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Control of protein activity and gene expression by cyclofen-OH uncaging

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ABSTRACT

The use of light to control the expression of genes and the activity of proteins is a rapidly expanding field. While many of these approaches use a fusion between a light activable protein and the protein of interest to control the activity of the later, it is also possible to control the activity of a protein by uncaging a specific ligand. In that context, controlling the activation of a protein fused to the modified estrogen receptor (ERT) by uncaging its ligand cyclofen-OH has emerged as a generic and versatile method to control the activation of proteins quantitatively, quickly and locally in a live organism. We here present that approach and its uses in a variety of physiological contexts.

Key words: Optogenetics, Optical control; Cre-ERT, caged molecules, cyclofen-OH

INTRODUCTION

The cells in living organisms are dynamical systems capable of responding to external signals by modifying their internal state and subsequently their external environment. As for all dynamical systems, the best way to study them is to investigate their response to local spatio-temporal perturbations[1]. To investigate these dynamical processes in a live organism, various methods (gathered under the term of optogenetics) have been developed to spatially and temporally perturb these processes using light, while monitoring the cells' response on a fast (sub-minute) timescale and single cell resolution. To photocontrol the activity of proteins (and the expression of genes), two main approaches have been adopted. One is based on light-sensitive proteins and the other on light-sensitive protein ligands.

The first light-sensitive proteins to be used in an optogenetic context were rhodopsins[2,3], which consist of a chromophore, retinal or one of its derivatives bound to a seven-domains transmembrane protein. Upon illumination, the bound retinal molecule undergoes photo-isomerization, which induces conformational changes in the opsin backbone. Channelrhodopsins [4,5] conduct cations and depolarize neurons upon blue light illumination, leading to neuronal activation. Conversely, halorhodopsins[6] pump chloride ions into the cytoplasm upon yellow light illumination, leading to hyperpolarization and inhibition of neuronal activity. These rhodopsins allow for control of neuronal networks in-vivo with unprecedented spatio-temporal resolution (reviewed in [7-9]).

Following on these pioneering works, various light-sensitive proteins were adapted as a means to control the activity of fused proteins. Flavoproteins[10-12] 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. Three major flavoproteins were used: light-, oxygen- or voltage-sensing (LOV) proteins[13]; blue light–utilizing flavin (BLUF) proteins[14]; and the light-sensitive cryptochromes (CRYs)[15]. Another two systems exploited for photo-dimerization of fused proteins is the plant phytochromes (PHY)[16] and UV-B resistance 8 (UVR8)[17]. In these systems, illumination of the chromophore induces its isomerization and triggers a protein conformational change, which can be used to control the activity of a fusion protein either directly by unmasking a protein function or indirectly through the control of protein-protein interactions.

These approaches based on the fusion between a protein of interest and a light-sensitive one have the advantage of reversibility and genomic design. However, to reduce leakage and improve signal to noise ratios may require extensive tweaking of the fused complex. Moreover, the activation of the fused protein sometimes requires prolonged illumination which defeats the purpose of the use of light as a means to improve spatio-temporal resolution[18].

To address some of these issues, another approach based on the use of caged ligands has been pursued by various groups. There exists a large variety of small molecules that bind to specific proteins, which are often transcriptional activators. These small ligands (ecdysone, doxycycline, IPTG, rapamycin, tamoxifen-OH, cyclofen-OH) can be caged and released upon illumination at an appropriate wavelength (often in the near UV - around 375 nm - but also in the visible, see below). Thus a caged ecdysone[19] was developed to create a photo-inducible gene expression system. Upon 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. Similar systems were developed based on caged-IPTG[20] (allowing for photo-induction of genes under control of the Lac operator) and caged-doxycycline[21] (photo-inducing the expression of genes under the Tet operator).

Other systems have been developed to control the activity of cytoplasmic proteins. Thus, caged rapamycin[22,23] was designed to promote the light-induced heterodimerization of two proteins fused to FK-506 binding protein (FKBP) and to the FKBP-rapamycin binding protein (FRB), enabling the photocontrol in live cells of signaling proteins (such as the small GTPase Rac involved in membrane ruffling) and the regulation of the activity of protein kinases.

In the context of the control of cytoplasmic proteins, steroid hormones and their receptors[24,25] have long been used as tissue-specific inducible systems. In absence of ligand, a protein fused to the hormone binding domain (and expressed under a tissue-specific promoter) is sequestered by cytoplasmic chaperones and therefore usually inactive. Introduction of appropriate steroids releases the fused protein from its chaperone complex and activates its function. That approach was used to induce the activity of a great variety of proteins: transcription factors[26-29] (such as Gal4, GATA or c-Jun), recombinases[30,31](such as Flp or Cre), kinases[32] (such as Erb1 or Src), oncogenes [33,34](such as B-Raf or cMyc), tumor suppressors [35,36](such as p53) and enzymes [37](such as β-Galactosidase). The caging and subsequent photo-release of steroid hormones thus offers a versatile, generic and proven approach to the quick photo-control of the activity of many proteins at a cellular level.

The advantage of caged ligands is that they build on existing inducible systems and are often characterized by a quick localized release of the ligand. However, they have to satisfy demanding physico-chemical constraints among which:

- the caged compound has to be soluble and permeate the tissues and the cells of the target organism;
- the active uncaged molecule has to be stable when illuminated;
- the illumination characteristics (intensity, wavelength, time-lapse) and both the caged inducer and the uncaged products have to be non-toxic to the cells.

A drawback of caged ligand systems is that they are irreversible. Once released they diffuse out and maybe degraded which limits their action in both space and time (though in many cases this may be desired). Moreover, issues of leakage and background activity (of the photo-activated protein) have to be addressed separately (for example by controlling the concentrations of caged-ligand, chaperones and/or receptor).

In the following we shall focus on a caged steroid we are most familiar with (caged cyclofen), which we have used in a live animal to gain a better understanding of a variety of physiological networks (cancer, somitogenesis, apoptosis, etc.). The lessons learned with this particular hormone-like molecule should be readily applicable to other steroids of the same family.

The caged-cyclofen-OH/ERT system

Within the steroid class of activators, tamoxifen-OH has long been used as the ligand of the estrogen receptor (ER) binding domain or more precisely, the ERT peptide: a binding domain engineered to interact specifically with tamoxifen-OH (in order to reduce possible interference with the endogenous estrogen pathway[38,39], one of the reason why caged-estradiol[40] is not much used). Tamoxifen-OH has been caged[41-44], but its photochemical reactivity[45,44] (isomerization) requires efficient caging groups[46] and accurate control over illumination for quantitative liberation[41,42] which may complicate its use in physiological conditions[47]. To address that issue, caged-cyclofen-OH[48] has been developed as a stable alternative to caged-tamoxifen-OH, see Fig.1. Cyclofen-OH has similar affinity to the ERT binding domain as tamoxifen-OH, is stable when illuminated and is easier to synthesize (see Supporting Information).

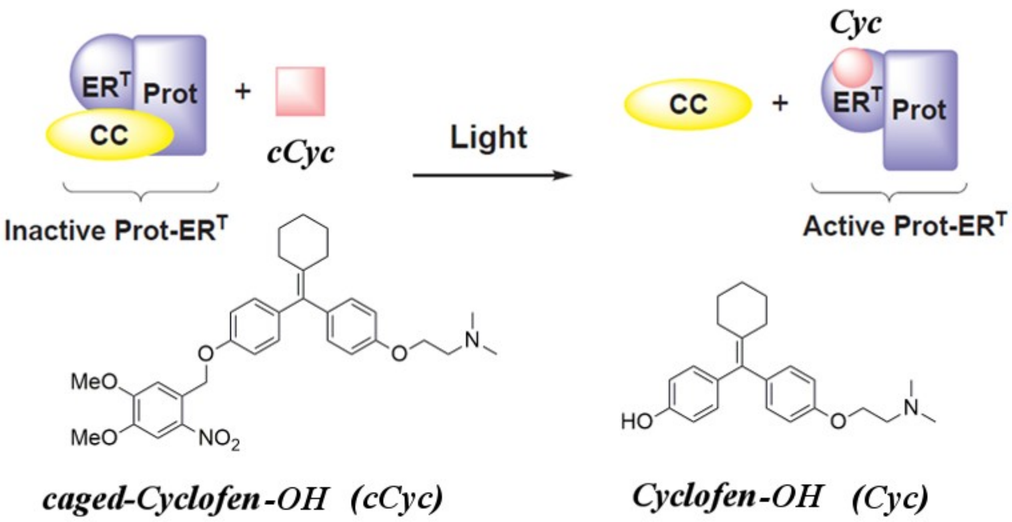


Fig.1: The caged-cyclofen-OH/ERT system. Proteins fused to the estrogen receptor (engineered to bind specifically to tamoxifen-OH, ERT) are sequestered by cytoplasmic chaperones (CC). Illumination of caged 4,5-dimethoxy-2-nitrobenzyl cyclofen-OH (cCyc) with UV (375 nm) light or multiphoton excitation (at 750 and 1064 nm with two- and three-photon excitation respectively) releases the hormone-like steroid cyclofen-OH (Cyc). Its binding to the ERT domain releases the fused protein from its cytoplasmic complex with chaperones. Adapted from Sinha et al.[48].

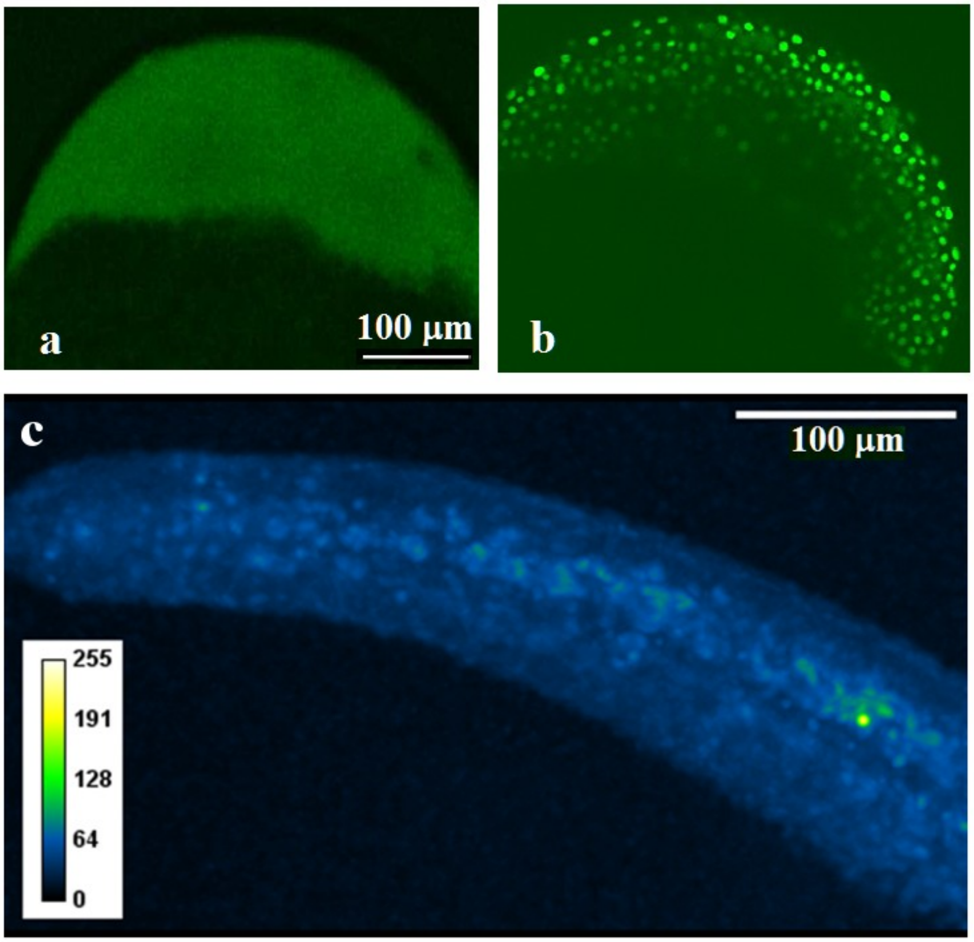
We first caged cyclofen-OH with 4,5-dimethoxy-2-nitrobenzyl alcohol, 6-bromo-7-hydroxy-4-hydroxymethyl-coumarin and 7-dimethyl-amino-4-hydroxymethylcoumarin[48,50]. In particular, the 4,5-dimethoxy-2-nitrobenzyl caged cyclofen-OH has been subsequently used in biological applications where it has been successfully uncaged with one-, two-, and three-photon excitations[48,50,51]. Since its original synthetic pathway was not compatible with production at large scales, we subsequently simplified its synthesis, which is here reported as Supporting Information[52]. The present alternative preparative method is now compatible with the production of caged precursors of cyclofen-OH up to the ten grams scale. Cyclofen-OH has been subsequently caged with coumarin-based caging moieties absorbing up to 500 nm[53]. The resulting blue light absorbing caged cyclofen-OH enabled us to achieve in living zebrafish embryos chromatic orthogonal photoactivation of two biologically active species controlling biological development with UV and blue-cyan light sources, respectively[54].

Proof of principle

To be of any use a caged-ligand system has to be demonstrated in the animal model where it will be applied. Namely it must be shown to allow control of protein activity or gene expression at the single cell level.

Transient activation must be characterized and permanent activation demonstrated (usually via the activation of a photo-controlled recombinase). These proof-of-principle experiments are our subject next.

The caged-cyclofen-OH/ERT system was first tested in model conditions[48]. Thus a fusion construct (GFP-nls-ERT) between a nuclear localized GFP and the ERT domain was injected in zebrafish embryos at the one-cell stage. The embryos were incubated in caged cyclofen-OH and the GFP fluorescence was observed to be diffused as expected from its cytoplasmic localization in a complex with chaperones, see Fig.2(a). The embryos were then illuminated for a few minutes with UV light (at 375nm), the cyclofen-OH was uncaged and the GFP released from its chaperone complex diffused within 10-15min into the cell nuclei, see Fig.2(b). Short (few seconds) two-photon illumination of caged-cyclofen-OH was sufficient for its uncaging and was shown to lead to the subsequent localization of GFP in the nucleus of the illuminated cell, Fig.2(c).



<u>Fig.2:</u> Photo-induction of GFP nuclear localization. A protein consisting of a GFP labeled with a nuclear localization signal (nls) is fused to the ERT domain. The mRNA of this construct (GFP-nls-ERT) is injected in zebrafish

embryos at the one-cell stage. (a) When the embryos are incubated in caged-cyclofen-OH, the GFP fluorescence is diffused as the construct is sequestered by cytoplasmic chaperones. (b) When they are illuminated with UV light, cyclofen-OH is released in the whole embryo, the GFP-nls-ERT protein construct is released from its chaperone complex and diffuses into the cells nuclei. (c) When illuminated with a two-photon laser for a few seconds, cyclofen-OH is released in a single cell and frees the GFP-nls-ERT protein in that cell only with subsequent increase in the fluorescence of that cell nucleus. Adapted from Sinha et al.[48].

In another set of experiments photo-activation via cyclofen-OH uncaging of a Cre-ERT recombinase led to the irreversible excision of a GFP gene flanked by loxP sites and the expression of a downstream dsRed gene[50]. Both global activation by UV illumination and local activation by two-photon illumination were demonstrated. In that case, it was shown that within one hour of photo-activation, the recombinase had floxed 50% of its targets. That experiment demonstrated the use of the caged-cyclofen-OH/ERT system for cell-specific permanent genetic modifications and in particular cell labeling.

To achieve transient genetic activation, we used a fusion between the ERT domain and a transcription factor (Gal4) which can similarly be activated by cyclofen-OH uncaging[55]. Diffusion of the transcription factor to the nucleus activates genes under control of a UAS promoter. As the released cyclofen-OH diffuses out of the cell, the Gal4-ERT proteins are re-sequestered by cytoplasmic chaperones and the transcription activity is shut off (which incidentally might give access to the kinetics of transcription bursts).

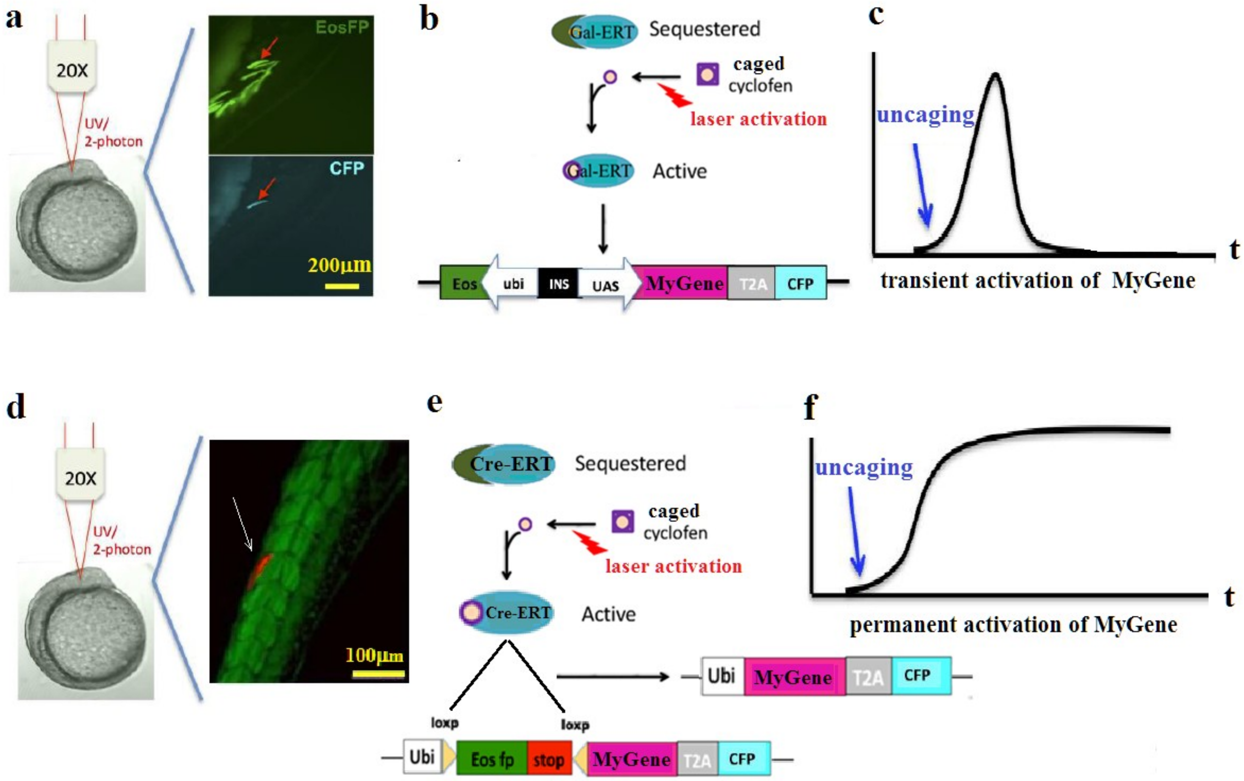


Fig.3. Transient and permanent activation of a gene of interest (MyGene). (a) Using UV or two-photon illumination, a Gal4-ERT construct is released (b) and transiently activates a gene of interest under a UAS promoter. A fluorescent CFP protein is used as a marker of activation while an EosFP serves as a marker of expression of the genetic construct injected as a plasmid at one cell stage. (c) Due to diffusion of the released cyclofen-OH, the activation of transcription lasts a few hours, but the translated protein may linger for a few days. (d) Using similar illumination on a Cre-ERT construct (e) may permanently label a single cell (shown here in red) since diffusion of the released cyclofen-OH will not alter the gene excision (e) performed by the Cre-recombinase. This approach can be used to permanently activate a gene of interest (f). Adapted from Feng et al.[55].

Applications in physiological contexts.

The caged-cyclofen-OH/ERT system has been used to control the expression of genes and/or the activity of proteins in order to investigate a number of physiological networks in various organisms.

Tumorigenesis is supposed to originate from rare single cell mutational event(s), however the probability of tumorigenesis when a single oncogene is turned on in a single cell has never been assessed. We decided to use our optogenetics approach to address that issue. Carcinogenesis was investigated in zebrafish by initiating the expression of a single oncogene (kRasG121V) in 2-3 days old embryos[55]. We showed that transient activation of the oncogene, Fig.3(a-c), did not result in cancer Fig.4(a-c), whereas permanent activation, Fig.3(d-f), led in some cases to tumors, Fig.4(d-f) albeit with a very low probability (about 0.5% of induced cells gave rise to a tumor). This work suggests that a single somatic mutation may not be enough to initiate a tumor but that multiple factors (and mutations) maybe required for a single cell to develop a tumor. The caged-cyclofen-OH/ERT system may help one address that issue by simultaneously inducing more than one oncogene in one (or a few possibly confluent) cell(s) and in various backgrounds (such as p53$^{-/-}$ and immuno-deficient embryos).

Somitogenesis was also investigated using the caged-cyclofen-OH/ERT system[56]. In this instance, we were interested in studying the network sustaining the propagating wavefront in the clock and wavefront model of somitogenesis[49]. The expression of an exogenous Fgf8 was induced via the activation of protein fusion between ERT and a Gal4 transcription factor binding to a UAS promotor driving the expression of Fgf8. It was shown that induction of Fgf8 expression led to severe developmental defects and a decrease in the size of somites, Fig.4(g,h). This assay allowed (in conjunction with more established approaches) to quantitatively investigate the Godbeter-Pourquié model of the somitogenetic wavefront[49].

To monitor the development of various organs, it is desirable to have a means to irreversibly label specific cells at a given developmental stage and monitor their progeny. The optogenetic method described here is ideally suited to that purpose. The caged-cyclofen-OH/Cre-ERT system was used to label cell clusters, with UV light (365nm) in a mouse context (skin and mammary gland)[57], and single cells with a 2-photon (750 nm) in zebrafish[50], see Fig.3(d) and with a 3-photon (1064nm) pulse-excitation in ~200 μm deep heart muscle of a zebrafish embryo[51]. These experiments demonstrate that photo-activation of caged-cyclofen-OH can be implemented in a variety of biological contexts using different illumination methods to label specific cells in the animal. These experiments open the way for cell labeling and monitoring in live animals over extended periods of time.

During embryonic development, many genes are involved at different time points and classical gain or loss of function by genetic approaches often fail to decipher protein function(s) at later stages of development[58,59]. Cyclofen-OH release overtakes this problem by allowing protein activation at any chosen developmental stage. A good example is the homeoprotein Engrailed 2 (En2), known to be important for brain regionalization[60,61], but which may interfere with earlier axis formation[62]. To decipher the time window during which En2 is active on the patterning of mesencephalic boundaries, we took advantage of the time-controlled activation of a fusion protein En2-ERT[62]. A mRNA coding En2-ERT was injected in zebrafish embryos at one cell stage and embryos incubated in caged-cyclofen-OH. Cyclofen-OH was then released upon time by one photon uncaging, and phenotypes were scored 48 hours post-fertilization. En2-ERT photo-activation prior to gastrulation induced a widespread insult resulting in abnormal axis and heart development defects in 40% of the embryos, while En2-ERT photoactivation at 50% and 70% epiboly impaired eye formation without any sign of axis abnormality. This impairment of eye formation (eye with reduced size or no eye phenotype) is clearly due to an expansion of the mesencephalon and a reciprocal reduction of the diencephalon. Later on, activation of En2-ERT at the beginning of somitogenesis (1- 2 somites) induced almost no phenotype. The time window at which En2 controls the diencephalic-mesencephalic boundary was thus defined in between 50% and 70% epiboly.

Another application is the activation of a protein that is deleterious for the cell. Apoptotic cells release signaling molecules that are of importance for developmental programs and homeostasis. Failure of apoptosis is one of the main contributions to tumour development and autoimmune diseases. To better understand this signaling pathway, one would like to induce apoptosis with a good spatiotemporal resolution, the ultimate goal being to do so at the single-cell level and at minute scale resolution. We took advantage of the cascade of proteases involved in apoptosis and used a fusion between one of the first protease in the cascade (Casp9) and the ERT domain[63]. The Casp9-ERT fusion protein has no activity when expressed in zebrafish embryo but induces apoptotic cell death in the presence of cyclofen-OH. Using caged-cyclofen-OH and two-photon uncaging allowed us to trigger apoptosis in a single cell of a developing embryo.

Stop codon suppression (particularly the amber stop codon) has become a popular strategy to introduce unnatural amino acids (Uaas) into a protein at a specific site, which is commonly referred as the genetic code expansion. The methodology involves the read-through of an amber stop codon inserted into the gene of the targeted protein by a suppressor tRNA aminoacylated with a desired Uaa. Varieties of Uaas containing side-chains that serve as photoactive, spectroscopic and redox probes have been successfully introduced into proteins, which facilitate the development of novel detection methods of specific protein function *in vivo*[64,65]. Recently, we and others have generated zebrafish with the gene of orthogonal aminoacyl-tRNA synthetase (RS)/suppressor tRNA pairs incorporated into the host genome[66,67]. These transgenic fish can utilize *p*-azido-L-phenylalanine (AzF)[66] or lysine derivatives[67] for protein synthesis in response to the amber stop codon.
We have used our caged-cyclofen-OH/ERT system to activate a RS (AzFRS) aiming to control the genetic code expansion scheme at the single cell level and to alleviate possible long-term physiological impairment to zebrafish resulting from global or constitutive suppression of genomic stop codons. In preliminary experiments with cell cultures, we have seen that a fusion

between AzFRS and ERT could indeed be activated by cyclofen-OH to initiate amber suppression and AzF incorporation.

The previous examples demonstrate the variety of applications and usefulness of the caged-cyclofen-OH/ERT approach, which provides for a generic approach to the control of protein activity and gene expression in a variety of physiological contexts and animal models.

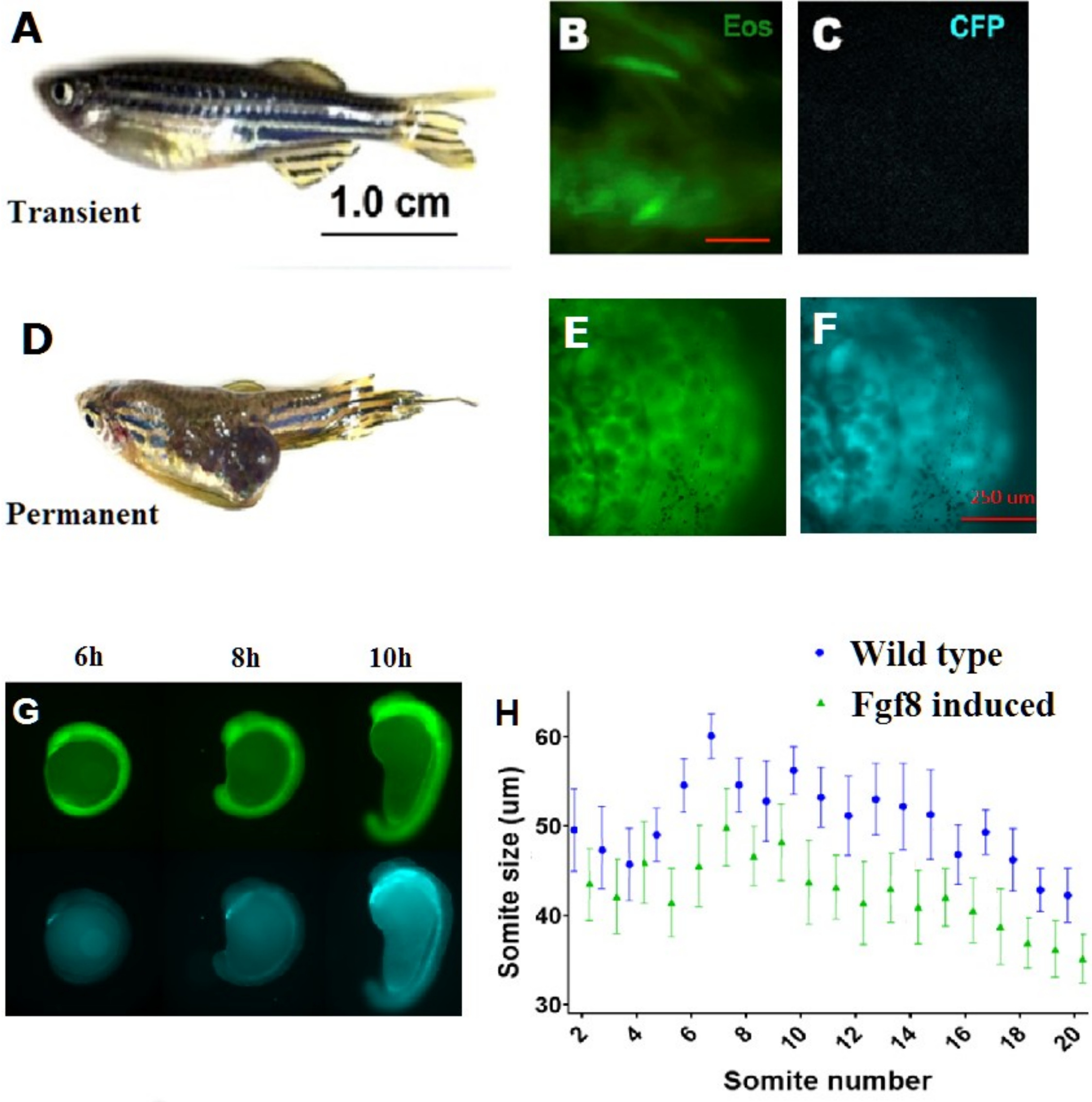


Fig.4: Examples of the use of the caged-cyclofen-OH/ERT system in a variety of physiological contexts. (A-C) transient activation of an oncogene (kRasG12V), see Fig.3(a-c), in a zebrafish embryo does not lead to cancer. On the other hand permanent induction (D-F, see also Fig.3(d-f)) can lead to tumorigenesis as demonstrated by the tumor growth (D) and the presence of the oncogene marker (CFP, F). In the context of development permanent activation of Fgf8 yields to an increase in the concentration of the protein

and its fluorescent marker (CFP) during somitogenesis with observable phenotypic consequences (G) and in particular a shortening of the somite size. (H). Adapted from Feng et al.[55].

CONCLUSIONS

In this article, we have described a generic and versatile approach to the control of gene expression and protein activity in a live animal at the level of a single cell, and with temporal resolution of a few seconds. This approach builds on the well-established conditional ERT/tamoxifen-OH genetic induction system. That approach has been used to induce the activation of specific genes (permanently using a Cre-ERT/loxP or transiently using a Gal-ERT/UAS system) in different organisms (zebrafish, mice, fly, etc.). The conditional activation of many proteins has also been achieved by fusing them with the ERT peptide. Thus many transgenic lines of various organisms exist that allow for this conditional activation globally or locally in specific tissues. We have shown that the use of caged cyclofen-OH together with a well-established conditional expression system can be utilized to induce rapid activation of genes and proteins at the single cell level in a live organism. On the basis of the similarity between our ligand and other steroids, we believe that similar results could be obtained with other caged steroids of the same family (estradiol, tamoxifen-OH, etc.).

The main advantage of the approach sketched here is its versatility and ease of implementation within an existing context of appropriate transgenic lines. It allows for fast activation (with a few seconds of illumination) at intensities and wavelengths that are not detrimental to the cell. Its main drawbacks are:

1) The lack of control on the time lapse of activation (once the caged steroid is photo-activated it cannot be de-activated). Transient activation relies on the steroid unbinding from the ERT peptide and its diffusion out of the induced cell(s) within an ill-controlled timescale (typically a few hours). This is in contrast with some photo-activable proteins which dimerization can be induced and reversed by illumination at appropriate wavelengths.
2) The lack of sub-cellular control. Once released the steroid diffuses throughout and out of the cell (though the protein-ERT construct may be targeted to specific organelle). This is in contrast with some photo-activable proteins which dimerization can be induced locally within a cell.

Nonetheless, we believe that the caged-cyclofen-OH/ERT system provides an alternative and complementary approach to the optogenetic methods that rely on photo-induced conformational changes in some proteins to control the activity of their fused partner.

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