocity for each isomeric state of **1b** clearly reveals that the action of ATPase could be controlled dynamically through irradiation with light in the presence of the photochromic ATP analogue. We did not observe any significant changes in the sliding velocities of the MTs before or after UV irradiation of **1a**, which lacks the terminal *tert*-butyl group on the azobenzene moiety (Table 1). Thus, the nature of the substituent on the azobenzene unit of the ATP-Azo is a crucial factor affecting the photo-controlled functions of ATPases. The presence of the hydrophobic group presumably enhanced the difference in one or more of the properties (e.g., binding affinity, hydrolysis rate) of the *trans* and *cis* states of **1b** as substrates for an ATPase, relative to that of **1a**, ultimately resulting in observable changes in the motile velocities of the kinesin/MT/**1b** system.

As mentioned above, the sliding velocity of the MTs was clearly photo-controllable in the presence of **1b** at or below 300 μ M (Figure 2). At high concentrations of **1b**, however, we observed interesting behavior for the interactions between the MTs and the kinesin units coated on the glass surface, providing another approach toward photo-controlling the motility of the kinesin/MT system, as described below.

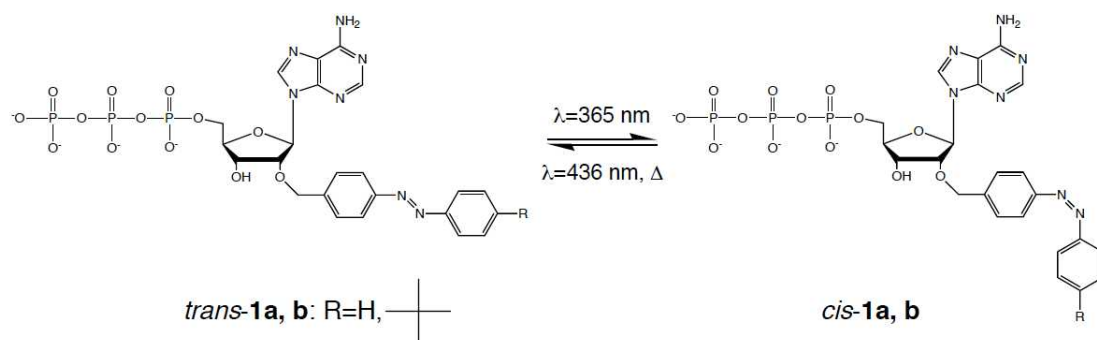
At 650 μM of *trans*-**1b**, the number of MTs attached to the glass surface was less than that at or below 300 μM ; these MTs tended to glide on the kinesin-coated glass surface in a discontinuous manner or detach from the surface. In the presence of 1 mM of *trans*-**1b** in the buffer, we observed only drifting MTs—that is, none were bound to the kinesin units coated on the glass surface (Figure 3a). After converting *trans*-**1b** into *cis*-**1b** through irradiation with UV light ($\lambda = 365\text{ nm}$), the MTs were bound to and slid on the kinesin-coated glass surface (Figure 3b). Subsequent sequential irradiation with visible ($\lambda = 436\text{ nm}$) and UV ($\lambda = 365\text{ nm}$) light resulted in the MTs detaching from (Figure 3c) and reattaching to (Figure 3d) the glass surface, respectively. In contrast, for **1b** at or below 300 μM , we did not observe any clear difference in the attachment behavior of the MTs in the presence of *trans*-**1b** or *cis*-**1b** (see Figure S3, Supporting Information).

Although the exact mechanism of such attachment and detachment remains unclear, this phenomenon can be explained by considering that **1b** functions as a photoresponsive inhibitor of the intermolecular interactions between kinesin and the MTs in addition to behaving as a photoresponsive energy source when it is a substrate of ATPase kinesin. The amphiphilic nature of **1b**, with its hydrophilic triphosphate unit and hydrophobic *p*-*tert*-butylazobenzene moiety, suggests that it might form a complex with proteins via hydrophobic interactions, forming a triphosphate-presenting surface, at sufficiently high concentrations in water. We speculate that the formation of such complexes between *trans*-**1b** and either kinesin or the MTs inhibited the binding between the MTs and the kinesin units. The tendency of **1b** to form such complexes was confirmed by the observation of turbid buffer solutions when we performed the motility assay with *trans*-**1b** at 1 mM in the presence of proteins (e.g., casein). The same solution became to transparent after irradiation with UV light induced the photoisomerization of *trans*-**1b** to *cis*-**1b**; no such turbidity appeared for *trans*-**1b** at a concentration equal to or less than 300 mM.

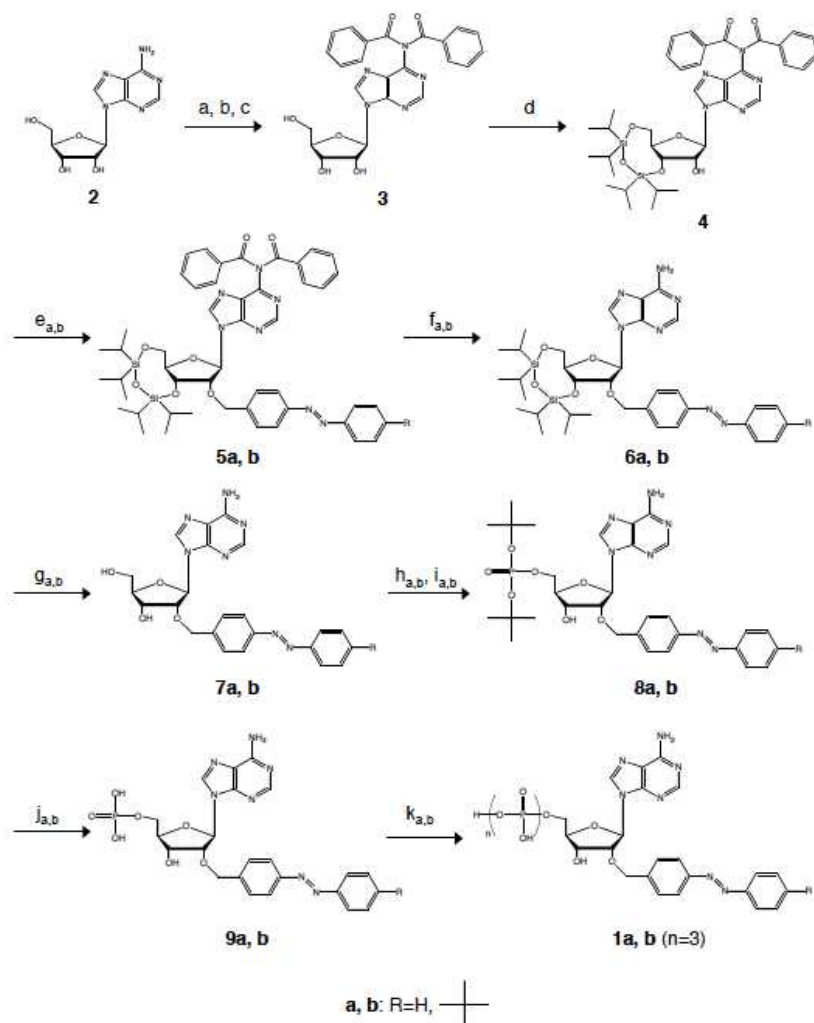
The reversible and repeatable detachment and attachment of the MTs on the glass surface coated with kinesin units in the presence of **1b** at 1 mM can be regarded as a novel photoswitch for controlling the motility of kinesins.

In conclusion, we have realized reversible photo-control of the motility of MTs driven by kinesins in the presence of a novel ATP analogue, ATP-Azo **1b**. We anticipate that this technique might find wide applicability in nanobiotechnology, particularly in regard to the growing utility of bio-nanomotors.^[18]

ATP-Azo derivatives, prepared with a simple strategy of, for example, changing only the substituent at the terminus of the tethered azobenzene unit, have potential applications when combined with other ATPases. Furthermore, replacing ATP with another nucleotide triphosphate might allow related systems to find applicability with NTPases. For example, GTP-Azos might be particularly suitable candidate molecules for photo-controlling GTPases because some GTPases and ATPases, with GTP hydrolyzing G-proteins and ATP hydrolyzing motor proteins, have structures and hydrolysis mechanisms similar to those of “switch” proteins.^[19]



Scheme 1. Photoresponsive ATP analogues 1a and 1b. Reversible *trans* (left)/*cis* (right) isomerizations of the azobenzene and *p*-*tert*-butylazobenzene moieties in **1a** and **1b**, respectively.



Scheme 2. Synthesis of ATP-Azo 1a and 1b. a) TMS-Cl, dry pyridine, rt, 4.5 h; b) benzoyl chloride, ca. 0 °C, rt, 3 h; c) H₂O, ca. 0 °C, rt, 1 h, 81%; d) TIPDS-Cl, dry pyridine, rt, 8 h, 76%; e) 4'-bromomethylazobenzene (**a**) or 4-*tert*-butyl-4'-bromomethylazobenzene (**b**) (see Scheme S1, Supporting Information), NaH, dry DMF, –20 to ca. 0 °C, >4.5 h, 25% (**a**) or 27% (**b**); f) NH₄OH, dioxane, ca. 0 °C, rt, overnight, 78% (**a**) or 46% (**b**); g) TBAF, dry THF, ca. 0 °C, 1.5 h, 88% (**a**) or 93% (**b**); h) di-*tert*-butyl *N,N*-diisopropylphosphoramidite, ¹H-tetrazole, dry DMF, rt, >3 h; i) MCPBA, ca. 0 °C/55 min then rt/25 min, 75% (**a**) or 60% (**b**); j) trifluoroacetic acid, dry CH₂Cl₂, rt, >6 h, quant.; k) phosphoric acid, DCC, tri-*n*-butylamine, dry pyridine, rt, >2 days, 10% (**a**) or 8.8% (**b**).

Concentration	Sliding velocity of MTs				
	[average $\pm$ s.e.m. (nm/s), n = 15–50]				
	*estimation (see Figure S2, Supporting Information)				
	<i>trans-1a</i>	<i>cis-1a</i>	<i>trans-1b</i>	<i>cis-1b</i>	ATP
1 mM	372 $\pm$ 7	383 $\pm$ 7	nd	236 $\pm$ 14	656 $\pm$ 26
100 μ M	179 $\pm$ 4	175 $\pm$ 3	75 $\pm$ 3	117 $\pm$ 3	ca. 390*

Table 1. Sliding velocity of the MTs in the presence of each substrate

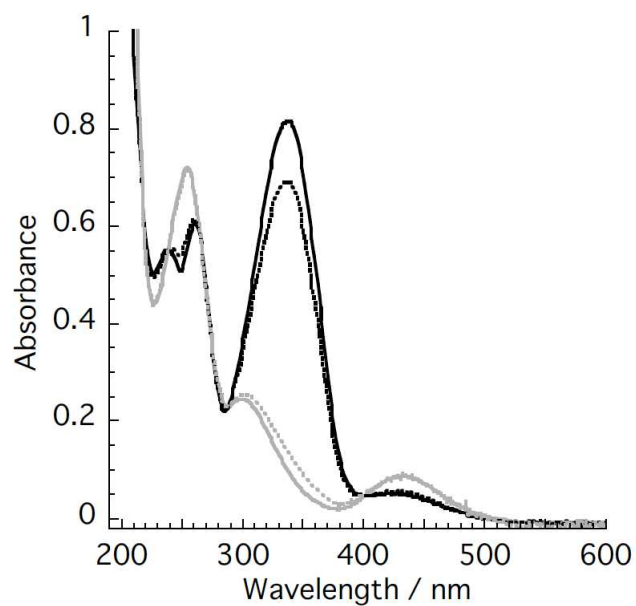


Figure 1. Photoisomerization of **1b** (32.5 μ M) at 23.5 ± 0.5 $^{\circ}$ C in water. Spectral changes of **1b** before (black solid line) and after (gray solid line) irradiation (1 min) with UV light, followed by irradiation with visible light (black dotted line). Thermal recovery after 270 min in the dark is revealed by the gray dotted line.

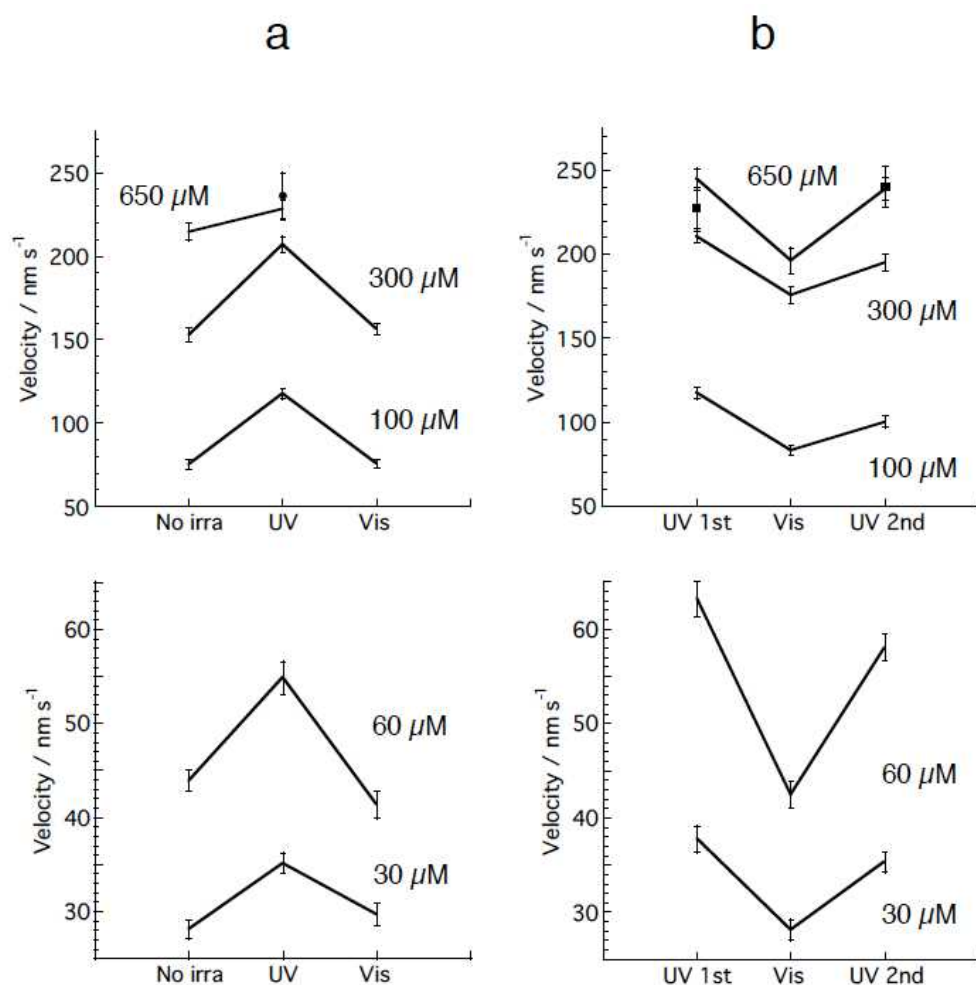


Figure 2. Photoresponsive sliding velocities of MTs driven by kinesins coated on glass at various concentrations of **1b**. The concentration of **1b** corresponding to each curve is presented, except for 1 mM concentration [circles for (a), squares for (b)]. The error bars represent standard errors of the mean. (a) “No irra”: sliding velocities of MTs prior to irradiation; “UV”: irradiation with UV ($\lambda = 365$ nm) light after “No irra”; “Vis”: irradiation with visible ($\lambda = 436$ nm) light after “UV.” MTs bound to the glass surface were not observed in following cases: Vis for 650 μ M; No irra for 1 mM ($n = 50$ for 30 μ M to 300 μ M; $n = 35$ for 650 μ M; $n = 11$ for 1 mM) (b) “UV 1st”: Sliding velocities of MTs upon irradiation with UV light; “Vis”: irradiation with visible light after “UV 1st”; “UV 2nd”: irradiation with UV light after “Vis.” MTs bound to the glass surface were not observed in the following case: 1 mM, Vis [$n = 50$ for 30 μ M to 300 μ M; $n = 35$ (UV 1st), 12 (Vis), 18 (UV 2nd) for 650 μ M; $n = 17$ (UV 1st) or 8 (UV 2nd) for 1 mM].

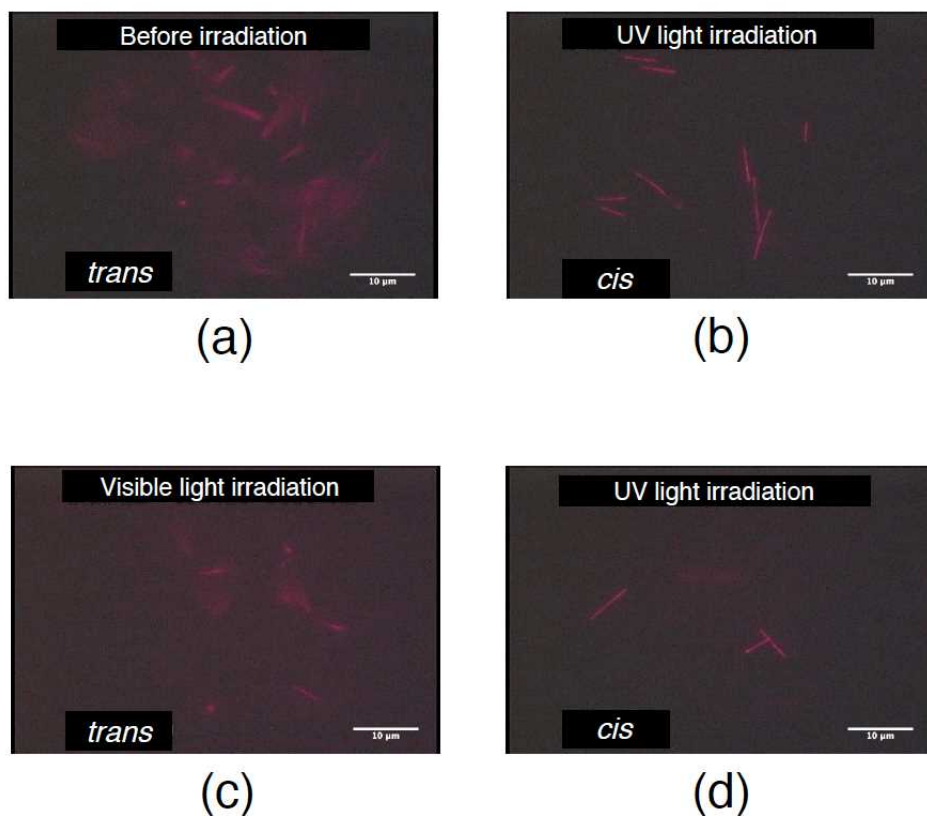


Figure 3. Photo-responsive interactions between MTs and kinesins coated on the glass in the presence of 1 mM **1b**. (a) Prior to irradiation. (b) Irradiation with UV ($\lambda = 365$ nm) light after (a). (c) Irradiation with visible ($\lambda = 436$ nm) light after (b). (d) Irradiation with UV light after (c). Scale bar: 10 μm .

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