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A *light* way for nuclear cell biologists

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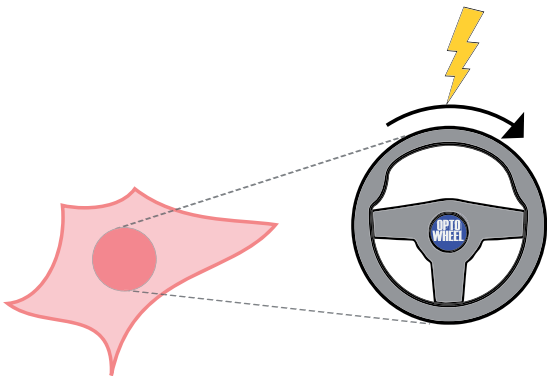
The nucleus is a very complex organelle present in eukaryotic cells. Having the crucial task to safeguard, organize and manage the genetic information, it must tightly control its molecular constituents, its shape and its internal architecture at any given time. Despite our vast knowledge of nuclear cell biology, much is yet to be unravelled. For instance, only recently we came to appreciate the existence of a dynamic nuclear cytoskeleton made of actin filaments that regulates processes such as gene expression, DNA repair and nuclear expansion. This suggests further exciting discoveries ahead of us. Modern cell biologists embrace a new methodology relying on precise perturbations of cellular processes that require a reversible, highly spatially confinable, rapid, inexpensive and tunEable external stimulus: light. In this review, we discuss how optogenetics, the state-of-the-art technology that uses genetically encoded light-sensitive proteins to steer biological processes, can be adopted to specifically investigate nuclear cell biology.

Keywords: dynamics; nucleus; optogenetics; photosensors; protein engineering.

Abbreviations: DBD, DNA binding domain; dCas9, nuclease-dead Cas9; eIF4E, eukaryotic initiation factor 4E; FMN, flavin mononucleotide; GFP, green fluorescent protein; IDRs, intrinsically disordered regions; LOV, light-oxygen-voltage; NES, nuclear export signal; NLS, nuclear localization signal; PA-Rac1, photo-activatable Rac1; TAD, transactivation domain; TEV, tobacco etch virus; TFs, transcription factors.

Graphical Abstract



Investigating Nuclear Cell Biology by Controlling Protein Function in Space and Time

Eukaryotic cells evolved a special organelle, the nucleus, into which the chromosomes are stored, used, repaired if necessary, and replicated (*1*, *2*). Unsurprisingly, the majority of nuclear processes are related to gene expression, DNA replication and repair. However, several other processes also occur in the nucleus, such as post-translational modification of proteins (*3*, *4*), proteasomal degradation (*5*), nuclear protein import and export (*6*), as well as nuclear shape establishment and maintenance (*7*, *8*). As true for every research topic, novel exciting discoveries continue to be made in the field of nuclear cell biology as the molecular tools and instruments at our disposal to investigate biological pathways progressively become more sophisticated. A discovery that exemplifies the paramount importance of advances in technologies and molecular tools is that of the actin nucleocytoskeleton (*9*–*12*). A well-recognized concept when talking about the cytoplasm, the existence of dynamic actin filaments in the somatic cell nucleus had been long ignored, despite an interesting study indirectly suggested their existence (*13*). Dynamic actin filaments in healthy, living mammalian cells have been visualized for the first time and connected to a biological function by the Grosse lab when the researchers had the idea to localize the genetically encoded probe LifeAct (*14*) to the nucleus by adding a nuclear localization signal (NLS) to it (*15*). This smart stratagem allowed focussing on the small fraction of nuclear actin (about 20% of the total) avoiding the visualization of the overwhelming cytoplasmic actin pool, which would have otherwise obscured the less prominent nuclear structures. The discovery was further facilitated by new generation confocal and super-resolution fluorescence microscopes, as obviously any study relying on microscopic visualization is intrinsically limited by the resolution of the instrument.

Seeing is believing, we all agree. Biology relied on observations as its main methodology for centuries.

After all, isn't nature what we aim to unravel? We simply need to observe what naturally occurs, make hypotheses, test and refine them to finally understand how nature works. Cell biology has been no exception to this general trend. However, in recent decades, a novel methodology has emerged, one that prompts us to take action. While the concept of perturbing a biological process to understand the mechanism(s) behind it is relatively old, with gene knock-out and overexpression, for instance, dating back to the late 80s (*16–18*), nowadays, we recognize that temporal changes in the localization, interactome and activation of biomolecules (proteins, RNAs, lipids, etc.) greatly contribute to their function (*19–21*). Therefore, modern cell biologists apply perturbations of a more sophisticated kind than gene knock-out or overexpression.

Light: the Perfect External Stimulus to Apply tunEable and Dynamic Perturbations

Let us assume that we want to understand the role that molecule X plays in biological process Y. We have already performed first analyses depleting or giving to the cells an excess of X. The data have given us a first glimpse of what X might be doing. However, at this stage, we lack important information, such as: does X function at a specific time during biological process Y? Would the result be different if X were depleted only after time point t, instead of from the beginning on? Does X need to be localized to cellular address Z to function? Does X perform different—or even contradictory—functions if localized to different cellular addresses? Is the function of X dependent on its interaction with W? Does this interaction require a special localization and timing? Does the function of X depend on whether it is active in a sustained or oscillating manner? What happens if X is active only in a subpopulation of cells (this question being particularly relevant when studying entire tissues or organs)? We clearly need a technology that allows us to control the localization, activation and interaction pattern of molecules in individual cells in a temporally defined manner. We use the term 'molecules' and not 'proteins' to acknowledge the complexity of cellular regulation that is achieved by biomolecules others than proteins, such as lipids, second messengers, RNAs, etc. However, since proteins are prominent regulators of biological processes, are easily produced in cells by delivery of the appropriate genetic constructs, and directly regulate other biomolecules, most methods have been developed to control proteins or their encoding genes.

Chemical inducers such as doxycycline, tetracycline or rapamycin have been widely used in cell biology to control the expression level, localization and interaction status of proteins (*22–25*). While being adjustable in terms of concentration and timings of their administration, these chemical inducers cannot be applied in a spatially confined manner, nor are they easily removable from the cells, thus making dynamic perturbations difficult or impossible to achieve. Light, on the other hand, is a perfect external stimulus: it can be focussed on individual cells or even subcellular structures, it can be readily removed, it reaches its target immediately, typically does not interfere with endogenous processes and is harmless (unless wrongly applied). Photosensitive proteins abound in nature, since the rotation of our planet on its axis causes the regular alternation of day and night, which has forced organisms to evolve ways to regulate their behaviour differently depending on the availability of the light stimulus. These proteins sense light via a chromophore that is either covalently or non-covalently bound to them, and typically undergo a conformational change upon absorbance of a photon of a specific wavelength that is coupled to a biological function, such as ion conductance, kinase activity or second messenger production (*26*). The field of optogenetics, that is, the use of genetically encoded light-sensitive proteins naturally able or engineered to steer biological processes, is commonly believed to have been born when neuroscientists Zhuo-Hua Pan, Edward Boyden and Karl Deisseroth proved that overexpressing channelrhodopsin-2—a natural light-gated cation-selective channel from the green alga *Chlamydomonas reinhardtii,* which was discovered by Georg Nagel, Ernst Bamberg and Peter Hegemann (*27*, *28*)—made it possible to excite neurons with blue light (*29*, *30*). However, strictly speaking, the first proof of light control of a biological process precedes the work in neurons, and dates back to 2002, when the Quail lab controlled gene expression in yeast using the light-inducible interacting PhyA-PIF3 and PhyB-PIF3 pairs (*31*). Interestingly, it took several years after this seminal paper for other labs to demonstrate that light-sensitive proteins other than opsins could be expressed in non-excitable cells to control and/or investigate intracellular biological processes (*32–34*). Importantly, alongside studies relying on the expression of minimally modified natural light-sensitive proteins (*e.g.* fused to a localization tag or to a protein partner) (*31*, *33–36*), novel, unnatural proteins were engineered by genetically fusing light-sensing domains to functional domains or even entire proteins to turn these latter ones into light-inducible units (*32*, *37–42*). Nowadays, the engineering of light sensitivity onto a protein of interest normally blind to light has become widespread and novel methods have been developed, such as the caging of peptides into the photosensor or the introduction of the photosensor into surface-exposed loops of the protein of interest (*43–45*). The optogenetic toolkit is extremely well-equipped, allowing us to control a great variety of biological processes selecting the method and the wavelength most appropriate to the specific case.

Ways to Control Protein Function with Light

As mentioned above, proteins are involved in the regulation of almost all biological processes; thus, it is possible to steer practically any one of them by controlling the activity of one or more proteins. For instance, even if we wished to study the role of lipids in some signalling pathway, we could manipulate the lipid species of choice by controlling the enzyme(s)

required for its biogenesis/turnover. There are truly many ways to control the function of a protein of interest (Fig. 1). This could potentially discourage those (nuclear) cell biologists who want to adopt optogenetics for studying a particular biological problem. We suggest them to approach the great variety of existing tools enthusiastically, seeing it as an opportunity to select the tool that is most suited to their

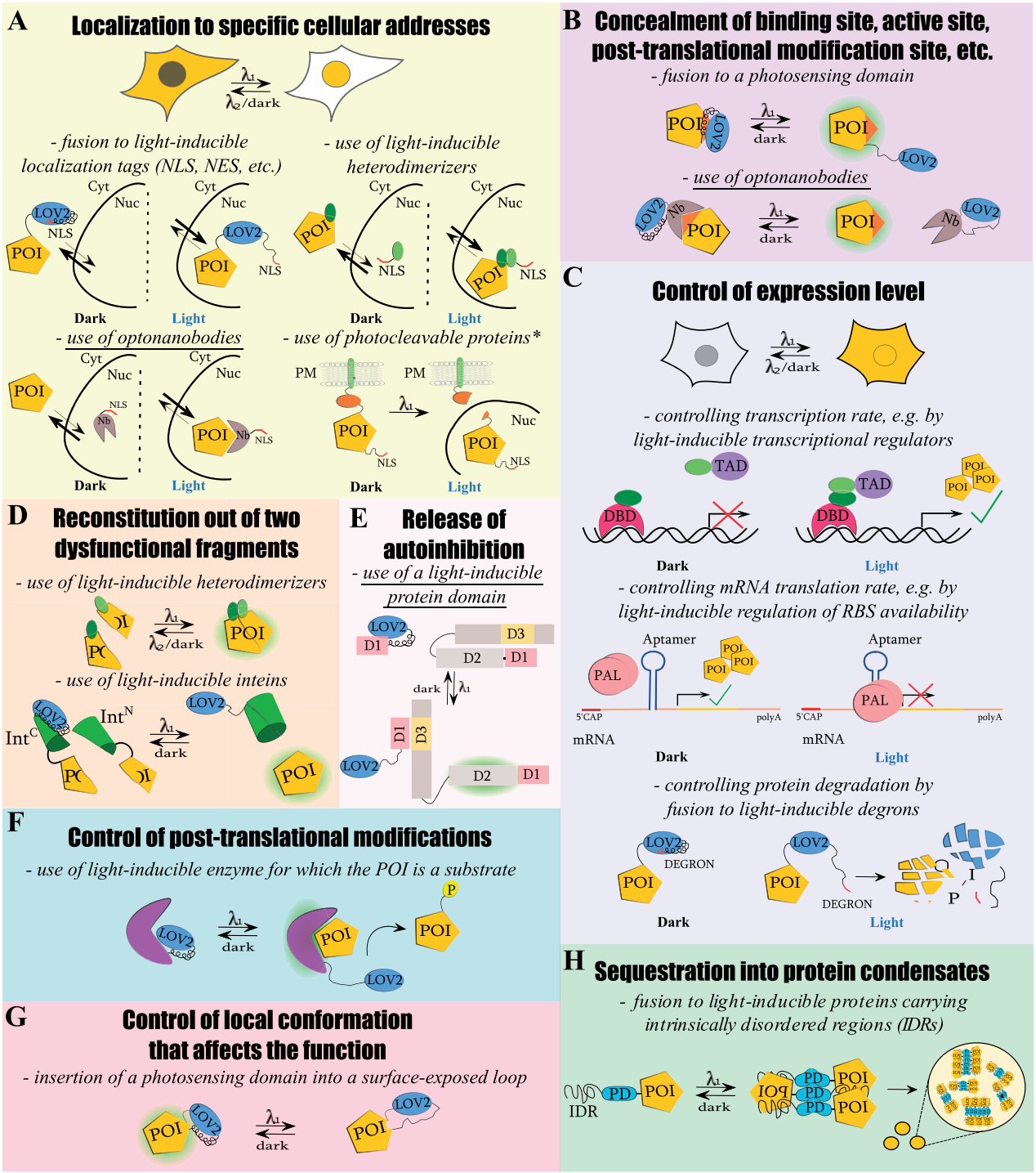


Fig. 1. Overview of optogenetic strategies to control a protein of interest. Shown are schematic illustrations of selected optogenetic strategies. Since some photosensors revert back to their inactive form only in the dark, while others can be switched back using a second wavelength or if kept in the dark, we wrote 'dark/λ_2'. Underscored are strategies that allow controlling endogenous proteins. The asterisk indicates an irreversible strategy. Symbols and abbreviations: λ_1, activating wavelength; λ_2, deactivating wavelength; Cyt, cytoplasm; Nuc, nucleus; *As*LOV2, LOV2 domain of *Avena sativa* phototropin 1; POI, protein of interest; NLS, nuclear localization signal; NES, nuclear export signal; dark green/light green ovals, heterodimerizing protein pair; Nb, nanobody; PM, plasma membrane; Int^C, C-terminal fragment of a split intein; Int^N, N-terminal fragment of a split intein; D1 and D3, interacting domains involved in autoinhibition; D2, functional domain; P, phosphorylation; DBD, DNA binding domain; TAD, transactivation domain; PAL, photoreceptor from *Nakamurella multipartite*; DEGRON, degradation signal; IDR, intrinsically disordered region; PD, photosensing domain.

application or laboratory equipment. Here are some considerations that can direct the choice of the method:

1. *the time-scale of the biological process under consideration*
If the process we are interested in occurs on the timescale of few seconds or minutes, we should preferentially select a method that allows us to control the activity of the protein of interest directly and not indirectly via controlling its gene expression, since this takes a longer time.
2. *the type of cells or organism into which the optogenetic tool is to be used*
If we work with primary cells or organisms that require the use of viruses for delivery of the construct(s), the construct size may be a limitation, since many viruses impose a limit on the size of the transgene. Thus, one-component systems may be preferable to multi-component ones. Among multi-component systems, those based on smaller proteins may be preferable. Moreover, the cell type/organism further dictates which chromophores are endogenously available and, consequently, which optogenetic tool can be used without external supply of the chromophore (an important consideration mainly when working with organisms).
3. *the microscopic set-up available*
While fluorescence microscopes are commonly equipped with a green fluorescent protein (GFP) filter set or laser—being therefore immediately compatible with blue light-inducible optogenetic tools—they typically lack a light source and filter sets compatible with far-red light (740 nm). To make optogenetic experiments at the microscope with tools triggered by red and far-red light we thus often need to invest into the right equipment.

Sometimes, even after having narrowed down the suitable tools due to the above-mentioned considerations, there are still a number of candidates. For example, once we have clarified that we want to control the localization of our protein of interest with blue light, we still need to decide which specific tool to adopt. There are indeed various ways to localize a protein of interest to a desired cellular address with blue light-responding tools. In these cases, it is useful to dig deeper into the specifications of each tool, such as the dark/light fold change that is achievable or the extent of leakiness (*i.e.* the extent of localization prior to the light stimulation).

Controlling localization

Protein function depends on localization. This is evident for transcription factors (TFs), which must enter the nucleus to gain access to the genes they regulate (*46*, *47*). However, this concept is general and every protein has its own specific place inside the cell. Thus, having a way to regulate a protein's localization equals to regulating its function. There are many methods to control with light protein localization (Fig. 1A). Some are general and can be used to localize the protein of interest anywhere in the cell. Others have been developed for specific cases, such as nuclear import or export.

Light-inducible localization tags Since many proteins are regulated by their entry into or exit from the nucleus, we developed two tools, called LINuS and LEXY, to control nuclear protein import and export, respectively (*48*, *49*). Interestingly, the Kuhlman group developed around the same time two other tools, called LANS and LINX, for the same purpose (*50*, *51*). They are all based on the second light-oxygen-voltage (LOV) domain of *Avena sativa* phototrophin 1 (*As*LOV2) that uses as light-absorbing moiety (chromophore) flavin mononucleotide (FMN), which is produced by all cells. Thus, optogenetic tools based on this domain respond to blue light and do not require the external supply of the chromophore. In the tools designed for controlling nuclear protein import and export, the C-terminal helix of the *As*LOV2 domain—called Jα helix—was modified to bear an NLS or a nuclear export signal (NES) (Fig. 1A). In the dark, the Jα helix is folded and bound to the core *As*LOV2 domain (*52–54*), and consequently the NLS/NES is not accessible to the endogenous import/export machinery. After blue light absorption, the Jα helix unfolds and undocks from the core *As*LOV2 domain (*52–54*), exposing the NLS/NES, which can be recognized and mediates the translocation of the protein of interest fused to the modified *As*LOV2 domain to the designated compartment (nucleus or cytoplasm, respectively). These tools for controlling nuclear import and export built upon two previous optogenetic tools that showed for the first time how short peptides could be photocaged in the Jα helix of the *As*LOV2 domain (*43*, *44*). An example of mCherry nuclear/cytoplasmic accumulation that can be achieved with LINuS/LEXY in U2OS cells is shown in Fig. 2A and B. Since the *As*LOV2 domain re-acquires its closed conformation within less than a minute (*55*), when light exposure ceases the protein of interest fused to LINuS/LANS or LEXY/LINX can go back to the original compartment thanks to the presence of a constitutive NES (for LINuS and LANS) and NLS (for LEXY and LINX) on the construct (Fig. 1A).

Similarly to these tools, the natural LOV domain from the fungus *Botrytis cinerea* (BcLOV4) mediates the blue light-inducible association of a protein of interest fused to it to the plasma membrane (*56*). BcLOV4 bears a polybasic amphipathic helix downstream to the C-terminal Jα helix of the LOV domain, which gets released after blue light absorption and associates with anionic plasma membrane phospholipids in a reversible manner.

While at the moment only nuclear import and export and plasma membrane localization can be readily achieved with single component, LOV-based tools, the concept is generalizable and other localization tags could be photocaged in the Jα helix. This would require, however, some engineering.

Light-inducible heterodimerizers Protein localization can be powerfully controlled by light-inducible heterodimerizers. These are full proteins or domains that either naturally or due to protein engineering bind to

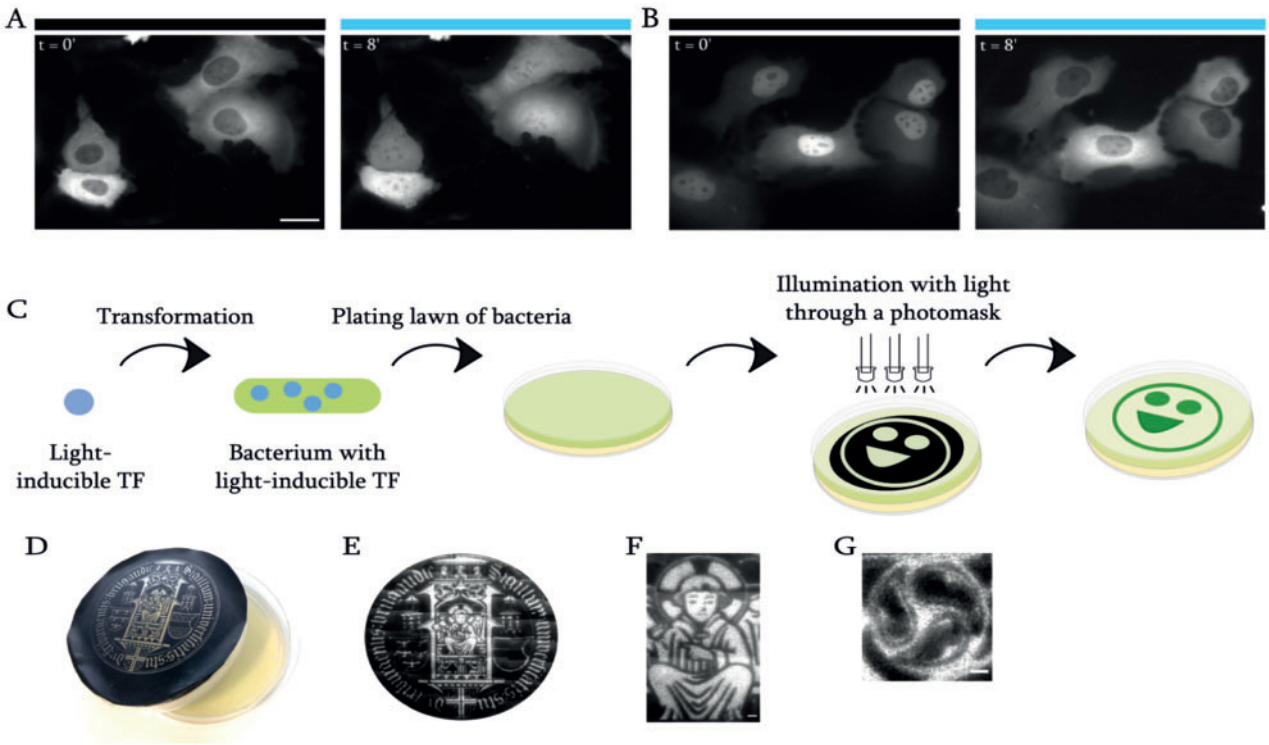


Fig. 2. Optogenetic control of nuclear protein import and export and gene expression in living cells. (**A** and **B**) Representative fluorescence images of U2OS cells transiently transfected with NES-mCherry-LINuS (A) and NLS-mCherry-LEXY (B) before (left image) and after (right image) 8 min of blue light illumination. Blue light was administered in short pulses of 300 ms every 30 s for 8 min. Scale bar, 30 µm. (**C**) Schematic illustration of the bacterial photography workflow. For simplicity, the reporter construct, consisting of the *gfp* gene under a promoter activated by the light-inducible TF, was omitted. (**D**) Photomask used to produce the bacteriograph in (E). The photomask representing the logo of the University of Freiburg was printed out and attached to the lid of a common agar plate. (**E**) Bacteriograph created following the procedure described in (C) and using the photomask described in (D). Images of the resulting bacterial lawn are taken with a fluorescent microscope and subsequently assembled in a single image. (**F** and **G**) Zoom-in on two parts of the bacteriograph in (E). Scale bar, 1 cm.

each other only after absorption of light of a specific wavelength and dissociate either spontaneously after a certain decay time or when absorbing a second wavelength. There are truly many such heterodimerizers, for use with UV, blue, green and red light (*31*, *33*, *34*, *44*, *57–66*) (for a review dedicated to this topic, we refer the reader to Ref. *67*). In some cases, the heterodimerizer is composed of two proteins that naturally interact with each other following absorption of light of a specific wavelength, *e.g.* PhyB-PIF, Cry2-CIBN, Gigantea-FKF1 and BphP1-PpsR2. In other cases, the interacting pair is made of a full-length protein or protein domain and a peptide, which is photocaged in the Jα helix of the *As*LOV2 domain, as in TULIPs and iLID. In one case, the so-called Magnet system, the heterodimerizer was engineered from a natural homodimerizer, the LOV-domain protein from *Neurospora crassa* VVD, introducing mutations that created a positively and a negatively charged version of VVD which would attract one another but not undergo homodimerization.

The concept behind their usage is the same for all tools based on heterodimerizers: the protein of interest is fused to one of the two proteins in the heterodimerizing pair and is localized to some cellular address other than the desired one (*e.g.* the cytoplasm), while the other protein in the pair is localized to the cellular address to which the protein of interest should localize after activation (*e.g.* the nucleus) (Fig. 1A). Upon light absorption, the protein pair interacts, effectively retaining the protein of interest to the desired cellular address, provided the localization signal on the other construct (the 'localizer') is stronger. It is important to understand that the two interacting proteins should find each other in order to interact. This means that the protein of interest is necessarily temporarily found in the desired cellular address, albeit typically for a brief time. In the example above, the construct of the protein of interest fused to one component of the heterodimerizer would have to constantly shuttle between the nucleus and the cytoplasm. We wish to point out that, while all other photodimerizers are based on partners that interact under light of a certain wavelength, there are three tools—two based on the *As*LOV2 domain (*65*, *66*) and one based on the photoactive yellow protein (YPP) (*66*)—that allow for the interaction to happen in the dark.

Optonanobodies Nanobodies are the variable domain (VHH) of the heavy chain-only antibodies expressed in camelids (*68*). They are small (~15 kDa) compared to conventional antibodies (~150 kDa) and fold stably in reducing cellular environments making them well-suited for studies in live cells (*69*). Some nanobodies have been engineered to have affinity for their targets

in the nM range (*70*, *71*). Recently, nanobodies have been controlled by light following three strategies: (i) the *As*LOV2 domain (or, more precisely, an optimized version of it called sLOV) was inserted into a surface-exposed loop of the nanobody following a previously reported method (*45*) whereby the blue light-triggered unfolding of the Jα helix brings about a local conformational change, which, in turn, results in either loss or gain of binding capacity by the nanobody towards its antigen (*72*). The same method was shown to work also with monobodies (*73*), which are synthetic customizable protein binders constructed using a fibronectin type III domain as molecular scaffold; (ii) the localization of the nanobody was controlled by a light-inducible heterodimerizing pair (*74*); (iii) the nanobody was split into two fragments, which were brought into physical proximity using a light-inducible heterodimerizer (*75*). Importantly, optonanobodies offer the opportunity to control the localization of selected endogenous proteins, as long as nanobodies specific for them can be developed (Fig. 1A).

Photocleavable proteins When reversibility is not necessary, the localization of the protein of interest can be controlled by fusing it to PhoCl, a photocleavable protein that was engineered from the photoconvertible fluorescent protein mMaple (*76*). Upon exposure to violet light (~400 nm), PhoCl undergoes a cleavage of the polypeptide backbone, which leaves two protein fragments behind: a large empty barrel and a short peptide, which spontaneously dissociate from one another. Zhang and colleagues used circular permutation to obtain a short peptide after photocleavage that most likely does not disturb the function of the protein of interest to which it remains fused. The photocleavage can be exploited to eliminate from the protein of interest a localization tag, which remains bound to the large empty barrel post-cleavage. For instance, the protein of interest can be retained to the plasma membrane in the dark by creating a fusion construct consisting of a transmembrane protein, PhoCl and the protein of interest (Fig. 1A). After exposure of the cells to violet light, the protein of interest dissociates from the rest of the construct, thus also from the plasma membrane and can accumulate into the nucleus provided it bears an NLS (either endogenous or genetically fused to it) (Fig. 1A). The disadvantage of this optogenetic tool, beyond its irreversibility, is the need for violet light, which is more phototoxic than light of higher wavelengths. Moreover, full cleavage is not achievable (*76*), thus it is important to determine if the amount of protein of interest whose localization changes upon illumination is sufficient for functionality or not.

Controlling the availability of a binding site, active site, post-translational modification site

The activity of proteins is often regulated by the binding of protein partners or by post-translational modifications (*77–79*). One way to control a protein is, therefore, to control the availability of a binding site, or a post-translational modification site. There are two methods to achieve this: one requires the engineering of the protein of interest and the other the development of a nanobody, which is then to be engineered to be light-inducible (Fig. 1B).

Fusion to a photosensing domain One of the first optogenetic tools to have been developed was the so-called photo-activatable Rac1 (PA-Rac1) (*32*). PA-Rac1 was constructed by genetically fusing a constitutive active form of Rac1 to the *As*LOV2 domain in a way that this latter would sterically hinder the binding of the effector PAK to Rac1 only in its dark conformation. Indeed, unfolding and undocking of the Jα helix from the core LOV2 domain would consent the binding site to be accessible again. This is a general strategy that was used, for instance, to engineer a light-inducible version of the DID domain of the formins mDia1 (*80*) and mDia2 (*15*), as well as of inhibitors of the RE1-silencing TF REST (*81*). We envisage that it could be used to conceal in the dark not only binding sites for protein partners, but also post-translational modification sites or active sites, as long as these are located in the protein structure at positions that the fused *As*LOV2 domain would reach (Fig. 1B).

Use of optonanobodies As mentioned previously, nanobodies can be made light-responsive. We, therefore, speculate that optonanobodies could also be used to conceal an important site on the protein of interest, as long as a nanobody that recognizes the protein of interest via interaction with such site can be developed (Fig. 1B). The advantage of this strategy is that endogenous proteins could be controlled.

Controlling the expression level

A protein needs to be expressed to exert its activity. Moreover, its expression levels may dictate its function (*82*, *83*). Clearly, one way to control a protein of interest is to control its expression level. A myriad of optogenetic gene expression systems exist, suggesting that this way of controlling a protein of interest must appear as the most natural or useful to researchers. Interestingly, as previously mentioned, the very first optogenetic tool ever created was one to control gene expression in yeast (*31*). Currently, the available optogenetic tools act at three steps of the gene expression process: gene transcription, mRNA translation and protein degradation (Fig. 1C). Other steps in the process, such as RNA maturation or export, mRNA splicing or degradation, or protein folding could also be put under light regulation using one of the strategies presented in this review to render a key enzyme in the process light-responsive.

Gene transcription Without doubt, this is the step that has been most extensively targeted, be it by regulating with light TFs or transcriptional activators (*63*, *84–99*), the RNA polymerase (*100*), DNA recombinases (*101–106*), DNA looping (*107*) or epigenetic modifiers to act upon the chromatin state (*108*). The majority of the tools falling in this category is based on the consideration that a TF can be separated in two parts: one containing the DNA binding domain (DBD) and the other containing the transactivation domain (TAD) (Fig. 1C). Transcription is initiated only when the TAD is recruited to the promoter by the DBD. This idea is actually behind the yeast or bacterial two-

hybrid technique, which is extensively used to validate protein–protein interactions in living cells (*109*, *110*). In this case, the DBD is fused to protein A and the TAD to protein B. A and B are the proteins whose interaction we wish to test. The DBD and the TAD will get physically close—allowing transcription of a reporter such as GFP or beta-galactosidase to occur—provided proteins A and B interact with each other. In the case of optogenetic gene expression systems, the interaction between the DBD and the TAD is controlled through light-inducible heterodimerizers (see above). Other methods to control gene expression consist in controlling with light: (i) homodimerization, since several TFs contact their cognate DNA response element in dimeric form (*85*, *88*, *89*, *96*, *98*, *111*); (ii) a post-translational modification that regulates the localization or activity of the TF (*37*, *94*, *112*); (iii) the nuclear localization of the TF (*48–51*) and (iv) the stability of the TF (*113*).

Importantly, methods based on nuclease-dead Cas9 (dCas9), Zinc fingers or TALENs allow controlling transcription of selected endogenous genes. This is true also for those methods whereby an intact, natural TF is light-regulated, for instance NFAT (*112*) or p53 (*49*, *113*). However, in this case, all target genes of the TF are, at least potentially, activatable—some may not be activated due to missing post-translational modifications on the TF, which may require pathway activation, or specific TF dynamics (*19*, *114*).

An interesting application of light-inducible TFs is bacterial photography (Fig. 2C). Typically, GFP—or brighter variants thereof—is cloned under a promoter that is activated by the light-inducible TF. The bacteria transformed with the plasmids for the reporter and the light-inducible TF are plated as a uniform lawn. The plate is then illuminated with light of the appropriate wavelength, but instead of homogenously, light reaches the plate through a desired pattern, applied by means of a so-called photomask. In Fig. 2D, the photomask with the logo of the University of Freiburg is shown, with which we illuminated a lawn of bacteria transformed with pBLADE, a plasmid carrying super folder GFP under the control of the pBAD promoter and BLADE, a novel, light-inducible TF engineered by us and the Khammash group fusing the DBD of the bacterial transcriptional regulator AraC and the light-inducible homodimerizer VVD (*111*). BLADE allows obtaining high quality bacteriographs, as seen in Fig. 2E–G.

mRNA translation mRNA translation has been so far controlled in four ways: by controlling translation initiation using light to (i) recruit eukaryotic initiation factor 4E (eIF4E) to the mRNA of interest (*115*, *116*) or (ii) release inhibition of eIF4E using a light-inducible form of an eIF4E binding protein (opto-4EBP) (*117*); (iii) by interfering with translation recruiting to an aptamer sequence placed between the mRNA CAP and the Kozak sequence a light-inducible RNA binding protein called PAL (Fig. 1C) (*118*) and (iv) by sequestering with light the target mRNA into protein clusters, which makes them less accessible to the ribosomes (*119*). In all cases, specific RNA binding proteins are necessary to mediate the interaction with the target mRNA, PAL being the only one that naturally binds RNA in response to light (*118*).

Protein degradation Protein degradation is mediated by degrons, small modifications that target proteins for proteolysis. Perhaps the best known degron is ubiquitin, followed by the PEST sequence. Other amino acid sequences act also as degrons and mediate ubiquitin-independent protein degradation. By controlling a degron strategically fused to the protein of interest it has been possible to control the stability of this latter one. One method consists in photocaging the degron in the Jα helix of the LOV2 domain, so that, only upon light exposure, the degron becomes accessible (Fig. 1C) (*120*, *121*). The other strategy is more complex and combines two layers of light regulation, one to control the exposure of a tobacco etch virus (TEV) protease recognition site (tevS) and one to control the nuclear export of the TEV protease (*122*). The protein of interest is fused to the light-inducible tevS and to three repeats of a degron and is cytoplasmically localized. The TEV protease is fused to LEXY and is localized to the nucleus in the dark. Upon blue light illumination, tevS is exposed as well as the NES within LEXY; consequently, the TEV protease re-localizes into the cytoplasm, where it cleaves the construct bearing the protein of interest at tevS, eliminating the three degrons from it. This strategy, therefore, leads to protein stabilization with light instead of protein degradation. Notably, a natural LOV protein that mediates protein stabilization with light has recently been discovered, which can also be used to stabilize a protein of interest with light (*123*).

Controlling reconstitution of two dysfunctional halves

Conditional reconstitution of protein functionality dates back to the beginning of the 90s, when Johnsson and Varshavsky showed that ubiquitin split in two dysfunctional fragments could regain its function when these latter ones were fused to interacting protein pairs that would bring the two fragments back in physical proximity (*124*). It is surely evident to the reader that this method can be readily made light-responsive by selecting light-inducible heterodimerizers as those described above instead of constitutively interacting protein pairs (Fig. 1D). This approach has been indeed used to control with light, for instance, the Cre recombinase (*102*, *125*) and dCas9 (*126*). It is, however, general and it can be adopted as long as two dysfunctional fragments of the protein of interest can be expressed in the cells. A slightly different method consists in using light-inducible inteins (*127*, *128*). Inteins are proteins that splice themselves out of host proteins and connect the flanking polypeptides with a new peptide bond (*129*). They come in two flavours: contiguous and split (either naturally or artificially). Inteins mostly spontaneously splice themselves out of the host protein, yet conditional inteins have been discovered or created (*129*). Two optogenetic tools based on inteins have been developed so far to control with blue light the reconstitution of proteins of interest in mammalian cells: one in which a mutated *As*LOV2

domain was used to sterically hinder in the dark the C-terminal fragment of the split *Npu* DnaE intein, which was additionally truncated to reduce background splicing (Fig. 1D) (*128*), and one in which a single fusion construct was created in which the two fragments of *Npu* DnaE were separated by the *As*LOV2 domain in way that their association would be favoured in the light (*127*). The advantage of using light-inducible inteins over light-inducible functional reconstitution is that the protein of interest gets reassembled in its full-length form, rather than simply functioning due to the close proximity of the two fragments. On the other hand, since protein splicing is irreversible, these methods lead to the accumulation of the protein of interest, which would exert its function for a long time (until degraded or diluted out due to cell division). Methods based on functional complementation allow, instead, turning the protein of interest on and off at will.

Controlling autoinhibition

Several proteins are kept in a latent state via intramolecular interactions between two of their domains that result in a conformation non-permissive of activity (*130*). We speak in this case of protein autoinhibition. One way to control with light the activity of a protein of interest that undergoes autoinhibition is to engineer a light-inducible version of one of the domains involved in the autoinhibition, for instance by fusing it to the *As*LOV2 domain as previously explained, and to overexpress it in cells. In the dark, the overexpressed, heterologous domain is sterically hindered by the LOV domain which is in its closed conformation, thus the protein of interest remains autoinhibited (Fig. 1E). Upon light absorption, the domain becomes available for binding *in trans* to its cognate domain, sequestering it away from the intramolecular interaction, thus releasing autoinhibition (Fig. 1E). This method was followed by the Zaider-Bar and Grosse groups to regulate with light the autoinhibition of the actin polymerization-promoting formins mDia1 (*80*) and mDia2 (*15*), respectively.

Controlling post-translational modifications

An alternative way to control a post-translation modification of a protein of interest is via a light-inducible version of the enzyme responsible for the modification (Fig. 1F). Once again, any of the strategies described here to control protein function can be adopted to create such light-inducible enzyme. It should be noted that this approach is very different from the one described above (Fig. 1B), since typically enzymes have more than one substrate. Therefore, when using a photocaged version of an enzyme, it is not possible to direct its activity exclusively towards the protein of interest. The advantage is, however, that in this case the endogenous version of the protein of interest gets activated and not a heterologous, overexpressed form of it, as with the method that targets the post-translational modification site directly on the protein of interest. Following this approach, for instance, we controlled the ubiquitylation status, and consequently, the expression levels, of endogenous p53 in HCT116 cells using a light-inducible version of the PMI peptide that blocks p53–Mdm2 interaction (*113*). Since Mdm2 has many other targets than p53 (*131*), however, we cannot exclude that, when bound to PMI, Mdm2 is sequestered away also from other proteins, not only p53.

Controlling local conformational changes that are coupled to activity

One of the very first optogenetic endeavours consisted in coupling a protein of interest to a photosensing domain in a way that the conformational change of the latter, upon light absorption, would propagate down to the first, generating an allosteric light control of the protein of interest (*41*). Later, the Hahn group expanded this concept and demonstrated that the photosensing domain can be inserted directly into the protein of interest at strategic, non-conserved, surface-exposed loops that are allosterically linked to a faraway active site (*45*). The authors used the *As*LOV2 domain, which in the dark is characterized by termini in close spatial proximity (≈10 Å). Upon blue light absorption, the unfolding and undocking of the Jα helix causes local extrinsic disorder to the structure of the protein of interest, which propagates down to the active site, typically resulting in inhibition of the activity (Fig. 1G). This method was recently used by the Niopek group to create a light-inducible version of the Cas9 inhibitor AcrIIA4 (*132*) and by the Avalos and Toettcher groups to obtain optonanobodies and monobodies (*72*, *73*). Interestingly, the authors found variants whereby the *As*LOV2 domain disrupted the binding of the nanobody to its target in the dark rather than in the lit state (*72*). Thus, with this strategy it is theoretically possible to also achieve light inhibition of a protein of interest. A more detailed description of optogenetic tools for allosteric control of proteins of interest can be found in this recent review (*133*).

Controlling localization in protein condensates

Lately membraneless organelles (also called protein condensates) in both prokaryotic and eukaryotic cells have emerged as central mechanism to control biological processes (*134*, *135*). Few optogenetic tools are already available to regulate with light the formation or disassembly of such protein condensates (Fig. 1H) (*136–138*). These tools exploit the notion that intrinsically disordered regions (IDRs) of proteins mediate phase separation by weak and transient interactions among them (*139*). The association or dissociation among IDRs is regulated by light using proteins that oligomerize in the light or dark, respectively. In some cases, the protein of interest will have higher activity in the condensates (*e.g.* enzymes that function in the same metabolic pathway) (*138*). In other cases, sequestration into the condensate may inhibit function (*140*).

Nuclear Cell Biologists have a *Light* Way Ahead of Them

Optogenetics is a powerful technique that is destined to become widely used by nuclear cell biologists. Many discoveries in the field of nuclear cell biology have been already supported by optogenetic experiments (*15*, *51*, *107*, *141–145*). In theory, there is no limit to the processes that can be controlled with light using any of the strategies presented here as well as others relying for instance on photoswitchable small molecules (*146*, *147*) or chemically synthesized photoswitchable peptides (*148*). Even though some optimization is most likely required for an optogenetic tool to work as wished with a specific protein of interest, the effort is surely worth it. We envisage that in the future more emphasis will be put on developing novel optogenetic tools to control endogenous proteins, since overexpression may lead to unwanted effects or artefacts. This will go hand-in-hand with advances in the production of protein binders (DARPins, nanobodies, monobodies, peptides) with high specificity for a protein of interest (*149*). However, we wish to remind the reader that, thanks to the CRISPR/Cas genome editing technology, an endogenous gene can be exchanged with a synthetic one that codes for the protein of interest fused to the optogenetic tool; thus, the problem of protein overexpression could be bypassed even for existing tools that require the engineering of the protein of interest. In many cases, the optogenetic tool is cloned upstream or downstream of the gene of interest, which remains unaltered. Thus, the DNA fragment to insert in the genome is of acceptable size for homologous recombination-based genome editing. The Kuhlman group nicely demonstrated the feasibility of this approach by inserting their optogenetic tool for nuclear protein import LANS into the *Caenorhabditis elegans lin-1* locus using CRISPR/Cas (*50*). While it is understandable that, during the engineering phase of an optogenetic tool, plasmid-borne constructs transiently transfected in cells are a reasonable choice, when using an optogenetic tool to answer a biological question it would be more appropriate to better mimic the natural expression levels of the protein of interest.

We hope to have sparked the interest of nuclear cell biologists for optogenetics and encourage them to embrace it to unravel novel exciting mechanisms behind the organization and function of an incredibly fascinating organelle: the nucleus.

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Conflict of Interest

None declared.

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