Optical control of protein activity and gene expression by photoactivation of caged cyclofen

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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 activatable protein and the protein of interest to control the activity of the latter, 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. Here, we present the experimental details behind this approach.

1. Introduction

The study of physiological processes at the single cell level is a blooming field that is driving the development of many tools and approaches, from super-resolution (Gao et al., 2012) and light-sheet microscopy (Mickoleit et al., 2014) to optogenetics (Kralj, Douglass, Hochbaum, Maclaurin, & Cohen, 2011; Miesenböck, 2009). To investigate physiological processes non-invasively, various methods have been developed that use light to spatially and temporally perturb these processes, while monitoring the cells' response on a fast (sub-minute) timescale and single cell resolution (Hochbaum et al., 2014). In particular 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 (Sakmar, 2002; Zemelman, Lee, Ng, & Miesenböck, 2002). These light sensitive ion channels were first used as actuators of neuronal networks, i.e., to depolarize (Lórenz-Fonfría & Heberle, 2014; Nagel et al., 2002) (activate) or hyperpolarize (Bamberg, Tittor, & Oesterhelt, 1993) (inhibit) neuronal activity. These rhodopsins allow for control of neuronal networks in vivo with unprecedented spatio-temporal resolution (Fenno, Yizhar, & Deisseroth, 2011; Hegemann &

Nagel, 2013; Zhang et al., 2011). More recently these channels have been modified to be used as sensors of the transmembrane voltage (Hochbaum et al., 2014).

Following on these pioneering studies, various light-sensitive proteins were adapted as a means to control the activity of fused proteins. These photo-sensitive proteins undergo a conformational change via the photo-isomerization of a bound chromophore naturally present in most cells, such as riboflavin (Conrad, Manahan, & Crane, 2014; Crosson, Rajagopal, & Moffat, 2003; Hemmerich, Nagelschneider, & Veeger, 1970; Kim & Lin, 2013; Masuda, 2013), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) or cryptochromes (Chaves et al., 2011) (CRY), phytochromes (PHY; Rockwell, Su, & Lagarias, 2006). This conformational change can induce the dimerization of the photo-sensitive protein with its partner (and consequently the coupling of appropriately fused proteins). Alternatively, the conformational change can directly control the activity of the fused protein.

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, reducing leakage and improving signal-to-noise ratios often requires extensive optimization 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 (Gautier et al., 2014).

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) or infrared via two-photon excitation). Thus, a caged ecdysone (Lin, Albanese, Pestell, & Lawrence, 2002) 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 (Young & Deiters, 2007) allowing for photo-induction of genes

under control of the Lac operator and caged-doxycycline (Cambridge, Geissler, Keller, & Cürten, 2006) photo-inducing the expression of genes under the Tet operator.

Other systems have been developed to control the activity of cytoplasmic proteins when it depends on dimerization. Thus, caged rapamycin (Karginov et al., 2011; Umeda, Ueno, Pohlmeyer, Nagano, & Inoue, 2011) 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 (Beato, Herrlich, & Schütz, 1995; Norman, Mizwicki, & Norman, 2004) 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 large variety of proteins: transcription factors (Braselmann, Graninger, & Busslinger, 1993; Briegel et al., 1993; Hyder, Nawaz, Chiappetta, Yokoyama, & Stancel, 1995; Kulessa, Frampton, & Graf, 1995) such as Gal4, GATA or c-Jun, recombinases (Metzger, Clifford, Chiba, & Chambon, 1995; Nichols, Rientjes, Logie, & Stewart, 1997) such as Flp or Cre, kinases (Picard, 2000) such as Erb1 or Src, oncogenes (Gandarillas & Watt, 1997; McMahon, 2001) such as B-Raf or cMyc, tumor suppressors (Jia et al., 2013; Roemer & Friedmann, 1993) such as p53 and enzymes (Wang et al., 1995) 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:

- As any drug, 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 uncaging is irreversible, though the effect of the released ligand can be limited in time via diffusion and degradation. Moreover, issues of leakage and background activity have to be addressed separately by controlling the concentrations of ligand and/or receptor.

2. 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, ER^{T2} (hereafter shorten as ERT) peptide: a binding domain engineered to interact specifically with tamoxifen-OH in order to reduce possible interference with the endogenous estrogen pathway (Feil et al., 1996; Feil, Wagner, Metzger, & Chambon, 1997). Tamoxifen-OH has been caged (Link, Shi, & Koh, 2005; Shi & Koh, 2004; Wong et al., 2017) but its instability under illumination hinders its use in physiological conditions (Inlay et al., 2013). To address that issue, caged-cyclofen-OH (Sinha, Neveu, Gagey, Aujard, Benbrahim-Bouzidi, et al., 2010a) 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 and is stable when illuminated.

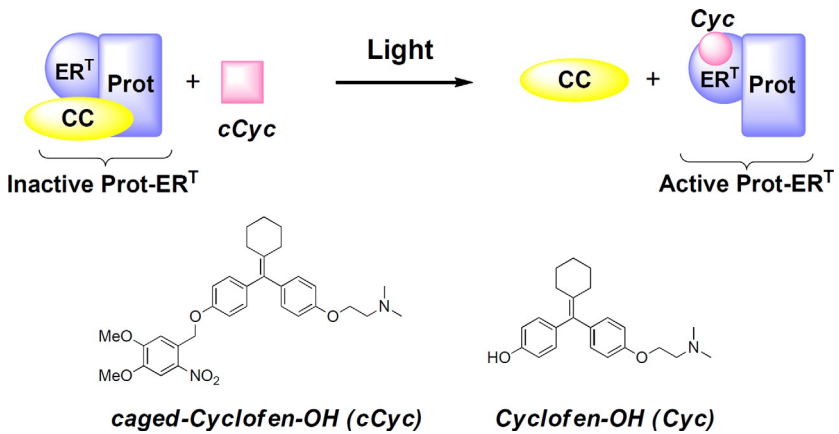


Fig. 1 The caged-cyclofen-OH/ERT system. Proteins fused to the estrogen receptor engineered to bind specifically to tamoxifen-OH (ERT) are sequestered by cytoplasmic chaperones (CC). Illumination of caged 4,5-dimethoxy-2-nitrobenzyl cyclofen-OH (**cCyc**) with UV (375 nm) light or multiphoton excitation (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 frees the fused protein from its cytoplasmic complex with chaperones.

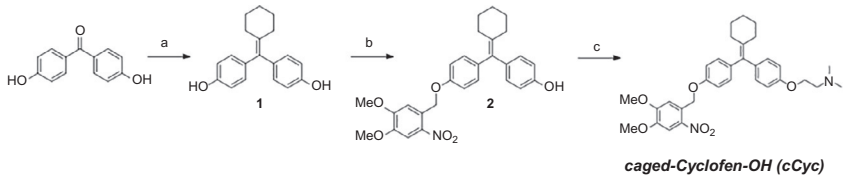
Cyclofen-OH has been caged with a 4,5-dimethoxy-2-nitrobenzyl moiety (Sinha, Neveu, Gagey, Aujard, Benbrahim-Bouzidi, et al., 2010a; Sinha, Neveu, Gagey, Aujard, Le Saux, Rampon, et al., 2010b) and has been used in various biological contexts to activate proteins fused to ERT with one-, two-, and three-photon excitations or to modify the spectral properties of fluorescent proteins (Sinha, Neveu, Gagey, Aujard, Benbrahim-Bouzidi, et al., 2010a; Sinha, Neveu, Gagey, Aujard, Le Saux, Rampon, et al., 2010b; Jullien & Aujard, 2017; Rampon et al., 2015). Since its original synthetic pathway was not compatible with production at large scales, we subsequently modified its synthesis (Jullien & Aujard, 2017). The present alternative preparative method (see below) is now compatible with the production of caged precursors of cyclofen-OH up to the 10 g scale. Cyclofen-OH has been subsequently caged with coumarin-based caging moieties absorbing up to 500 nm (Fournier et al., 2013). The resulting blue light absorbing caged cyclofen-OH enabled us to achieve in live zebrafish embryos chromatic orthogonal photoactivation of two biologically active species controlling biological development with UV and blue-cyan light sources, respectively (Fournier et al., 2013).

In the following we shall first describe how caged cyclofen-OH is synthesized, how its decaging can be calibrated against illumination duration and intensity and how it is used in the context of zebrafish physiology.

3. Synthesis of caged cyclofen-OH

Since the original synthesis reported in (Fournier, Aujard, et al., 2013) was not compatible with large scale production and required a final careful purification step to isolate the caged compound from its active precursor, we developed an alternative preparative method, which is compatible with the production of caged precursors of cyclofen-OH up to the 10-g scale.

The new synthetic pathway starts with a McMurry coupling on 4,4′-dihydroxybenzophenone, enabling to introduce the cyclohexyl ring. The second step introduces the caging arylalkyl moiety by phenol monosubstitution. The second phenol group is then alkylated (in the original protocol, the order of the latter steps was reversed). The overall yield for the synthesis of compound **cCyc** from 4,4′-dihydroxybenzophenone is 47% and intermediate purifications now rely on precipitations only. This route of synthesis is summarized in Scheme 1.



Scheme 1 Improved synthesis of the caged cyclofen-OH **cCyc**. (a) cyclohexanone, $TiCl_4$, Zn, THF, reflux, 2 h (yield: 81–85%); (b) 4,5-dimethoxy-2-nitrobenzyl bromide, K_2CO_3, acetonitrile/H_2O, r.t., 16 h; (c) 2-(dimethylamino)ethyl chloride hydrochloride, Cs_2CO_3, acetone, reflux, 18 h (yield: 70%).

4. Protocol for the synthesis of caged cyclofen-OH (cCyc)

4.1 Equipment

1. Three-necked, round-bottomed flask (500 mL) equipped with a magnetic stirring bar, a thermometer, a condenser connected to an argon inlet, a septum/an addition funnel (100 mL); 5, 10, 50 mL syringes, funnel (100 mL) containing a plug of glass wool; separating funnel, fritted glass; round-bottomed flask (250 mL)
2. Stirring hotplate
3. Analytical balance
4. Ice bath; oil bath
5. Vacuum pump
6. Rotary evaporator
7. Merck silica gel 60F254 precoated plates for thin layer chromatography.; UV lamp (254/365 nm)
8. Aluminum foil
9. NMR spectrometer

4.2 Chemicals

General procedures: Commercially available reagents were used as obtained. The ^{1}H and ^{13}C NMR spectra were recorded at room temperature on Bruker AM 250, 300 or 400 spectrometers; chemical shifts are reported in ppm with protonated solvent as internal reference (^{1}H, $CHCl_3$ in $CDCl_3$ 7.26 ppm, CHD_2SOCD_3 in CD_3SOCD_3 2.49 ppm; ^{13}C, $^{13}CDCl_3$ in $CDCl_3$ 77.0, $^{13}CD_3SOCD_3$ in CD_3SOCD_3 39.7 ppm); coupling constants (*J*) are given in Hz. Microanalyses were performed by the Service de

Microanalyses de Gif sur Yvette. Mass spectra (chemical ionization and high resolution with NH_3 or CH_4) were performed by the Service de Spectrométrie de Masse de Chimie ParisTech (MS) and by the Service de Spectrométrie de Masse de l'ICOA (HRMS, Orléans).

1. Dry tetrahydrofuran (Sigma-Aldrich)
2. Titanium chloride (Sigma-Aldrich)
3. Zinc powder (Sigma-Aldrich)
4. Argon gas
5. 4,4′-Dihydroxybenzophenone (Alfa Aesar)
6. Cyclohexanone (Sigma-Aldrich)
7. Potassium carbonate (K_2CO_3) (VWR)
8. Ethyl acetate (VWR)
9. Brine (saturated solution of NaCl in water)
10. Magnesium sulfate ($MgSO_4$) (VWR)
11. Dichloromethane or cyclohexane/ethyl acetate: 3/1, v/v; (VWR)
12. Silica gel 60 (0.040–0.063 nm; Merck) for column chromatography (Sigma-Aldrich)
13. DMSO-d_6 and $CDCl_3$ for NMR experiments (Sigma-Aldrich)
14. Acetonitrile (ACN)/H_2O (2/1, v/v).(VWR)
15. 4,5-Dimethoxy-2-nitrobenzyl bromide (Sigma-Aldrich)
16. Phosphorous pentoxide (P_2O_5) (VWR)
17. 2-(Dimethylamino)ethyl chloride hydrochloride (Alfa Aesar)
18. Acetone (VWR)
19. Cesium carbonate (Cs_2CO_3) (VWR)
20. Methanol (VWR)

4.3 Protocol

Synthesis of 1: **4-(cyclohexylidene(4-hydroxyphenyl)methyl) phenol**

1.1 Working at −10 °C, add titanium chloride (6.2 mL, 56 mmol; 6 equivalents in 4,4′-dihydroxybenzophenone) dropwise under argon to a stirred suspension of zinc powder (8.20 g, 126 mmol; 14 equivalents in 4,4′-dihydroxybenzophenone) in 80 mL of dry tetrahydrofuran.

1.2 Warm the mixture to room temperature and reflux for 2 h.

1.3 Working at 0 °C, add a solution of 4,4′-dihydroxybenzophenone (2.0 g, 9 mmol) and cyclohexanone (4 mL, 38 mmol; 4 equivalents in 4,4′-dihydroxybenzophenone) in 120 mL of dry tetrahydrofuran to the cooled mixture and reflux for 2 h.

1.4 Bring the reaction mixture to room temperature and add 10% (w/v) aqueous potassium carbonate (30 mL). Filter and extract with ethyl acetate.

1.5 Wash the organic layer with brine, dry over $MgSO_4$, and concentrate in vacuo.

1.6 Load the concentrate on a column chromatography (cyclohexane/ethyl acetate, 3:1, v/v) to yield **1** as a white powder (2.15 g, 85%). Alternatively, precipitation of the concentrate in dichloromethane affords **1** (2.1 g, 81%). Precipitation can also be carried out in cyclohexane or in a cyclohexane/ethyl acetate mixture.

1.7 Perform NMR analysis of the white powder **1** to obtain: ^{1}H NMR (250 MHz, DMSO-d_6, δ): 9.28 (s, 2H), 6.82 (d, 4H, $J=8$ Hz), 6.65 (d, 4H, $J=8$ Hz), 2.14 (m, 4H), 1.52 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6, δ): 155.5 (2C), 136.2, 133.8, 133.6 (2C), 130.3 (4C), 114.6 (4C), 31.9 (2C), 28.1(2C), 26.3. These data are in line with the reported values for the same compound (Sinha, Neveu, Gagey, Aujard, Benbrahim-Bouzidi, et al., 2010a; Sinha, Neveu, Gagey, Aujard, Le Saux, Rampon, et al., 2010b).

Synthesis of 2: **4-((4-(4,5-dimethoxy-2-nitrobenzyloxy)phenyl) (cyclohexylidene)methyl)phenol**

2.1 Prepare a mixture of **1** (1.0 g, 3.5 mmol) and potassium carbonate (470 mg, 3.4 mmol, 0.95 eq.) in acetonitrile/H_2O (60 mL, 2/1, v/v).

2.2 Add portion-wise 4,5-dimethoxy-2-nitrobenzyl bromide (940 mg, 3.4 mmol, 0.95 eq.) over a period of 1 h (about 150 mg every 10 min).

2.3 Stir the resulting mixture for 16 h at room temperature in the dark.

2.4 Filter the precipitate, wash with acetonitrile and then dry in vacuo over P_2O_5.

2.5 Analyze the white powder obtained **2** (1.4 g containing 96% of **2**, 4% of the product of **1** dialkylation, and traces of **1**; this mixture was used without further purification for the next step) by NMR to yield: ^{1}H NMR (ppm, 300 MHz, $CDCl_3$, δ): 7.77 (s, 1H), 7.35 (s, 1H), 7.04 (AA′XX′, 2H, $J=9$ Hz), 6.97 (AA′XX′, 2H, $J=9$ Hz), 6.91 (AA′XX′, 2H, $J=9$ Hz), 6.73 (AA′XX′, 2H, $J=9$ Hz), 5.48 (s, 2H), 4.68 (s, 1H), 3.97 (s, 3H), 3.95 (s, 3H), 2.23 (m, 4H), 1.57 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6, δ): 156.4, 155.9, 153.3, 148.0, 139.9, 137.1, 136.2, 133.7, 133.5, 130.7 (2C), 130.5 (2C), 122.5, 114.9 (2C), 114.5 (2C), 111.5, 108.4, 66.7, 56.3, 56.2, 32.1 (2C), 28.3 (2C), 26.4; MS (CI, NH3): m/z 493.30 (calculated average mass for $[C_{28}H_{29}NO_6+NH_4]^+$: 493.23; HRMS (TOF MS ES+): m/z 476.2077 (calculated mass for $[C_{28}H_{29}NO_6+H]^+$: 476.2073.

Synthesis of cCyc: 2-(4-((4-(4,5-dimethoxy-2-nitrobenzyloxy) phenyl)cyclohexylidene)methyl)phenoxy-*N*,*N*-dimethyl-ethanamine

3.1 Prepare a solution of **2** (800 mg, 1.68 mmol) in acetone (50 mL) and Cs_2CO_3 (1.64 g, 5 mmol, 3 eq.).

3.2 Add 2-(dimethylamino)ethyl chloride hydrochloride (484 mg, 3.36 mmol, 2 eq.) portion-wise over a period of 30 min and stir the resulting suspension at reflux temperature in the dark for 18 h.

3.3 Cool the reaction mixture to room temperature and filter it through a funnel containing a plug of glass wool or cotton.

3.4 Wash the precipitate with dichloromethane and evaporate the solvent.

3.5 Load the concentrate on a column chromatography (CH_2Cl_2/Methanol: 9/1) to yield **cCyc** as a light brown powder (640 mg, 70% yield). **cCyc** can be obtained as yellowish crystals after recrystallization from isopropyl ether (m.p.: 116–117 °C) (Sinha, Neveu, Gagey, Aujard, Benbrahim-Bouzidi, et al., 2010a; Sinha, Neveu, Gagey, Aujard, Le Saux, Rampon, et al., 2010b).

3.6 Analyze product with NMR to yield: ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.76 (s, 1H), 7.35 (s, 1H), 7.04 (AA′XX′, 2H, J = 9 Hz), 7.00 (AA′XX′, 2H, J = 9 Hz), 6.90 (AA′XX′, 2H, J = 9 Hz), 6.82 (AA′XX′, 2H, J = 9 Hz), 5.47 (s, 2H), 4.03 (t, 2H, J = 6 Hz), 3.96 (s, 3H), 3.94 (s, 3H), 2.70 (t, 2H, J = 6Hz), 2.32 (s, 6 H), 2.23 (m, 4H), 1.58 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 157.0, 156.2, 153.8, 147.7, 138.9, 138.5, 136.8, 137.7, 133.2, 131.0 (2C), 130.7 (2C), 129.6, 114.3 (2C), 113.8 (2C), 109.4, 107.9, 67.0, 65.8, 58.3, 56.3, 56.3, 45.8 (2C), 32.4 (2C), 28.6 (2C), 26.8. elemental analysis calcd (%) for $C_{32}H_{38}N_2O_6$ (546.66): C 70.32, H 7.00, N 5.12; found: C 70.11, H 7.24, N 5.02.

5. Calibration of caged-cyclofen photo-activation

The liberation of the cyclofen-OH inducer necessitates photo-activation, which involves light absorption by the caged precursor **cCyc** followed by effective bond breakage leading to **cyclofen-OH** and the nitrosobenzaldehyde by-product from the caging moiety (Zhang et al., 2018). The corresponding process can be written as the chemical reaction (1).

$$\textbf{cCyc} + \mathrm{n}\,h\nu \rightarrow \textbf{cyclofen-OH} + \text{by-product} \quad (1)$$

where n $h\nu$ (n = 1 or 2 with one- and two-photon excitation) designates the photon(s) effectively driving the reaction. These photons are provided by a light source and the experimentalist should provide enough photons to exhaust the conversion of the caged precursor but without generating detrimental side-effects on the biological sample. Therefore one should always characterize a light source to achieve and optimize any photoactivation experiment. Several criteria should be taken into account.

5.1 The wavelength range of the light source

To be effective, the photoactivation wavelength should be within the absorption range of the caged precursor or more precisely of the caging moiety. With respect to **cCyc**, this information has been provided in (Aujard et al., 2006) for one- and two-photon excitation. The wavelength of the light source should thus be comprised between 325 and 425 nm with one-photon excitation and between 700 and 800 nm with two-photon excitation. Possible relevant light sources are benchtop UV lamps (generally used to read out thin layer chromatography plates in the chemical laboratory; to photoactivate biological samples over large areas), or light sources installed on microscopes. With one-photon excitation, light sources delivering photons at 405 nm are usually available but some setups provide access to excitation wavelengths down to 365 nm (as used for DAPI imaging). With two-photon excitation, the microscope should be equipped with a femtosecond pulsed laser source delivering 150–200 fs laser pulses around 750 nm.

5.2 The power range of the light source

Once the light source has been identified, it needs to be calibrated for the photon flux it generates at the biological sample. A light source is generally characterized by its power P. This power can be directly measured with a power-meter when it is available (e.g., from Thorlabs). The sensitive element of the power-meter should be located at the position of the biological sample (e.g., at the front of a microscope objective). Alternatively, one can rely on a calibration exploiting the photoconversion of a reference (see below).

5.3 Tuning the illumination for uncaging experiments

With one-photon excitation, the power P (in Watt or Joule s^{-1}) first has to be transformed into a photon flux J (in mol m^{-2} s^{-1}). Knowing the

frequency ν (expressed in Hz) of the light source (or its wavelength: $\lambda = c/\nu$) and the surface S (expressed in m^2) of the illuminated zone, one has

$$J = P/N_A h\nu\, S \tag{2}$$

In Eq.(2), the denominator is the energy of 1 mol of photon and it involves the Avogadro number N_A ($= 6 \times 10^{23}$ photons/mole) the Planck constant h, and the light frequency ν (for $\lambda = 350$ nm: h ν = h $c/\lambda = 5.68 \times 10^{-19}$ J). Then the rate constant k of the uncaging reaction (1) (expressed in s^{-1}) can be calculated using Eq.(3)

$$k = \varepsilon(\lambda)\phi J \tag{3}$$

where $\varepsilon(\lambda)$ is the molar absorption coefficient of the caging moiety at wavelength λ (expressed in $m^2\ mol^{-1}$; it can be calculated by writing $\varepsilon(\lambda)$ $[m^2\ mol^{-1}] = 0.1\ \varepsilon'(\lambda)$ $[M^{-1}\ cm^{-1}]$) and ϕ is the quantum yield of uncaging, which measures the probability of releasing the caged substrate once light has been absorbed by the caged precursor. For **cCyc**, one has $\varepsilon'(350\,nm) = 6000\ M^{-1}\ cm^{-1}$, $\varepsilon'(400\,nm) = 2000\ M^{-1}\ cm^{-1}$, and $\phi = 6 \times 10^{-3}$. To fully achieve uncaging of **cCyc**, one should illuminate the sample for a typical duration of about $5/k$ (in seconds). With a typical laser excitation power $P = 10\,\mu W$ (at the focal plane of the microscope objective) focused on a spot of diameter 10 μm (the minimal spot size should be commensurate with the typical size of the cell cross-section): $k(350\,nm) \sim 1\,s^{-1}$. Hence illumination of a few seconds is enough to achieve full uncaging of **cCyc** in the few cells along the optical path.

Eq.(3) can also be used to calibrate the light source when no power-meter is available. One should use a photoactive reference (characterized by $\varepsilon_{ref}(\lambda)$ and ϕ_{ref}) the fluorescence of which strongly changes upon illumination. In a first step, the light source is applied on a sample containing the photoactive reference while recording its fluorescence decay. The fluorescence decay is then fitted to an exponential form:

$$I_F(t) = I_F(0) + [I_F(\infty) - I_F(0)]\left[1 - \exp\left(-k_{ref}t\right)\right] \tag{4}$$

in order to retrieve k_{ref}. In Eq.(4), $I_F(t)$, $I_F(0)$, and $I_F(\infty)$ designate the recorded fluorescence intensity at time t, time zero, or at steady-state (infinite time). The photon flux is then deduced from Eq. (3):

$$J = \frac{k_{ref}}{\varepsilon_{ref}(\lambda)\phi_{ref}} \tag{5}$$

In particular, Quérard et al. (2017) describe how fixed cells expressing genetically encoded reversibly photo-switching proteins can be used as references for calibrating light fluxes at 405 nm at the focal plane of microscope objectives.

With two-photon excitation, the uncaging rate constant within the excitation volume V_{exc} can be expressed (Sinha, Neveu, Gagey, Aujard, Benbrahim-Bouzidi, et al., 2010a) as:

$$k_{exc} = 0.737\delta_u \frac{T}{\tau_P}\left(\frac{P}{\pi h\nu\omega_{xy}^2}\right)^2 \tag{6}$$

where δ_u is the two-photon uncaging cross *sec*tion of the caging group at the excitation wavelength λ (for the 4,5-dimethoxy-2-nitrobenzyl caging group $\delta_u(750\,\text{nm}) = 2\times10^{-52}\ \text{cm}^4\,\text{s photon}^{-1} = 2\ 10^{-60}\ \text{m}^4\,\text{s photon}^{-1}$). T is the period of the laser pulses (typically 10 ns for a pulse rate of 100 MHz) of incident power at the focal point P, duration τ_P (typically 200 fs) and beam waist ω_{xy}. P can be measured with a power-meter, T and τ_P are specific to the laser source and are provided by the manufacturer. ω_{xy} can be measured by fluorescence correlation spectroscopy (using a reference fluorophore) or alternatively estimated (Zipfel, Williams, & Webb, 2003) using the numerical aperture NA of the microscope objective (typically NA ~1):

$$\omega_{xy} \approx \frac{0.23\,\lambda}{NA} \tag{7}$$

For a typical power $P = 10\,\text{mW}$, $k_{exc} \approx 2\ 10^4\ \text{s}^{-1}$. When uncaging with two-photon excitation is performed within a single cell of volume V, the rate constant for uncaging within the cell is given by:

$$k = \frac{V_{exc}}{V} k_{exc} \tag{8}$$

where $V_{exc} \approx 5.57\ \omega_{xy}^2\,\omega_z$ can be measured by FCS or alternatively estimated from the wavelength λ, the numerical aperture NA and the refractive index n of the medium, (Zipfel et al., 2003; $n = 1.33$ for water) with:

$$\omega_z \approx 0.38\,\lambda\left(\frac{1}{n-\sqrt{n^2-NA^2}}\right) \approx 0.8\,\lambda \tag{9}$$

For the values quoted above $V_{exc} \approx 0.1\ \mu\text{m}^3$. Thus for a typical cell of volume $V = 100$–$1000\ \mu\text{m}^3$, the uncaging rate $k = 2$–$20\,\text{s}^{-1}$. To achieve full uncaging of **cCyc** in one cell, one should illuminate the sample for a duration of about $5/k$, typically about 1 s.

6. One- and two-photon activation in zebrafish

Based on the previous considerations and estimates one should first test the uncaging of **cCyc** in an appropriate line of zebrafish, such as Tg(ef1a:loxP-GFP-loxP-dsRed2), available from Zfin (line ne2466tg) where gene activation is readily observed as a change in fluorescence (from the green fluorescence of GFP to the red fluorescence of dsRed). Injecting mRNA of Cre-ERT (about 3 pg) at the one cell stage (or crossing with a Cre-ERT expressing transgenic line such as cmr1tg from Zfin) allows for activation of the recombinase upon uncaging of **cCyc** (at a typical concentration of 4–6 μM), followed by excision of the GFP gene and expression of dsRed. Measuring the intensity of the illumination source (UV lamp, UV or two-photon laser) at the position of the embryo allows for a quick estimate of the time required to uncage **cCyc** and observe the appearance of dsRed in the illuminated embryos, see Fig. 2. Illumination with various time-spans around that estimate allows one to determine the optimal illumination parameters for uncaging of **cCyc** and gene activation which may then be used in other contexts (see below).

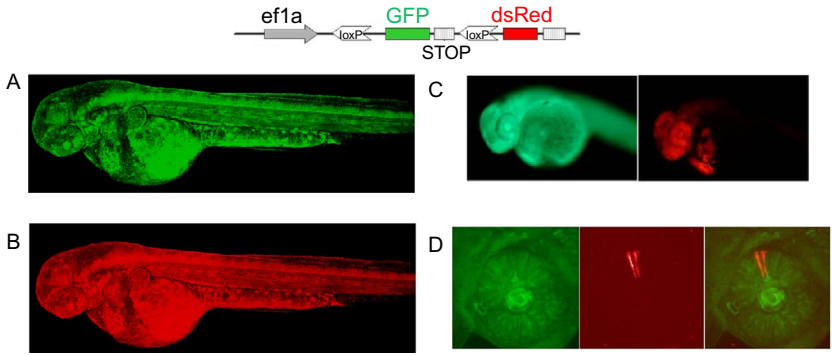


Fig. 2 Global and local activation of Cre-ERT by **cCyc** uncaging. 3 ng of Cre-ERT mRNA was injected at the one cell stage in a transgenic line Tg(ef1a:loxP-GFP-loxP-dsRed2) and incubated 1 h in 3 μM of **cCyc**. (A) image of the embryo in the GFP channel prior to illumination. (B) Expression of dsRed following transfer of the embryos into fresh embryo medium (no **cCyc**), followed by global 5 min uncaging with a UV lamp (intensity: $J = 2.4 \times 10^{-3}$ mol/m^2 s). (C) same as in (B) but with 30 s local illumination using the Hg-lamp of the microscope to uncage **cCyc** in the embryo head and turn on expression of dsRed in the head only. (D) same as before but illuminating at a diffraction-limited fixed spot for 10 s with a two-photon laser (200 fs, 76 MHz, 750 nm; Mira-Coherent) to uncage **cCyc** in a single cell of an eye at 10 hpf (and observing the embryo at 48 hpf, where the initial activated cell divided into two cells).

6.1 Equipment

UV lamp (365 nm, 6 W, Fischer-Bioblock) for global activation.
Microscope with a 25× (or 40×) water immersion objective (Leica or Olympus) equipped for local activation with a Hg lamp or a UV laser line (from a 365 nm CW; Crystal Laser).
Two-photon laser source (200 fs, 76 MHz, 750 nm, provided by a mode-locked Ti-Sapphire laser, Mira-Coherent) illuminating the embryo via a 25× (or 40×) microscope objective for two-photon local activation.
Power meter (NOVA II Laser Measurement Instruments or Thorlabs S170C Microscope Slide Power Sensor) for measurement of the illumination power.

6.2 Chemicals

Embryo medium (EM): NaCl (29.4 g/100 mL), KCl (1.27 g/100 mL), $CaCl_2 \cdot 2H_2O$ (4.85 g/100 mL), and $MgSO_4 \cdot 7H_2O$ (8.13 g/100 mL) (The Zebrafish Book, (Westerfield, 2007)).
Embryo medium as above supplemented with 3–6 μM of **cCyc**.

6.3 Biological materials

Transgenic zebrafish embryos, allowing for expression of a fluorescent protein marker upon photo-activation (see below the protocols for the engineering of such embryos).

6.4 Protocol

Using a power-meter measure the illumination intensity of the source (UV lamp or laser) at the position where the embryos will be placed. Estimate, with Eqs. (5) or (8), the uncaging rate and illumination time required for the experiment. Incubate (preferably dechorionated) transgenic embryos (at few hpf up to 2 dpf) in EM supplied with 3–6 μM **cCyc** for 1–2 h away from light. Just before photo-activation transfer the embryo to fresh EM and illuminate them with a UV or two-photon light source. If global activation is sought, illuminate the embryos for about 5 min. With a UV lamp positioned a few cm above a Petri dish holding them. If local photo-activation is sought place the embryo under a water immersion microscope objective and illuminate the desired area with a UV laser or the UV lamp (∼365 nm) of a microscope for a timespan estimated from Eq.(5) (typically a few seconds). If activation in a single cell is sought illuminate the desired cell with a focused two-photon laser (at ∼750 nm) for a timespan estimated from Eq.(8) (about 1 s).

Let the embryo grow for a few hours to a few days before observing the activated fluorescent protein marker using a fluorescent microscope or binocular.

7. Transient and permanent gene activation

A quantitative description of the molecular networks that sustain morphogenesis is one of the challenges of developmental biology. In addition the induction of tumors (by turning on some oncogenes and/or turning off tumor suppressor genes) in specific cells of a growing animal could illuminate the mechanisms of oncogenesis. These challenges require the precise spatial and temporal control over the expression of the target gene(s). The caged-cyclofen-OH approach described above allows one to do that in transgenic lines expressing the appropriate plasmid construct, see Fig. 3.

To achieve transient genetic activation, we use a fusion between the ERT domain and a transcription factor (Gal4) which is activated by cyclofen-OH uncaging (Feng et al., 2017). A transgenic line is engineered which expresses that fusion protein ubiquitously. A second line is engineered which expresses the desired gene under control of a UAS promoter. By crossing those lines, an embryo is generated in which local photo-activation of **cCyc**, leads to release of Gal-ERT from its chaperone complex and activation of the gene under the UAS promoter. As the released cyclofen-OH diffuses out of the cell, the Gal4-ERT is re-sequestered by cytoplasmic chaperones and the transcription activity is shut off (unless **cCyc** is globally uncaged and left in the medium in which case permanent global activation can be achieved).

To achieve local and permanent genetic activation, we fuse the ERT domain with a Cre recombinase. A transgenic line is engineered which expresses that fusion protein ubiquitously. A second line is engineered which expresses the desired gene downstream of an insertion marker and a stop codon flanked by loxP sites and under a ubiquitous promoter. By crossing those lines, an embryo is generated in which local photo-activation of **cCyc**, leads to the release of Cre-ERT from its chaperone complex, the excision of the DNA fragment flanked by the loxP sites and the permanent activation of the desired gene by the ubiquitous promoter. In contrast with the cyclofen-induced activation of Gal-ERT which is reversible, out-diffusion of the released cyclofen-OH does not reverse the Cre-induced excision of the DNA fragment between loxP sites.

In the following we will briefly describe how the required transgenic zebrafish lines can be engineered. More details can be found in the available literature (Westerfield, 2007).

7.1 Equipment

Micro injection machine (FemtoJet, Eppendorf)

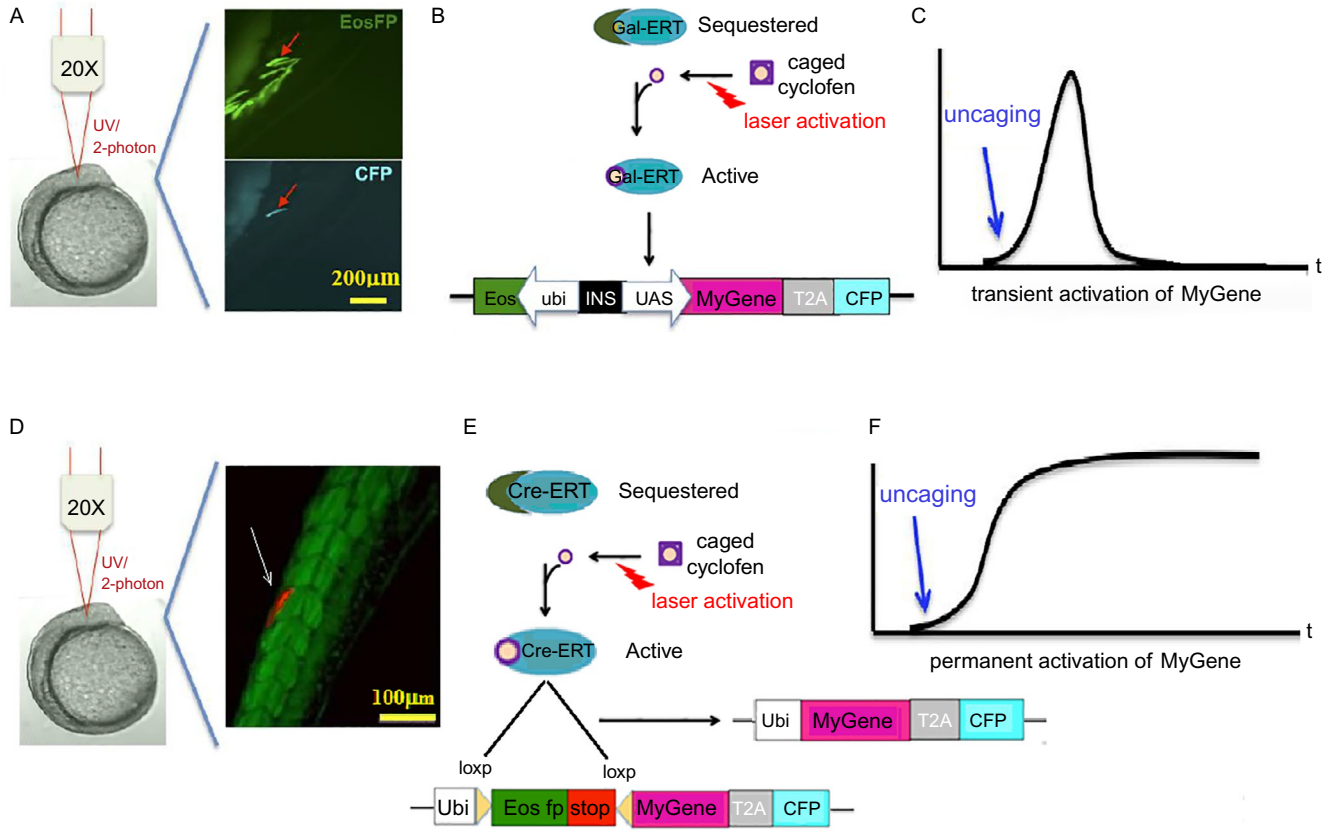


Fig. 3 See legend on next page.

7.2 Chemicals

Embryo medium: NaCl (29.4 g/100 mL), KCl (1.27 g/100 mL), $CaCl_2 \cdot 2H_2O$ (4.85 g/100 mL), and $MgSO_4 \cdot 7H_2O$ (8.13 g/100 mL) (The Zebrafish Book, (Westerfield, 2007))

7.3 Biological materials

Zebrafish embryos

The host Tol2 (Kawakami, 2007) plasmid *pT24-uas:Prot-T2A-cfp-ubi:Eos* prepared with standard molecular biology protocols (not detailed here) which contains the homologous cDNA sequence of the gene of interest *(Prot)* from *Danio rerio*.

The tol2 transposase mRNA synthesized by in vitro transcription from the linearized template pCS2tol2 digested with *Not*I using the mMESSAGE mMACHINE SP6 Kit (Ambion).

7.4 Protocol

Generation of the double transgenic line ubi:Gal4-ERT (or ubi:Cre-ERT); uas:Prot-T2A-cfp (or ubi:loxP-Eos-loxP-Prot-T2A-cfp). Prot stands for the gene of interest (in the example below Fgf8).

For the injection method please refer to (Rosen, Sweeney, & Mably, 2009). For injection, approximately1 nL of a solution (10 ng/μL) of the plasmid pT24-uas:Prot-T2A-cfp; ubi:Eos (or ubi:loxP-Eos-loxP-Prot-T2A-cfp) was injected into the cytoplasm of one-cell stage eggs together with the tol2 transposase mRNA (100 ng/μL) in 0.2 M KCl. Potential F0 fish were identified via the mosaic expression of EosFP at 3–5 days post fertilization and were raised to sexual maturity (3 months). The F1-progeny obtained from the cross of the putative F0 carrier with WT animals were screened for germ-lineage transmission via the fluorescence of the EosFP marker.

Fig. 3 Transient and permanent activation of a gene of interest (MyGene). (A) Using UV or two-photon illumination, a Gal4-ERT fusion is released (B) and transiently activates a gene of interest under a UAS promoter. A fluorescent CFP protein is used as a photoactivation marker while an EosFP serves as an insertion marker. The genetic construct shown in (B) is injected as a plasmid at one cell stage to generate a mosaic embryo and/or engineer a transgenic line. (C) Due to diffusion of the released cyclofen-OH, the activation of transcription lasts a few hours, but the translated protein may linger on for a few days. (D) Using similar illumination on a Cre-ERT fusion may permanently label a single cell (shown here in red) since out-diffusion of the released cyclofen-OH will not reverse the Cre-induced excision of a stop codon and its insertion marker, putting the expression of MyGene under a ubiquitous promoter (E). This approach can be used to permanently activate a gene of interest (F).

The ubi:Gal4-ERT (or ubi:Cre-ERT) fish were obtained in the same way using CFP as a marker expressed in the eye of the embryos 3 days after their birth.

A double transgenic line ubi:Gal4ERT and uas:Prot-T2A-cfp;ubi:Eos (or ubi:Cre-ERT and ubi:loxP-Eos-loxP-Prot-T2A-cfp) was created by crossing the ubi:Gal4-ERT (ubi:Cre-ERT) fish and the uas:Prot-T2A-cfp;ubi:Eos (ubi:loxP-Eos-loxP-Prot-T2A-cfp) fish, and screened for blue eyes and green body, see Fig. 4.

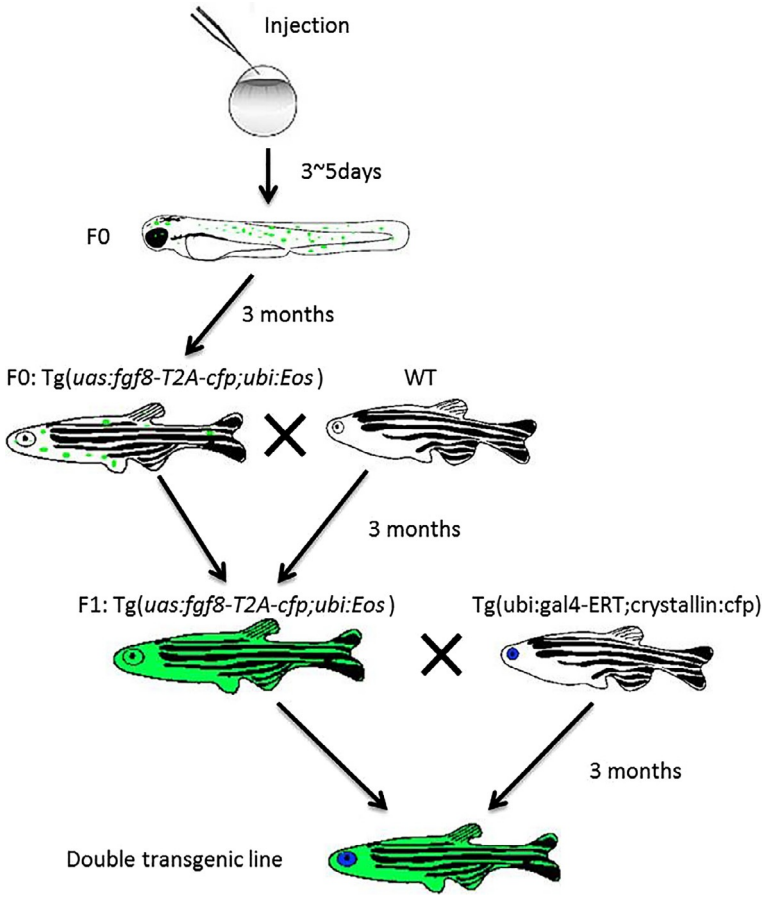


Fig. 4 Generation of a double transgenic line: the plasmid of interest (here uas:fgf8-T2A-cfp; ubi:Eos) is injected with the mRNA of the tol2 transposase at the one cell stage. F0 embryos are identified by the mosaic expression of EosFP at 3–5 dpf, they are raised to sexual maturity, crossed with WT embryos and the F1 progeny selected by the uniform expression of EosFP. The F1 progeny is crossed with Gal4-ERT embryos expressing CFP under a crystallin promoter. Double transgenic embryos are identified by their uniform EosFP expression and CFP expressing crystalline.

8. Conclusions

The photo-activation of caged-cyclofen-OH (**cCyc**) has been used to control the activity of a variety of proteins (the Gal4 transcription factor, Cre-recombinase, caspase, GFP, etc.) fused to the ERT domain of the estrogen receptor. Activating a transcription factor has been used to transiently turn on a target gene, while the activation of a Cre-ERT recombinase has been used to permanently turn on a target gene (behind a stop codon flanked by loxP sites). The uncaging of cyclofen and the consequent activation of the protein fused to the ERT receptor can be performed within a time-span of seconds to minutes globally (using a UV lamp) or locally (within a few cells using one or two-photon excitation). This approach is versatile and easy to implement in various organisms (zebrafish, mice, etc.).

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