

# How to control proteins with light in living systems

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**The possibility offered by photocontrolling the activity of biomolecules *in vivo* while recording physiological parameters is opening up new opportunities for the study of physiological processes at the single-cell level in a living organism. For the last decade, such tools have been mainly used in neuroscience, and their application in freely moving animals has revolutionized this field. New photochemical approaches enable the control of various cellular processes by manipulating a wide range of protein functions in a noninvasive way and with unprecedented spatiotemporal resolution. We are at a pivotal moment where biologists can adapt these cutting-edge technologies to their system of study. This user-oriented review presents the state of the art and highlights technical issues to be resolved in the near future for wide and easy use of these powerful approaches.**

To decipher a complex biological process, one needs tools to perturb the various actors involved to gain information about the important parameters. In this context, light seems like a very attractive actuator to perturb a system with high spatiotemporal resolution. The gain in resolution when compared to classical genetic or pharmacologic perturbations results from the trigger itself—light illumination—which can be controlled with millisecond and submicron resolutions<sup>1</sup> (Fig. 1a).

The specific perturbation of a biological process implies the ability to photocontrol its actors, which are typically proteins. To achieve this goal, one can first directly act at the protein level and photoinduce a change of its activity (Fig. 1b). This requires the presence of a light-sensitive module, which may already exist within the protein core (as in photoreceptors) or be attached, genetically or chemically, to the protein of interest. Apart from these *cis*-acting elements, light-activable *trans*-activators can also control a protein function by releasing an agonist or antagonist of a particular protein. A second option is to control the intracellular concentration of a protein of interest by inducing its production with light (Fig. 1b). Photocontrol of protein synthesis can be obtained using light-gated transcription factors, for instance. Alternatively, cellular protein concentration can be light-controlled by photoinducing protein degradation (Fig. 1b). When the function of a protein depends on its cellular location, a fourth strategy is to photoinduce its relocation (Fig. 1b). The choice between these different options depends on several parameters, such as the time resolution required to match the timing or rate of the process under study: a change in protein activity occurs much faster when it results from a conformational change of the protein rather than its synthesis or degradation (Fig. 1b); these considerations have to be kept in mind when choosing the appropriate method.

This user-oriented review aims to give hints on how to control proteins with light in living systems. Our presentation is far from exhaustive but gives a general overview of the cutting-edge approaches, distinguishing the genetically encodable photoactuators, which arose from the optogenetics field<sup>1</sup>, from hybrid approaches that take advantage of exogenous synthetic light-sensing molecules. We end this review by discussing the problems to be aware of and the challenges to be addressed.

## Genetically encodable photoactuators

To turn a protein into a light-responsive entity, one can rely either on a light-sensing molecule already present in the protein or on the addition of a light-sensitive module. To implement these ideas, natural photoreceptors such as rhodopsins, flavoproteins and phytochromes have been turned into genetically encodable optical actuators. In most cases, under illumination, the associated chromophore isomerizes and induces an overall conformational change of the protein (Fig. 2a–f), which can be rerouted to manipulate protein activity.

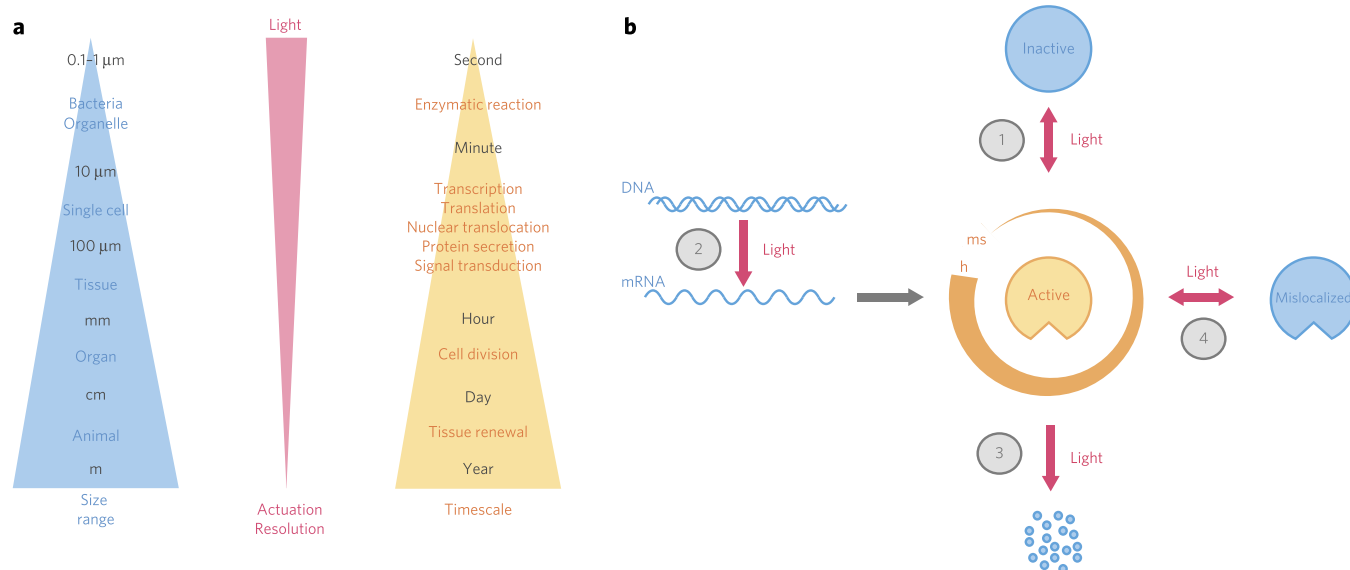
The first optogenetic tools exploited rhodopsins, which consist of a chromophore, retinal or one of its derivatives (Fig. 2d) bound to a seven-transmembrane protein. Upon illumination, the bound retinal molecule undergoes isomerization, which induces conformational changes in the opsin backbone (Fig. 2a,b). Rhodopsins are found across all kingdoms of life and fulfill a diverse range of functions.

In type I rhodopsins (microbial opsins), the chromophore is covalently linked to the protein, which allows for very rapid thermal resetting<sup>2</sup> following photoactivation. Some rhodopsins act as ion channels or pumps and are used to induce cell polarization<sup>3</sup> or depolarization<sup>4</sup> to control neuronal activity (Fig. 2a). Others are linked to enzymatic activity that is tunable by light<sup>5</sup>. Channelrhodopsins conduct cations and depolarize neurons upon blue light illumination, leading to neuronal activation<sup>4</sup>. Conversely, halorhodopsins pump chloride ions into the cytoplasm upon yellow light illumination, leading to hyperpolarization and inhibition of neuronal activity in adults<sup>6</sup>.

Type II rhodopsins are G protein-coupled receptors (GPCRs) found only in higher eukaryotes. Unlike type I rhodopsins, the chromophore detaches from the protein upon isomerization, and a new retinal must be recruited to reset the protein. Hence, the resetting time of type II rhodopsins is much longer than it is for type I<sup>2</sup>. Despite this drawback, the GPCR structure, which is common to many eukaryotic receptors, was attractive enough to prompt the development of chimeric receptors (collectively termed optoXRs; Fig. 2b), allowing the control by light of signal transduction via G proteins and thus the photocontrol of second messengers (such as cyclic AMP and InsP3) or ion channels<sup>7,8</sup>.

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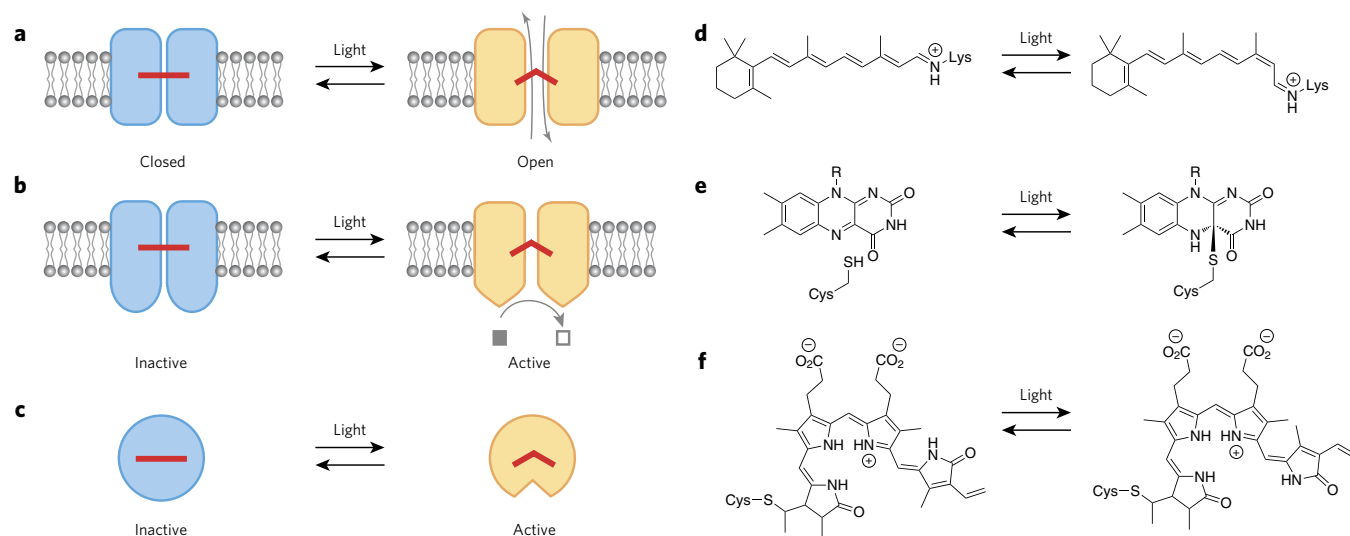


**Figure 1 | Using light to control proteins in living systems.** (a) The high spatiotemporal resolution of light actuation makes it possible to address biological processes with a wide range of temporality (from seconds for enzymatic reaction to days for tissue renewal) at various spatial scales (from less than micrometers for organelles to centimeters for animals). Upon increasing the illumination requirements (e.g., smaller field and/or shorter light pulse to access better resolution in space and time), it becomes possible to control proteins with light (actuation) at smaller spatial and temporal scales. (b) Light gives control over protein function either directly by changing its active state (1) or by modifying its effective concentration via its rate of synthesis (2), rate of degradation (3) or compartmentalization (4). The timing of the response varies from few milliseconds to hours, depending on the chosen method.

As the first use of these tools was in the control of neuronal activity *in vivo*, major efforts have been put into screening natural opsins and generating a variety of chimeric rhodopsin versions with various dynamic responses and light sensitivities<sup>2</sup> (Table 1). In the last five years, the study of freely moving animals in which the neuronal activity of specific cells can be controlled by light has provided truly unprecedented insights into neuronal connectivity and circuitry, cognition and behavior<sup>9–13</sup>. These tools have also been used in the field of developmental biology, enabling

precise mapping and control of the cardiac pacemaker<sup>14,15</sup> or the automated control of embryonic stem cell differentiation<sup>16</sup>.

These astonishing developments have motivated biologists to extend the optogenetic toolbox to soluble light-gated modules engineered from other natural light-sensitive proteins. Flavoproteins attracted particular interest because of their riboflavin-based chromophore, either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), which is naturally present in most cells. For some



**Figure 2 | Light control of proteins with genetically encoded photoactuators.** (a) Microbial rhodopsins can be expressed in neurons to regulate membrane potential; these proteins interact with the chromophore retinal, which is present in most cells. (b) The intracellular domain of vertebrate rhodopsins can be exchanged with the intracellular domain of specific GPCRs to photocontrol specific signaling cascade (IP<sub>3</sub>, DAG or cAMP). (c) Light-induced conformational changes in the LOV or in the CRY domain have been used to control protein localization, transcription or activity of a fused protein. (d) In rhodopsins, illumination drives isomerization of a double bond of the chromophore, thereby modifying its geometry. (e) In the case of the LOV domain, photoexcitation induces a covalent thioether bond between bound FMN and a highly conserved cysteine residue of the LOV domain. (f) Phytochromes contain a covalently bound chromophore (bilin or biliverdin). Upon exposure to light, isomerization of the chromophore induces a conformational change in the protein, modifying its interaction properties.

**Table 1 | Examples of cellular features controlled by genetically encoded photoactuators**

Light target	Light-gated module	Biochemical output	Targeted feature	$\lambda_1$ ( $\lambda_2$ ) (nm)	ALI <sub>1</sub> (ALI <sub>2</sub> ) (mW cm <sup>-2</sup> )	ID (s)	TR (s)	Ref no.
UVR8	VSVG-YFP-UVR8-UVR8	ER retention	Protein secretion	312	0.3	7	>3 × 10 <sup>4</sup>	28
Retinal animal opsin	NinaE* 'chARGe' GPCR	IP <sub>3</sub> , DAG	Neuron activation	White light	-	22	-	89
Retinal micr. opsin	ChR2 channel (Na <sup>+</sup> , K <sup>+</sup> ) <sup>a</sup>	Ion flux	Neuron activation	442	10 <sup>3</sup>	-	-	2,4
Retinal animal opsin	Rh-5HT1A GPCR	G-gated channel	Neuron activation	485	-	3	-	8
Retinal animal opsin	Rh-β2AR GPCR	cAMP	Signaling	504	500	6	-	7
Retinal animal opsin	Rh-α <sub>1</sub> AR GPCR	IP <sub>3</sub> , DAG	Signaling	504	500	6	-	7
Retinal micr. opsin	MChR1 channel (Na <sup>+</sup> , K <sup>+</sup> ) <sup>a</sup>	Ion flux	Neuron activation	531	-	2	-	90
Retinal micr. opsin	ChR1 channel (H <sup>+</sup> ) <sup>a</sup>	Ion flux	Neuron activation	500	400	0.2	-	91
Retinal micr. opsin	VchR1 channel (Na <sup>+</sup> , K <sup>+</sup> ) <sup>a</sup>	Ion flux	Neuron activation	589	1.4	1	-	92
Retinal micr. opsin	eNpHR3.0 pump (Cl <sup>-</sup> ) <sup>a</sup>	Ion flux	Neuron inhibition	590-660	0.4	-	-	93
Retinal micr. opsin	NpHR pump (Cl <sup>-</sup> ) <sup>a</sup>	Ion flux	Neuron inhibition	593	2,200	0.05	-	72
FAD CRY	CRY2-Gal4BD transcription factor	CIB1-Gal4AD binding	Transcription initiation	461-488	-	0.1	1,000	23
FAD CRY	CRY2-CreN recombinase	CIBN-CreC binding	Recombination	461-488	-	2	-	23
FAD CRY	Tale-CRY DNA binding	CIB1-VP64 binding	Transcription initiation	466	5	0.5	1,000	25
FAD CRY	Tale-CRY DNA binding	CIB1-Sin3IDx4 binding	Histone acetylation	466	5	0.5	1,000	25
FMN LOV2	DHFR(x2)-LOV2 DHFR	TH-folate synthesis	Nucleotide biosynthesis	White light	-	300	50	29
FMN LOV2	LOV2-TAP Trp repressor	DNA binding	Gene expression	470	20	30	40	21
FMN LOV2	Lov-Rac1 small GTPase	GTPase	Actin dynamics	458	-	300	43	94
FMN FKT1	FKT1-VP16AD transcriptional activator	GI-Gal4BD binding	Transcription initiation	450	0.3	300	>5,000	22
FMN FKT1	FKT1-Rac1 small GTPase	G1Cher-CAAX binding	Actin dynamics	450	0.3	300	>5,000	22
FMN LOV2	LOV2-degron-targeted protein	Ubiquitination	Protein degradation	465	0.8	15,000	100	33
FMN LOV	mPAC adenylate cyclase <sup>a</sup>	cAMP	Signaling	460	0.6	100	16	31
FAD VVD-LOV	Gal4-vivid transcription factor	DNA binding	Gene expression	460	0.1-1	80,000	8,000	24,26
FAD BLUF	bPAC adenylate cyclase <sup>a</sup>	cAMP	Signaling	455	0.5	1-10	12	30
Bilin PHY	PHY-Gal4BD transcript. factor	PIF-Gal4AD binding	Transcription initiation	664 (748)	0.02-0.2 (0.02-0.2)	<1	>10 <sup>3</sup>	20
Bilin PHY	PHY-Cdc42 small GTPase	PIF-WASP binding	Actin dynamics	656 (766)	-	-	-	32
Bilin PHY	PHY-mCherry-CAAX mb. anchor	PIF-YFP binding	Protein trafficking	650 (750)	0.4 (5)	<1/<4	-	19
Bilin PHY	PHY-mCherry-CAAX mb anchor	iSH-YFP-PIF PI3K activation	Signaling	650 (750)	-	30	-	27

<sup>a</sup>These microbial opsins were unmodified.  $\lambda_1$ , wavelength of activating light;  $\lambda_2$ , wavelength of deactivating light (for reversible systems); ALI<sub>1</sub>, activating light intensity; ALI<sub>2</sub>, deactivating light intensity (for reversible systems); ID, illumination duration for activation (ID<sub>1</sub>/ID<sub>2</sub> for reversible systems); TR, timescale for thermal resetting after activation. Asterisk denotes that, along with NinaE, it was necessary to express arrestin-2 and Gα. Micr., microbial.

of these enzymes, light activation induces the formation of a thiol adduct between the chromophore and a conserved cysteine residue (Fig. 2e), which triggers a marked modification of the protein structure (Fig. 2c). Three major flavoproteins were used: light-, oxygen- or voltage-sensing (LOV) proteins<sup>17</sup>; blue light-utilizing flavin (BLUF) proteins<sup>17</sup>; and the plant light-sensitive cryptochrome (CRY2)<sup>18</sup>. Among the other photoreceptors exploited to develop optogenetic tools, one can cite the plant phytochromes (PHYs), which make it possible to control with light the heterodimerization of proteins<sup>19</sup>.

The photochemical behavior of phytochromes depends on the light-catalyzed *cis-trans* isomerization of a bilin chromophore (Fig. 2f). A major attractive feature of this class of proteins is their activation with red light, which enables easier multiplexing. Another advantage is that infrared light can be used to regenerate the inactive state. By alternating red and infrared illumination, the active state can thus be turned on and off at will.

In all of the developed systems (LOV, BLUF, CRY2 and PHY), photoisomerization of the bound chromophore induces a

conformational change of the protein, which can be used to control the activity of a fusion protein either directly by unmasking a protein function through a conformational change or indirectly through the control of protein-protein interactions. This toolbox now offers the possibility to photocontrol a variety of cellular protein functions from chromatin modification to DNA transcription<sup>20–26</sup> or recombination<sup>23</sup>, protein translocation<sup>19,22,27,28</sup>, enzymatic activity<sup>29–31</sup>, cell morphology<sup>22,32</sup>, signaling pathways<sup>7,27,30,31</sup> and protein degradation<sup>33,34</sup> (Table 1). Recent works have focused on improving the response timescale and the signal-to-noise ratio and have expanded the spectrum of available wavelengths (Table 1), making multiplexing possible (Box 1 and Fig. 3).

### Chemical and genetic hybrid photoactuators

In parallel to the development of the toolbox described above, hybrid approaches have been developed that combine genetic

modifications of the protein of interest and the use of exogenous photoactive synthetic molecules. The interest of relying on synthetic light-sensitive molecules is that a large collection of photochemistries is available<sup>35</sup>. Various photolabile protecting groups can turn a biomolecule into a photoactivable entity<sup>36</sup>. These so-called caging groups temporarily block the interactions with biological partners and can be photolysed with very high spatiotemporal resolution to locally release the biologically active molecule. Photoswitchable synthetic platforms that can interconvert between two functional conformations upon illumination are useful alternatives when reversibility is needed.

In the context of the photocontrol of protein activity, various strategies have been developed to exploit the opportunities offered by caged and photoswitchable compounds, either as *trans*- or *cis*-actuators (Fig. 4a–c). The design of *cis*-actuators is, however, technically more challenging as it requires specific

### Box 1 | From light absorption to chromatically orthogonal photoactivation

Light control of protein activity originates from a photochemical reaction, which involves an effective interaction between the chromophore of a photoactive substrate and a reagent—the photon. The primary event is light absorption. It requires a match between the wavelength-dependent photon energy and the energy gap associated with the transition of the chromophore from a ‘ground’ to an ‘excited’ state, which can be compared to an electronic isomer: light within the wavelength range of 200 nm to 700 nm enables photoactivation of the organic chromophores driving protein photocontrol (Fig. 3a). The excited state subsequently releases its excess energy, yielding a photoproduct. Depending on the involved photochemistry, the photoactive substrate may yield a stable (irreversible photoactivation) or an unstable (reversible photoactivation) photoproduct. In the latter case, the photoactive substrate is regenerated, and several cycles of photoactivation can be applied.

The light source delivering photons is characterized by intensity  $I$ , quantified either by its power (measured with a power-meter in W) or by the number of photons emitted per unit of time (in mol photons per second or, equivalently, in Ein s<sup>−1</sup>), accessible by actinometry<sup>95</sup>. Conversion between both quantifications is easily performed upon considering that the energy contained in a single photon of wavelength  $\lambda$  is equal to  $hc/\lambda$ , where  $h$  is the Planck constant and  $c$  is the speed of light in a vacuum. At the sample, illumination is characterized by a flux  $I_s$  (in Ein s<sup>−1</sup> m<sup>−2</sup> or W m<sup>−2</sup>), which can be obtained upon dividing  $I$  by the surface  $S$  collecting the light. In epifluorescence microscopy with one-photon excitation, fluxes of 10<sup>−1</sup>–10<sup>3</sup> mW cm<sup>−2</sup> are typically applied, which correspond to 4 × 10<sup>−6</sup>–4 × 10<sup>−2</sup> Ein s<sup>−1</sup> m<sup>−2</sup> when exciting at 500 nm.

The ability of a chromophore to absorb a photon is quantified by its absorption cross-section  $\sigma$  (for a strongly absorbing organic chromophore,  $\sigma \sim 4$  Å<sup>2</sup>, close to the chromophore dimensions). A light beam of initial intensity  $I_0$ , propagating a distance  $l$  in a solution of chromophores (at concentration  $n$  molecules dm<sup>−3</sup> or  $C = n/N_A$  (in mol dm<sup>−3</sup> or mol l<sup>−1</sup> or M), where  $N_A$  is Avogadro’s number), will have its intensity dimmed owing to absorption by a factor  $e^{-\sigma nl} = 10^{-\epsilon Cl}$ , where  $\epsilon = N_A \sigma / 2.3$  is the molar absorption coefficient expressed in M<sup>−1</sup> cm<sup>−1</sup> (ref. 96). For a strongly absorbing chromophore with  $\sigma \sim 4$  Å<sup>2</sup>;  $\epsilon = 10^5$  M<sup>−1</sup> cm<sup>−1</sup>. The probability to yield a photoproduct following light absorption is the dimensionless quantum yield  $\phi$  of the photochemical reaction.

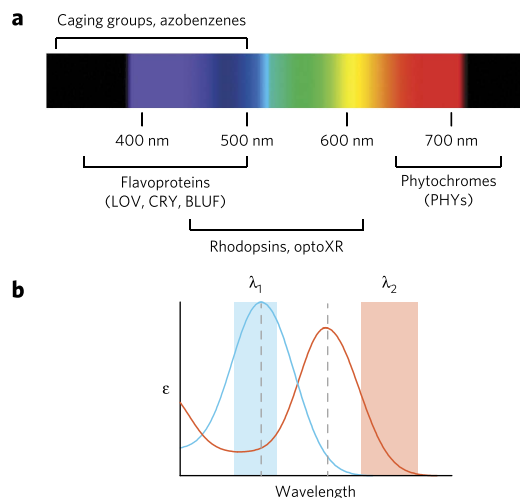
The rate constant of the photochemical reaction (in s<sup>−1</sup>) at the sample is  $k = N_A \sigma \phi I_s$ , where  $\sigma$  and  $I_s$  are respectively measured in m<sup>2</sup> and Ein s<sup>−1</sup> m<sup>−2</sup>. Although it involves inconsistent units mixing both centimeter-gram-second and meter-kilogram-second systems, the expression  $k = 2.3 \times 10^{-1} \epsilon \phi I_s$  may be useful to adopt, where  $k$ ,  $\epsilon$  and  $I_s$  are respectively measured in s<sup>−1</sup>, M<sup>−1</sup> cm<sup>−1</sup> and Ein s<sup>−1</sup> m<sup>−2</sup>. Illumination of a photoactive substrate  $S$  at concentration  $S_0$  generates a photoproduct  $P$ , whose concentration  $P$  exponentially increases with time  $t$  as  $P(t) = S_0 [1 - \exp(-kt)]$ . The illumination duration ( $\tau$  in s), enabling complete photoactivation of the sample, is typically taken as  $\tau \sim 5/k$ . If  $\epsilon = 10^4$  M<sup>−1</sup> cm<sup>−1</sup>,  $\phi = 0.1$  and  $I_s = 10^{-2}$  Ein s<sup>−1</sup> m<sup>−2</sup>, photoactivation is complete ( $P \sim S_0$ ) within  $\sim 2$  s.

It should be noted that when photoactivation is reversible, thermal return of the photoactivated state is characterized by a rate constant  $k^A$  (in s<sup>−1</sup>). Starting from a concentration  $P_0$ , the concentration of  $P$  exponentially decays in dark conditions as  $P(t) = P_0 \exp(-k^A t)$ , and the lifetime of the photoactivated state is typically taken as  $\tau^A \sim 5/k^A$  (in s).

The wavelength dependence of light absorption allows for selectively targeting a given photoactive precursor in a mixture of molecules differing by their spectral properties (chromatically orthogonal photoactivation). Figure 3b illustrates a typical situation involving two photoactive precursors 1 and 2 absorbing at different wavelengths. To achieve chromatically orthogonal photoactivation requires the identification of two wavelengths,  $\lambda_1$  and  $\lambda_2$ , such that the ratio  $\rho_{1/2} = \epsilon_1 \phi_1 / \epsilon_2 \phi_2$  of the photoactivation rates for 1 and 2 is respectively much greater than 1 at  $\lambda_1$  and much smaller than 1 at  $\lambda_2$  (to selectively photoactivate 1 and 2 with  $\lambda_1$  and  $\lambda_2$  excitation light, respectively). With respect to Figure 3b, this condition can be fulfilled by exciting in the red edge of the absorption band of the longer wavelength-absorbing chromophore (where only the latter chromophore absorbs light) and the blue edge of the shorter wavelength-absorbing chromophore (where the longer wavelength-absorbing chromophore exhibits low absorption).

The preceding paragraphs deal with light absorption relying on one-photon excitation, in which the reactive excited state is produced by the absorption of a single photon. Other excitation modes can be applied. In particular, two-photon excitation has recently gained popularity for highly localized photoactivation<sup>97,98</sup>. It is generally performed in the 700 nm to 900 nm range, where light absorption and scattering are lowest (in biological matter). The cross-section for two-photon absorption is much smaller than with one-photon excitation<sup>99</sup>. Hence, focused powerful light sources (pulsed lasers) have to be used. As with one-photon excitation, the rate constant for photoactivation with two-photon excitation within the excitation volume depends both on the photophysical and photochemical parameters of the chromophore as well as on features of the light source<sup>63</sup>. Chromatically orthogonal photoactivation with two-photon excitation is more demanding than with one-photon excitation owing to the rather large chromophore two-photon absorption bandwidth and the narrower accessible spectral range for discrimination<sup>100</sup>.





**Figure 3 | Light absorption for photoactivation.** (a) Absorption wavelength ranges of common chromophores allowing for photocontrol of proteins. (b) Favorable combination of absorption wavelengths (indicated by colored rectangles) of two chromophores that achieves chromatically orthogonal photoactivation of different proteins from the two photoactive precursors **1** (blue curve) and **2** (red curve).

functionalization of the protein with a non-natural photoactive moiety to obtain a light-sensitive adduct.

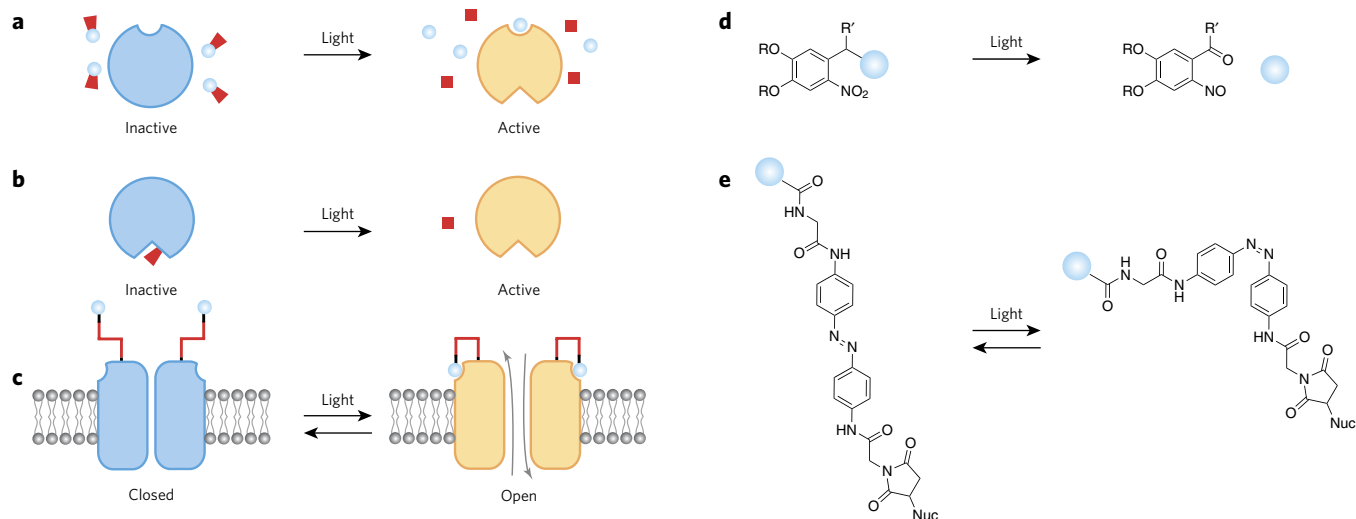
The caging concept (Fig. 4a,d) was first used to increase the spatial resolution of inducible gene expression platforms to photocontrol protein concentrations by acting at the transcriptional level. A caged ecdysone was developed to create a photoactivable ecdysone-inducible gene expression system<sup>37</sup>. Upon light illumination, the caged ecdysteroid is rapidly converted into active ecdysone, which binds and activates the ecdysone receptor, promoting its association to a responsive element and inducing the expression of the gene under its control. Caged selective estrogen-receptor modulators were used to control with light both gene expression and gene repression mediated by ERα and ERβ<sup>38</sup>. Photoactivable doxycycline derivatives were designed to activate with light transgenes on the basis of the tetracycline *trans*-activator ‘Tet-on’ system<sup>39</sup>. This technique

allows gene expression to be turned on in various organisms (mouse embryos and *Xenopus laevis* tadpoles) with very high spatiotemporal resolution by local illumination with ultraviolet light or by two-photon uncaging.

Strategies acting at the post-translational level have also been developed to improve temporal resolution. A specific strategy for the photocontrol of proteins that may be generally applicable proposes to use a caged 4-hydroxy-tamoxifen<sup>40</sup> or its analog, the caged cyclofen<sup>41,42</sup>, to control the function and cellular localization of proteins fused to a modified estrogen receptor ligand binding domain. Upon photorelease, the ligand binds the fused binding domain, releasing the protein from its complex with cellular chaperones and possibly redirecting it to the nucleus. Along the same line, caged rapamycin was designed to promote the light-induced heterodimerization of two proteins fused to FK-506 binding protein (FKBP) and FKBP-rapamycin binding protein (FRB)<sup>43</sup>, enabling the photocontrol of signaling proteins, such as the small GTPase Rac involved in membrane ruffling. Caged rapamycin was also used to regulate the activity of protein kinases in live cells<sup>44</sup>. Local photorelease of rapamycin could rescue the activity of protein kinases modified with an engineered catalytic domain containing iFKBP (a modified FKBP that renders the protein kinase inactive) by recruiting FRB<sup>44</sup>.

To control proteins at the post-translational level, the caging concept has also been extended to the direct caging of proteins to photocontrol their activity at the single-residue level. However, although caging groups can be introduced easily within small molecules by chemical synthesis, the insertion of a caging group within a protein sequence is a much more challenging task. To circumvent the issue of chemical derivatization and cell delivery, methods to genetically encode caged amino acids (lysine, tyrosine and cysteine) in mammalian cells were developed for the site-specific introduction of caging groups into protein sequence<sup>45–48</sup> (Fig. 4b). This technology made it possible to photocontrol protein localization<sup>45</sup>, signal transduction<sup>46,47</sup> and gene expression<sup>49</sup>. This approach, demonstrated initially in mammalian cells, should rapidly benefit from the recent upgrade of the unnatural mutagenesis strategy to multicellular organisms<sup>50</sup>.

The use of photoswitchable platforms for reversibly controlling protein function has been almost exclusively used so far to design light-gated ion channels and ionotropic receptors for applications in neuroscience (Fig. 4c,e). The light gate consists of a pore blocker



**Figure 4 | Light control of proteins with chemical and genetic hybrid photoactuators.** (a) Photolysis of caged compounds releases actuators interacting with a biological target, which becomes active after switching conformation and/or compartment and/or partner. (b) Proteins can be caged by site-directed insertion of caged unnatural amino acids. (c) Receptors can be rendered light sensitive by chemically coupling a synthetic photoswitchable agonist. (d) Light activation irreversibly breaks a bond in caged compounds. (e) The geometry of the photoswitchable chromophores is reversibly modified upon illumination.

(in the case of light-gated ion channels) or a ligand (in the case of ionotropic receptors) attached to the protein (via a nucleophilic amino acid side chain) with a photoswitchable azobenzene moiety, which acts as optical switch (Fig. 4c). A light-gated  $K^+$  channel was generated using a gate containing a quaternary ammonium as pore blocker<sup>51,52</sup>. Illumination with long-wavelength light converts the azobenzene into its *trans* configuration, enabling the blocker to reach the pore. Retraction and therefore conduction can be obtained by illumination with shorter wavelength. This technique made it possible to switch action potentials on and off in rat neurons. Similarly, a light-activated ionotropic glutamate receptor was obtained by covalently tethering a glutamate analog to the receptor with an azobenzene linker<sup>53</sup>. Photoisomerization provides the ability to reversibly control ligand binding, initiating allosteric domain closure and channel opening. This strategy, initially shown in non-neuronal cells<sup>53</sup>, enabled the remote control of neuronal activity in culture and *in vivo* on the millisecond timescale<sup>54</sup>. Recently, this concept was extended to the light-gated potassium-selective glutamate receptor<sup>55,56</sup>.

### Technical challenges and outstanding problems

**Matching the photoactivation characteristics to the question studied.** Light provides an exceptionally versatile method to perturb and read out protein activity. However, to fully exploit light's potential, the spatiotemporal characteristics and resolution of the chosen methods have to match those of the relevant biological question (Fig. 1).

Endogenous dynamics can be perturbed through a light-induced jump (or a periodic modulation) of the concentration of product in active molecule<sup>57,58</sup>. To analyze a phenomenon occurring on a timescale  $\tau$ , the light-induced concentration jump must be effective after a time distinctly shorter than  $\tau$ . Similarly, the concentration modulation must have a period smaller than  $2\pi\tau$ . Thus, if one wants to perturb and study transcription, one needs to bear in mind that the typical time for a gene to be transcribed and exported to the cytoplasm is about 10 min<sup>59</sup> (Fig. 1a). Hence, light activation of the relevant transcription factor must take place within 1–2 min. Similarly, if one is interested in interfering with translation, the photoactivation time must match the typical rate of translation, which is also in the range of tens of minutes<sup>59</sup> (Fig. 1a).

The illumination time associated with photoactivation can be estimated from the photochemical parameters of the substrate and the illumination power. Properly choosing a desirable photochemistry and a light source is crucial for reducing the time required to generate a change in concentration. In particular, increasing the light intensity is useful as long as it does not provoke an endogenous response. However, at the highest powers, one has to keep in mind that the photochemical step may not be rate limiting; in that case, further increasing the light intensity will not increase the temporal resolution. This may be the case with caged compounds for which photoinitiation is often followed by thermally driven elimination steps requiring up to few hundreds of seconds in unfavorable situations<sup>60</sup>.

In the case of reversible photoactivation, the relaxation time associated to thermal return has an additional role. Either steady or periodic illuminations are often required to keep photocontrol over protein function. For example, in the recently reported LITE system<sup>25</sup>, many hours of illumination were required to induce transcription. Related to that issue is the off-time (or resetting time) of the system. How quickly does the system shut off when illumination is interrupted? Does the shut-off mechanism depend on diffusion or degradation of the activated products (e.g., mRNA and proteins), or can it also be induced by light, e.g., via isomerization of a chromophore at a different wavelength<sup>19</sup> (Table 1)? When one relies on thermal return to the inactive state (typical for LOV fusions), degradation of the products or diffusion (and therefore dilution) of

the activated molecule (in the case of caged molecules), the resetting time can be minutes to hours.

Similarly, the spatial resolution of the photoactivation method used should match the problem investigated. If one attempts to selectively photocontrol a subcellular component (with typical 200-nm dimensions, e.g., a synapse, a pseudopod or a mitochondria; Fig. 1), a focused laser beam using two-photon photoactivation should preferentially be used. This contrasts with one-photon excitation, which activates all of the photosensitive molecules along the illumination path, thereby increasing photodamage of the sample. It should be noted that it is now possible to shape light in three dimensions to photoactivate volumes of various geometries<sup>61</sup>.

However, the spatial resolution of the photoactivation method will match the spatial resolution of the illumination profile only if the photoactivated substrate does not significantly diffuse in the cell on the timescale of the investigated phenomenon. In other words, the spatial resolution of photoactivation is considerably reduced when the photoactivated molecule can diffuse out of the illumination volume. This consideration rules out methods that rely on the irreversible photorelease of a small diffusible molecule to control protein with subcellular resolution. This decrease of resolution may be particularly detrimental when slow, thermally driven steps limit the photolysis rate. Then, not only is spatial resolution lost but also a highly localized illumination may not result in significant local increase of the product concentration<sup>62,63</sup>. Under such constraints, it is essential to favor photoactive substrates that have the largest photoactivation cross-sections (to increase the photoactivation rate; Box 1) and directly generate the desired state or product after photoactivation. In contrast, high spatial resolution can be obtained when photoactivation of a diffusible substrate is photochemically reversible at a red-shifted wavelength. Then, one can superimpose a localized light pattern to perform photoactivation with a uniform illumination at higher wavelength to drive return to the inactive state. In that case, the spatial resolution is essentially defined by the photoactivation illumination profile<sup>19,57</sup>.

When photoactivation of a diffusible substrate is performed within a cell, local illumination leads to a rise of product concentration within the whole cell at short timescales (typically 10 ms, the timescale a small molecule needs to explore a cell by diffusion). Then, the cell defines the spatial resolution. For activation at the single-cell level in an organism, two-photon illumination is often the method of choice as it ensures that only molecules within the focal volume are activated. However, when the product is the subject of catabolism, one should keep in mind that cellular processes are very often microcompartmentalized (as in the case of membrane rafts, nuclear speckles, mRNP granules and so on): as a result, protein photoactivation could remain heterogeneous within the cell. When dealing with slower biological phenomena, one may have to consider leakage of the activated product out of the cell, which leads to a further decrease in both the product concentration and the spatial resolution.

**Multiplexing.** The various methods developed to control protein with light, besides presenting different advantages and drawbacks, offer the possibility to independently control the activity of several proteins within the same cell or at different locations in a living organism. This feature will be particularly useful when analyzing feedback in both time and space.

Such multiplexing requires independent activation of different photochemical systems. The simplest way to do so relies upon using light sources at sufficiently different wavelengths to control orthogonal photoactivatable systems. As illustrated in Box 1, it is here essential to take advantage of a library of photoactivatable systems absorbing light in a broad range of wavelengths and exhibiting contrasted photoactivation cross-sections. Photoactivatable proteins with light absorption spanning the whole visible range are already available (Table 1). In contrast, despite recent progress<sup>64–70</sup>, most caging groups and photochromic molecules are still restricted to the

ultraviolet range. When exogenous inducers control protein functions, another possibility is to release two inducers by means of a single photoactivation step. Caged compounds based on branched self-immolative spacers are in that case promising substrates for the photorelease of two different molecules with a tunable delay<sup>71</sup>.

Another major issue related to multiplexing is the control of the expression level of the two (or more) photoactivatable engineered proteins and their stoichiometry. Adequate stoichiometry is generally achieved via bicistronic expression, either using an internal ribosome entry site or using skipping peptides (viral sequence of the 2A family). Clearly, improvements are needed if one wants to precisely monitor expression and stoichiometry in any cellular context.

Except for the control of different photosensitive ion channels<sup>72,73</sup>, we are not aware of many examples of multiplexing in the photocontrol of cellular processes<sup>74–76</sup>, but this will certainly be one of the future challenges of that nascent field.

**Quantification.** The quantification of the response to light activation has received little attention so far. Indeed, the photoactivation of a protein implies optical control of the concentration of its activated state, which depends on the concentration of the photoactive entity and the photoactivation rate (Box 1). For exogenous substrates, the concentration of photoactivatable molecules is chosen during the experiment. In contrast, the concentration of genetically encoded photoactivatable proteins is generally tuned by the strength of the promoter controlling their expression. The field would greatly benefit from a better control over the quantitative aspect of actuator expression (both to maintain it within physiological limits and, ideally, to fine-tune it) and downstream signaling (how much of the next component is activated in the cascade).

The photoactivation rate is governed by the photochemical properties of the chromophore and the intensity and duration of light illumination (Box 1). Many photochemical properties of chromophores are available in the literature<sup>60,77–80</sup>. Moreover, an order of magnitude of the light intensity at the sample can be obtained with a power meter (Box 1), enabling one to estimate the photoactivation rate *a priori*.

However, an accurate evaluation of the concentration of active state is often hampered by uncertainties associated to the optical characteristics of the medium and the concentration of photoactivatable substrate. In the uncaging context, one possible way to address this quantification issue relies on the simultaneous release of the active substrate together with a fluorescent reporter in a 1:1 molar ratio from a single precursor<sup>71,81–83</sup>. Quantitative analysis then simply implies analyzing the increase of the fluorescence emission after—or better, during—activation at the targeted site.

Alternatively, one can directly assess the biological activity of the photoactivated protein as a function of the intensity and duration of light illumination. For example, if the expression of a protein is placed under a photoactivatable transcription factor, the concentration of expressed protein for a given illumination energy can be estimated from the cotranslation of a fluorescent marker.

**Versatility and robustness.** In addition to the previous considerations, there are more general issues to be aware of when choosing or developing a photoactuator. Addressing these issues should ensure versatility and robustness of the methods for an easy transfer to any biological system of interest.

First and foremost is the issue of toxicity. The photoactivated molecules and/or proteins must not be toxic or interfere with cellular processes at the effective concentrations. This is not always the case, in particular with some of the light-sensitive channels<sup>84</sup>.

Then, there is the issue of leakage or contrast ratio. How active is the system when not illuminated, and how much does its activity

increase upon illumination? Light-sensitive transcription factors may have some activity even under dark conditions. Similarly, fusing a protein with a LOV domain does not always completely abolish its activity and may require extensive protein engineering to get rid of background activity<sup>85</sup>. Upon illumination, an increase in activity of the protein by an order of magnitude is usually considered excellent but is often difficult to ensure.

The issue of bioavailability is crucial when using a small exogenous molecule to photocontrol a protein, for example, when using a phytochrome or a caged activator. These molecules should permeate the cells of the organism to affect the desired pathway, and this may not always be possible with noninvasive means.

The localization of the targeted protein (cytoplasmic, membrane bound or exported) also constrains the type of photochemical methods used for control. For example, an exported protein cannot be controlled by a method relying on the complex that a protein forms with cytoplasmic chaperones.

Eventually, one of the main hurdles to overcome is the availability of the tools for protein light control. Whereas genetically encoded systems are easily accessible to the scientific community, dissemination of chemicals in large amounts may be hampered by sophisticated synthetic procedures. Targeting simple syntheses and setting up shared platforms would be useful, along with commercializing these chemicals.

To conclude, excellent tools for the control of protein activity with light already exist. Efforts should now be more explicitly focused on quantification to address the integrated dynamics of biological systems. This requires more efficient tools to sense physiological changes. Efforts have to be made, particularly in the design of fluorescent probes and biosensors allowing the quantification of changes with higher resolutions in space and time<sup>1</sup>. Another important avenue of development will be to overcome the drawbacks of light in the visible range (absorption and diffusion) to interrogate living organisms. From the latter point of view, it will be important to thoroughly explore other ways to trigger and read out physiological changes: other triggers that could be used to control proteins, such as X rays<sup>86</sup>, acoustic waves<sup>87</sup>, temperature jumps<sup>88</sup> and so on, may soon be added to the toolbox for protein control.

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## Competing financial interests

The authors declare no competing financial interests.

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