Resonance energy transfer sensitises and monitors in situ switching of LOV2-based optogenetic actuators

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Engineered light-dependent switches provide uniquely powerful opportunities to investigate and control cell regulatory mechanisms. Existing tools offer high spatiotemporal resolution, reversibility and repeatability. Cellular optogenetics applications remain limited with diffusible targets as the response of the actuator is difficult to independently validate. Blue light levels commonly needed for actuation can be cytotoxic, precluding long-term experiments. We describe a simple approach overcoming these obstacles. Resonance energy transfer can be used to constitutively or dynamically modulate actuation sensitivity. This simultaneously offers on-line monitoring of light-dependent switching and precise quantification of activation-relaxation properties in intact living cells. Applying this approach to different LOV2-based switches reveals that flanking sequences can lead to relaxation times up to 11-fold faster than anticipated. In situ-measured parameter values guide the design of target-inhibiting actuation trains with minimal blue-light exposure, and context-based optimisation can increase sensitivity and experimental throughput a further 10-fold without loss of temporal precision.
Optogenetic methods are increasingly important throughout biology, dissecting spatiotemporal properties of signalling networks and addressing fundamental questions in pathology, physiology and even psychology in intact organisms. Blue-light photoreceptors such as LOV2 are among the most popular cellular optogenetics photosensors. However, blue light can perturb cells, potentially complicating long-term (hours-days) optogenetic modulation. LOV2 can be indirectly sensitised by tuning relaxation, but this compromises time-resolution. Optogenetics has a unique potential to impose or modulate complex time-encoded inputs on cellular targets, and establish long-term manipulation and mimicry of periodic and pulsatile biological phenomena. It would be valuable to increase sensitivity while maintaining time-resolution. A second difficulty optimising optogenetic regulators for new targets is that real-time in situ detection of photosensor switching has not been reported. Except in specific cases where protein translocation or other visualised downstream phenomena are targeted, it is hard to know whether an actuator has been switched. Relaxation rate constants are obtained by cell-free spectroscopy and occasionally with heavily modified constructs not subsequently used for experimental actuation. LOV2 designs that modify Ja, the moiety regulated by light-dependent switching, can accelerate cell-free relaxation >30-fold. Rational design of illumination paradigms requires in situ relaxation and activation rates. Without this information, validation and use of optogenetic tools remain challenging tasks.

Here we present a simple method addressing these needs, simultaneously increasing light sensitivity of an optogenetic switch while providing on-line detection of activation and relaxation. Engineered resonance energy transfer (RET) between a mTurquoise2 fluorescent protein and FMN-bound AsLOV2 increases switch sensitivity. We demonstrate this with an optically-regulated nuclear export sequence (NES). Similar results are obtained with the optogenetic JNK inhibitor, optoJNKi. RET also causes mTurquoise2 quenching by dark-state AsLOV2. Blue-light generates the cysteine adduct that drives the actuator’s conformational change, leading to dequenching. LOV2 switching is thus directly observed as fluorescent tag dequenching. Darkness allows cysteine adduct decay and recovery of ground-state AsLOV2, regenerating tag quenching. Dequench-requench dynamics reveals activation and relaxation rates of AsLOV2 switching in intact living cells. This generalises to other fluorescent protein tags and AsLOV2-based actuators. Strikingly, LOV2 relaxation in actuators was up to 11-fold faster than expected from AsLOV2 spectroscopy. The differences in relaxation time could be explained by the impact of sequences flanking the LOV2 domain rather than an effect of the in vivo environment. Selection of fluorescent tags and consideration of activation-relaxation parameters facilitates minimal photon exposure protocols for JNK pathway regulation. Finally, comparison of LOV2 FRET-derived parameters with target pathway kinetics guides optogenetic actuator optimisation to further reduce blue light requirements tenfold and increase experimental throughput tenfold. This facilitates repeatable and relatively non-invasive interrogation of bidirectional pathway dynamics that could not be achieved by pharmacological approaches. We anticipate the methodology described could promote wider, more efficient application of cellular optogenetics in diverse experimental settings.

Results

Fused fluorescent protein determines actuation sensitivity.

Blue-light activated optogenetic tools are commonly tagged with red fluorescent proteins, verifying construct expression without activation. This occupies blue and yellow-green excitation channels, and multiplexing with fluorescent probes becomes difficult. A cyan tag allows recording of actuator expression without occupying other channels, permitting multiplexing with yellow to infra-red channels. However, there might be unintended consequences—spectral overlap with proximal (within 5 nm) photosensor chromophores (flavins in LOV2 and cryptochromes) may permit RET, influencing actuator sensitivity. Comparison of spectra from LOV2 and cyan, yellow and red proteins suggests potential RET in all cases (Supplementary Fig. 1). Consequences could include sensitised actuation by cyan proteins, suppressed actuation by yellow and red proteins or no effect (Supplementary Fig. 2).

Determining the influence of fusion tags requires an assay to quantify LOV2 switching. The optogenetic nuclear translocator LEXY interweaves a NES with the AsLOV2-Jα helix, providing a convenient actuation readout. Light-induced unfolding of the Jα exposes the NES, increasing nuclear export until LOV2 relaxation terminates NES exposure. We generated a panel of such constructs using different fluorescent tags (XFPs, Supplementary Figs. 3–4). Because of a minor sequence difference, we refer to these as optoNES to avoid possible confusion with the original LEXY. We only consider light sensitivity rather than dynamic range of translocation; these are independent parameters just as enzyme $K_m$ and $V_{max}$ are independent. Hippocampal neurons infected with AAVs encoding XFP-optoNES (Supplementary Fig. 4) show localisation to nuclear and cytoplasmic compartments, but translocation out of the nucleus in response to light (Fig. 1a, b; Supplementary Fig. 5 and Supplementary Movies 1–6).

To quantify light sensitivity, we provided cycles of ten light pulses, each partially switching the LOV2 domain. Translocation rate is quantifiable by fluorescence imaging (Fig. 1a–d). Four cycles were applied at each of four 438 nm photon doses, between ~14 and 420 μmol m$^{-2}$ flash$^{-1}$ (1 μmol m$^{-2}$ s$^{-1}$ = 27 μW cm$^{-2}$, Fig. 1c). Sensitivity was calculated from the nucleocytoplasmic ratio change per flash for each dose (Fig. 1e and Supplementary Fig. 6). Under these conditions, all constructs exhibited light-dependent nuclear export. But the mTurquoise2 fusion was notably more sensitive to blue light than the other fusion constructs (Supplementary Table 1).

To determine whether these differences result from RET, we separated fluorophores and LOV2 domain with 24 residues (Supplementary Fig. 7), increasing inter-dipole distances to which RET efficiency is exquisitely sensitive (6th power relation, Supplementary Fig. 1A). Excess positive charge in the spacer should favour an extended conformation, but even a random-coil conformation (with residue spacing of 0.38 nm) would add 1.9 nm. Generously estimating initial inter-dipole distance at 5 nm, a typical value for $R_0$, this equates to 1.4-fold distancing and a minimum sevenfold reduction of RET ($R/R_0$). These reduced-RET optoNES variants export with indistinguishable sensitivity (Fig. 1f and Supplementary Fig. 8). Thus, the spacer increased sensitivity where LOV2 can act as a donor (approximately threefold for Ypet or mScarlet constructs) and reduced sensitivity of the mTurquoise2 construct twofold (Fig. 1e–h and Supplementary Table 1). To evaluate whether differences in fusion protein sequences, and not RET, could be responsible for sensitivity differences, we replaced the constitutively fluorescent proteins with reversibly photoswitchable protein Droona. This protein can be switched between 400 nm absorbing and yellow fluorescent protein-like states (Supplementary Fig. 9A). Thus we compared actuation sensitivity using the same protein in either a dark or Ypet-like fusion (competent to act as a RET acceptor), and latter form showed significantly less sensitivity to light (Fig. 1i).

These results indicate engineering RET into optogenetic actuators can either increase or decrease sensitivity. In the specific case that a red fusion protein, commonly used in
Fig. 1 Fluorescent protein tags strongly influence the sensitivity of LOV2-based optogenetic nuclear export. a Blue-light induces export of mTurquoise2-tagged optogenetic nuclear export construct, mTq2-optoNES (Supplementary Fig. 4) in hippocampal neurons (n = 8 wells at 40× magnification, scalebar 10 μm, other examples in Supplementary Fig. 5). b Fluorescent protein tags (XFP) fused to optogenetic constructs might influence their sensitivity to light via RET. c Illumination scheme used to quantify light-dependency of nuclear export rates. d Example data from a single 10× field treated as shown in c (four cycles, at least 10 min apart, mean ± SEM, for 23 and 31 cells, respectively), showing faster export at higher photon flux. e Nuclear export rate dependence on photon flux is shown for optoNES constructs fused to fluorescent proteins mTurquoise2, Ypet and mScarlet (in turquoise, yellow and red, respectively) expressed in different cultures. The mTq2 construct is more sensitive (significant by F-test, F(2,66) = 41.34, P < 0.0001). Data are normalised to fitted maximal rates to facilitate sensitivity comparison (non-normalised versions in Supplementary Fig. 9). Corresponding experiments with a spacer between fluorescent protein and LOV2 domain to reduce resonance energy transfer (RET, Supplementary Fig. 8). All constructs are equally sensitive to light, thus in e, mTurquoise had increased sensitivity whereas Ypet/mScarlet decreased sensitivity. This suggests RET can either increase or decrease optogenetic switch sensitivity. In e, f, all channels were imaged regardless of which XFP was used, to ensure identical illumination conditions. Data are means ± SEM, n = 6 wells. Best-fit ED50s in μmol 438 nm photons.m⁻².flash⁻¹ indicated on the graphs for clarity and in Supplementary Table 1 with standard errors. g–i show best-fit values ± S.E. (**P = 0.0025, ***P < 0.0001) from curve-fitting of datasets in e, f and Supplementary Fig. 9F. Source data are provided as a Source Data file.
optogenetic actuators, is replaced with a cyan one, a sevenfold sensitivity increase may be achievable.

Direct visualisation of LOV2 switching in intact cells. RET from donor to LOV2 acceptor should quench donor emission (Fig. 2ai, left). Absorption of a photon by the ground-state (LOV-445) leads with high probability to formation of the triplet state LOV-660 within 2 ns. This subsequently forms the cysteine adduct LOV-390, which exhibits >50% lower absorption compared with LOV-445. RET to LOV2 should fall, resulting in measurable dequench of mTurquoise2 (Fig. 2ai, right). LOV-390 formation causes an immediate (~10 µs) partial unfolding of AsLOV2 flanking regions, via a glutamine lever mechanism acting
Fig. 2 Dequenching of mTurquoise2 as a measurement of LOV2 activation. a (i) LOV2 domain-fused mTq2 can act as a RET donor and transfer energy to FMN in the adjacent domain, and is partially quenched. Once LOV2 is activated directly or via RET, formation of FMN-adduct changes the absorption spectrum, reducing its ability to act as a RET acceptor. The LOV2 domain A′α helix extends, placing the FMN and LOV2 domain further from the fluorescent protein, also reducing RET. Thus mTq2 becomes dequenched; (ii) Placing a spacer between mTq2 and the LOV2 domain reduces RET from the outset, limiting initial quenching and the corresponding dequenching on LOV2 activation. b Changes in fluorescence emission of mTq2 fusion proteins, mTq2-optoNES, and mTq2-spacer-optoNES (Supplementary Fig. 8) expressed in HEK293 cells, are observed during 50 flashes of 14 μmol m−2 438 nm light at ~4 Hz. This replicate illustrates LOV2-dependent dequenching, with exponential fitting yield decay constants of 0.12 and 0.13 per flash i.e. ~8 s and differing levels of maximal dequench (or initial quench, A0); c, d Means ± SEMs (n = 3 wells) of samples as shown in b were exposed to photon doses per 438 nm flash as indicated. Dequenching curves were corrected in all cases to the signal from a parallel free mTurquoise2 sample exposed to identical illumination conditions (Supplementary Fig. 10), normalised to the first data point and then plotted against flash number. Data were fitted to exponentials to derive fluorescence prior to illumination, maximum fluorescence and rate constants. e Data from c, d, fitted to exponentials, show the relation between maximal dequench and photon dose. Fitting to a single site model provides estimates of dequench limit i.e. the extent to which the Tq2 is originally quenched, and ED50, the photon dose/flash to achieve half this dequenching at equilibrium when delivered at ~4 Hz. f All data points were fitted to a first order reversible kinetic model, providing more reliable estimates of g sensitivity (1/kf), h relaxation time constant during illumination, i Dequench limit and j ED50. **p<0.0001 and no significant difference by unpaired one-tailed multiple t-test with Holm-Sidak correction. Source data are provided as a Source Data file.

on both C-terminal Ja and N-terminal A′α helix14. Slower (250 μs12) and complete undocking of Ja follows12,15, resulting in the order-disorder transition used to generate actuation in optogenetic tools1. This N-terminal A′α helix unfolding reduces fluorescent tag-FMN proximity, contributing a second RET-limiting mechanism as LOV2 switches (Fig. 2ai).

As predicted, mTq2-optoNES emission increased during exposure to 438 nm light (Fig. 2b and Supplementary Fig. 10), revealing an initial quench (20% more than after equilibrium switching in intact cells; Fig. 2b, c). In contrast, the reduced-RET version (Fig. 2Aii and Supplementary Fig. 7) exhibited less dequenching (Fig. 2b–e). Dequenching is therefore sensitive to the relative placement of mTurquoise and LOV2 domains, consistent with RET. Nevertheless in both constructs, LOV2 domain-activating blue-light dequenches fused mTurquoise. Thus, dequenching curves indicate the switching of the LOV2 domain in intact cells. LOV2 switching has previously only been measured by photobleaching purified proteins in a cuvette. We can now infer the apparent sensitivity of activation from dequenching rate. Half-maximal de-quenching took ~1.3 s (5 readings, total ~140 μmol m−2 photons). Such a fast de-quench half-time may estimate in situ activation rate if cell-free LOV2Ja relaxation rates (~20–80 s6,16,17) are applicable and counteracting relaxation can be neglected.

We estimated dequenching limits and activation rates more directly with rounds of activation at different photon intensities, fitting data to exponentials to extrapolate pre-illumination and final intensity levels (Fig. 2c, d). Dequenching-dose curves (Fig. 2e) provide estimates of dequenching limit (i.e. in the absence of relaxation, as photon dose approaches infinity) and ED50 (photon dose that, at equilibrium, achieves half-maximal dequenching i.e. photoswitching). The entire data was also fitted to a first order reversible kinetic model (Fig. 2f), providing rate constants (Fig. 2g; h, for activation, 1/kf ~200 μmol m−2; for relaxation 1/k1 ~5 s) and additional estimates of dequench limit and ED50 (Fig. 2i, j). The activation sensitivity is comparable to observations in cells6. However, derived optoNES relaxation rates in situ were considerably faster than anticipated from cell-free spectroscopic measurements of LOV2Ja proteins16. This not only limits accuracy of the activation rate estimate shown in Fig. 2b but also has significant relevance for experimental use of LOV2-based optogenetic actuators.

Visualisation and direct quantification of LOV2 relaxation. The cysteine-adduct LOV-390 also absorbs activation wavelengths (425–475 nm). An unexpectedly short relaxation time during repeated light flashes (Fig. 2h) could potentially result from photochemical back-reactions16. Moreover cell-free LOV2 relaxation measurements showed a strong influence of temperature9, and Ja modifications used to generate some optogenetic actuators8. We therefore sought a simple direct method to determine relaxation time in situ in darkness, as accurate knowledge of relaxation rates within experimental settings is required to design illumination protocols.

The experimental paradigm used in Fig. 2b, in which dequenching reveals the protein fraction in activated state, can be repeatedly followed by defined periods of darkness. This shows that the duration of darkness determines loss of activated fraction (Fig. 3a–c), thereby revealing the relaxation rate of the LOV2-based actuator in intact living cells (Table 1a). The experiments confirmed that optoNES construct relaxation rates are indeed slower than those obtained by cell-free spectroscopy of LOV2Ja (tau 6–7 s vs. ~80 s5). The rates in darkness are slower than those derived from dequenching curves under repeated illumination (Fig. 2h), consistent with a photochemical back-reaction when LOV-390 absorbs blue light16. Regardless, a consistent conclusion from Figs. 2–3 is that actuators in intact cells relax much faster than expected from cell-free spectroscopy of LOV2, even when temperature is taken into account6,16.

The activation-relaxation paradigm in Fig. 3 provides a true relaxation rate (reverse rate constant) but only an apparent activation sensitivity (forward rate constant), which under-estimates the true sensitivity (Table 1a) due to the concurrent relaxation. However, based on first order reversible kinetics (Fig. 2e) the forward rate constant (sensitivity), dequench limit and photon dose required to achieve 50% maximal switch equilibrium (ED50) can easily be calculated from fitted parameters (see methods). Therefore we primarily use the activation-relaxation approach and derived the missing parameters by curve-fitting.

Optimisation of RET and sensitisation. Just as RET is reduced by increasing mTurquoise-LOV2 distance, removing sequence between mTurquoise and LOV2 could increase RET. RET from mTurquoise2 doubles activation sensitivity (Fig. 1g) and promotes LOV2 switching (Fig. 2g), allowing real-time activation/relaxation detection in living cells. We asked if RET could be further increased. A more photosensitive actuator could be switched more frequently and used for longer experiments with less phototoxicity. More sensitive detection permits more precise and frequent monitoring of actuator state.

Fluorescent protein structures reveal C-terminal residues outside the core beta-barrel structure. Deleting these residues can generate up to 95% RET in some applications18. We generated an mTurquoise2 deletion series lacking 7–11 C-terminal residues (Fig. 4a) fused to LOV2 residue 408. This
sequence was repeated three times per sample to reduce noise. Responses from each segmented cell (region of interest) in the dequenching phase was sample (without LOV2) exposed to the same illumination conditions (Supplementary Fig. 10). Means are plotted against reading number. Each were averaged. All wellwise-averaged data from transfected cells are normalised to an average of replicates for a corresponding mTurquoise-expressing sample (without LOV2) exposed to the same illumination conditions (Supplementary Fig. 10). Means are plotted against reading number. Each dequenching phase was fitted to an exponential as in Fig. 3, with independent size and common activation rate constant for each protein, providing offset and amplitude estimates for each cycle. These curves, together with the dark-phase changes are shown as a solid line between the data points, showing goodness of fit. Apparent activation rate constants at this photon flux were 0.12 and 0.11 per flash, equivalent to a photon sensitivity of 115 and 131 µmol/m².

The quench recovery estimated from the exponential curve fits in b/c, is plotted against the duration of dark time causing the quench. This reveals the time-dependence of recovery from dequenching. The data is fitted to an exponential to derive relaxation time constants (~7 s for both constructs) and dequenching maxima. As evident from b and c, the dequenching is at maximum ~19 and 5% of initial fluorescence for mTq2-optoNES and the spacer construct respectively. Source data are provided as a Source Data file. Data are presented as means ± SEM; n = 6 wells for mTq2-optoNES and n = 5 wells for mTq2-spacer-optoNES.

truncates the A’ helix that participates in Jα regulation but not the photocycle17. Confirming Jα perturbation, the NES21 motif is at least partially exposed as dC11–408LOV2-NES21 is mostly cytoplasmic (Fig. 4b), although the 408LOV2 can still optically regulate heterologous targets19. The deletions increase maximal dequench compared with the original mTq2-optoNES (Fig. 4c, d, Table 1a, and Supplementary Figs. 11–12). Directly fitting the entire dataset to a formula cast in terms of quench limit, sensitivity and relaxation rate, indicates the true initial quench of mTurquoise is as much as 24% (relative to LOV-390, Table 1a).

Sensitivity and dequench limit show significant correlation (Fig. 4e, Supplementary Fig. 12, and Table 2). The dC7 construct has the highest RET and sensitivity (lowest value in µmol m⁻²), suggesting further deletion either increased dipole distance or introduced unfavourable dipole angles and RET failed to increase. Nevertheless, increased RET allows optogenetic switching with fewer photons compared with the parental mTq2-optoNES construct.

Determinants of relaxation rate. The relaxation mismatch between actuators in situ and reports for cell-free purified recombinant LOV2 is evident, but the cause is not. One factor is temperature6,16, another is Jα editing8. The constructs in Fig. 4 have the same Jα sequence and fluorescent tag, yet in situ they exhibit threefold different relaxation rates (Table 1a). This suggests the tag-LOV2 linker can also have considerable impact. Although this potentially complicates interpretation of Fig. 1f...
where RET is limited with a spacer, Fig. 2 showed the spacer used did not affect relaxation rates. Whether the cellular environment also influences relaxation is unclear. We compared in situ relaxation rates with those determined in cell-free HEK293 lysates at different temperatures (Fig. 4f). This confirms the impact of temperature but, for the actuators tested, shows no evidence for contributions of the cellular environment per se (Fig. 4f).

In situ photocycle monitoring generalises to other actuators. Could activation-relaxation cycling report in-cell sensitivity and relaxation of other optogenetic actuators? We selected optoJNKi, a modified JNK inhibitor design that revealed resonance in activation and relaxation of other optogenetic actuators? We selected optoJNKi, 

| Actuator (Ypet fusion with LOV2 mutation) | Max quench at 140 μmol m⁻² (% of F₀) | Apparent activation rate (μmol m⁻²) at 140 μmol m⁻² | Relaxation time constant in situ (s) | Relaxation time constant reported cell-free (s) |
|------------------------------------------|-------------------------------------|-----------------------------------------------|-----------------------------------|-----------------------------------------------|
| optoNES wt                               | 57.4 ± 1.0                          | 492 ± 2                                       | 7.6 ± 0.6                         | 80 (Zoltowski et al. [23])                   |
| optoNES V416l                             | 54.1 ± 1.6                          | 584 ± 7                                       | 84.1 ± 1.7                        | 821 (Zoltowski et al. [23])                 |
| optoNES I427V                             | 28.2 ± 0.6                          | 272 ± 9                                       | 0.81 ± 0.14                       | 6 (Zayner et al. [3])                       |
| optoNES F434L                             | 23.8 ± 1.0                          | 237 ± 8                                       | 0.58 ± 0.25                       | 12 (Zayner et al. [3])                      |

Values shown are estimated best-fit ± standard error from curve fitting as described in the methods.

1. Maximal dequench or quench—is this the equilibrium impact on emission from the fluorescent protein on repeated illumination with 438 nm light (cf. Fig. 2). It is caused by resonance energy transfer to or from the nearby LOV2, but the equilibrium point depends also on continuous relaxation. For this reason the value depends on the rate (~4 Hz) and intensity of 438 nm light flashes, which is indicated.

2. The apparent activation rate is the measure of apparent sensitivity of switching, again influenced by concomitant relaxation and dependent on photon flux, which is indicated for each group. mTurquoise2 fusions were activated at 14 μmol m⁻² whereas Ypet and mScarlet fusions were less sensitive and were quenched at 140 μmol m⁻².

3. Relaxation time is calculated from periods of darkness and is independent of activating photon flux. In Table 1, the measured values of relaxation mutants in the optoNES context are compared to literature values of recombinant cell-free LOV2 mutant without actuator or fluorescent protein fusion.

4. The dequench limit is the estimated photon dose at the rate used (~4 Hz) predicted to achieve half-maximal switching at equilibrium, taking concomitant relaxation into account.

5. Dequench or quench limit—which is the estimated relaxation independent of maximal possible quench (for acceptors Ypet and mScarlet) or dequench (for mTurquoise2) at 140 μmol m⁻².

6. Sensitivity is the estimated sensitivity value in the absence of derivation from curve fit formulae that take the concomitant relaxation rate into account.

An asterisk denotes statistically significant difference in relation between optoNES and dC9 (P = 0.0364) and dC10 (P = 0.0004), and spacer-optoNES and dC10 (P = 0.001) constructs by one-way ANOVA and post-hoc Tukey test. No other significant difference was found among the mTurquoise2-LOV2 fusions. This is predictable from S4-state LOV-390 reduces Ypet emission, consistent with RET loss, thus traces are inverted compared with Figs. 2e and 445 to LOV-390 reduces Ypet emission, consistent with RET loss, thus traces are inverted compared with Figs. 2e and 4f.

Photocycle monitoring generalises to other fluorophores. In situ determination of activation and relaxation rates requires a RET-competent tag like mTurquoise (Figs. 2–5). Figure 1 shows RET also occurs with yellow and red proteins, and presumably any protein having spectral overlap with LOV2. Substituting mTurquoise with Ypet showed a larger RET effect (Fig. 6; quench limit up to 66%, Table 1a), consistent with greater spectral overlap (Supplementary Fig 1). As LOV-390 reduces Ypet emission, consistent with RET loss, thus traces are inverted compared with Figs. 2–5. A dark period recovers the sensitised Ypet signal. Activation and relaxation parameters can be obtained from Ypet-LOV2 fusions (Fig. 6e–g and Table 1a). RET from LOV2 to Ypet causes loss of energy from S1-state LOV-390 reduces Ypet emission, consistent with RET loss, thus traces are inverted compared with Figs. 2–5. A dark period recovers the sensitised Ypet signal. Activation and relaxation parameters can be obtained from Ypet-LOV2 fusions (Fig. 6e–g and Table 1a). RET from LOV2 to Ypet causes loss of energy from S1-state LOV* and activation sensitivities are >fivefold lower than mTurquoise2 versions. The RET-limiting spacer (Fig. 1 and Supplementary Fig 8) develops dequench limit. This is the opposite of the mTurquoise2 constructs, consistent with Ypet acting as acceptor. Red protein mScarlet has similar effects to Ypet (Supplementary Fig 13 and Table 1a). This is predictable.
given the spectral overlaps (Supplementary Fig. 1) and the longer wavelength proteins acting to limit LOV2 responsiveness. Dark state relaxation times, a thermal process of adduct decay, are generally insensitive to the fused fluorescent tag (Table 1a), in contrast to the strong effects of the linker sequence. However exceptions exist, as Ypet-fused optoJNKi has an unusually long relaxation time.

**Parameter-based minimisation of light dose for actuation.** The parameters applied allow prediction of activated state dynamics of the actuator during illumination trains, even if it does not translocate or provide visual cues of activation. AsLOV2 starts at leucine 404 or trimmed to 408 to increase RET; the trimmed constructs are typically constitutively cytoplasmic because disruption of α-helix by deleting LOV2 residues 404–407 renders the constructs functionally active as expected (the dC11 construct is shown; scalebar 20 µm, n = 3 wells in ×10 magnification, 1 example imaged at ×40). The C7–C11 deletion series of mTq2 fused to 408LOV2-NES21 were subjected to kinetic RET analysis, revealing characteristic dequench-relaxation cycles (mean, n = 3; SEM shown in Supplementary Fig. 11); Analysis of the dequench-relaxation as in Fig. 3 reveals that deletion of linker can almost double maximal dequench (at 14 µmol m⁻²) though relaxation rate is also affected. The dC7 construct has least deletion but greatest dequench, suggesting dipole angles or other parameters reduce RET in other constructs though mTq2-LOV2 distance in primary sequence is reduced. Best-fit parameters—maximum dequench, apparent sensitivity, relaxation time and ED50, dequench limit and sensitivity are compared in Table 1A. E Activation sensitivity of the mTurquoise-optoNES constructs from Table 1A correlates with dequench limit (equivalent to effective FRET efficiency; statistical analysis in Supplementary Table 2, best-fit ± S.E., n = 3 wells). F Best-fit relaxation rates obtained from the in situ data (green bars; Table 1A) are shown together with the corresponding values derived from measurements performed on lysates of cells expressing the same constructs (dC7, dC10, dC11 and the parental optoNES) at 25 and 37 °C (blue and red bars). This shows that temperature has a strong influence on relaxation rates of these constructs but, when the sequences used are identical, there is no significant difference between relaxation in intact cells and the cell lysates in any case. Mean ± standard error is shown (n = 8 cell-free, n = 6 and 3 for in-cell wild-type and variants respectively). *, **, *** denote P < 0.05, 0.01, and 0.0001, respectively (25 °C compared to either of the 37 °C values) by Tukey post-hoc test after two-way ANOVA. n.s. not significant. Source data are provided as a Source Data file.

**Fig. 4** Increased RET to LOV2 and sensitisation can be achieved by linker optimisation. a Alignments of XFP-optoNES variants compared with the original mCherry-fused LEXY, showing the end of the fluorescent protein β-barrel and intervening sequences. AsLOV2 starts at leucine 404 or trimmed to 408 to increase RET; the trimmed constructs are typically constitutively cytoplasmic because disruption of α-helix by deleting LOV2 residues 404–407 renders the constructs functionally active as expected (the dC11 construct is shown; scalebar 20 µm, n = 3 wells in ×10 magnification, 1 example imaged at ×40); b The C7–C11 deletion series of mTq2 fused to 408LOV2-NES21 were subjected to kinetic RET analysis, revealing characteristic dequench-relaxation cycles (mean, n = 3; SEM shown in Supplementary Fig. 11); c Analysis of the dequench-relaxation as in Fig. 3 reveals that deletion of linker can almost double maximal dequench (at 14 µmol m⁻²) though relaxation rate is also affected. The dC7 construct has least deletion but greatest dequench, suggesting dipole angles or other parameters reduce RET in other constructs though mTq2-LOV2 distance in primary sequence is reduced. Best-fit parameters—maximum dequench, apparent sensitivity, relaxation time and ED50, dequench limit and sensitivity are compared in Table 1A. d Activation sensitivity of the mTurquoise-optoNES constructs from Table 1A correlates with dequench limit (equivalent to effective FRET efficiency; statistical analysis in Supplementary Table 2, best-fit ± S.E., n = 3 wells). e Best-fit relaxation rates obtained from the in situ data (green bars; Table 1A) are shown together with the corresponding values derived from measurements performed on lysates of cells expressing the same constructs (dC7, dC10, dC11 and the parental optoNES) at 25 and 37 °C (blue and red bars). This shows that temperature has a strong influence on relaxation rates of these constructs but, when the sequences used are identical, there is no significant difference between relaxation in intact cells and the cell lysates in any case. Mean ± standard error is shown (n = 8 cell-free, n = 6 and 3 for in-cell wild-type and variants respectively). *, **, *** denote P < 0.05, 0.01, and 0.0001, respectively (25 °C compared to either of the 37 °C values) by Tukey post-hoc test after two-way ANOVA. n.s. not significant. Source data are provided as a Source Data file.
of binding targets. Nevertheless, a clear difference between mTurquoise2, Ypet and mScarlet versions is predicted as we likely avoid saturation of targets. These illumination conditions caused significant light-dependent inhibition by mTq2-optoJNKi, minimal light-dependent effect of Ypet-optoJNKi and no discernible impact of light on mScarlet-optoJNKi infected neurons (Fig. 7c–f).

This demonstrates the generality of the optoNES findings (Fig. 1), that sensitivity of optogenetic actuation under linear illumination conditions can be modulated by fluorescent fusion tags. It also demonstrates that in situ RET-derived activation and relaxation parameters aid the selection of minimal illumination conditions sufficient for switching (and hence actuation). This means that, when using optogenetic actuators, the blue-light toxicity artefacts can be limited even in long-term studies, and the likelihood of achieving intended outcomes is increased even where on-line validation assays are not available.

**RET guides optimisation of in situ relaxation time.** Relaxation of all optogenetic constructs tested in situ is consistently faster (up to tenfold) than for recombinant LOV2 measured by absorbance spectroscopy. This mismatch complicates the application of cellular optogenetics. For example, the high-frequency (0.13 Hz) illumination required (Fig. 7), if delivered through-the-objective, greatly limits throughput. Parallel trans-illumination devices (Fig. 7a), in providing an illumination path orthogonal to the epifluorescence microscope optical path, can resolve the sample throughput bottleneck. But rapid relaxation time also means higher blue-light doses are required to maintain switching (and hence actuation).

For these reasons we turned to relaxation-time mutants of LOV2. The impacts of mutating V416, I427 and F434 and others on decay rate of the FMN adduct and therefore cannot cycle, as shown. Means ± SEM (n = 3 wells) are plotted together with a series of exponential fits with a common activation constant (red line) to mTq2-optoJNKi, opto38i3 and opto38i5 are evaluated and the response curves show almost complete overlap. Raw averaged data (n = 3 wells) is shown for each construct; c, Best-fit amplitudes (± S.E.) of exponentials fitted to the data in b are plotted against preceding dark time; crosses plotted nominally at 200 s represent the amplitudes of the first exponential which was not preceded by any light exposure. Data are fitted to exponentials to derive the relaxation time in dark and maximal dequench (as % of initial signal) for each construct under the low illumination conditions used (14 μmol m−2 flash−1 at ~4 Hz). Max dequench (as % of total), apparent activation rates, and relaxation time constants in dark for the data in c, a are shown in Table 1a. From these values, ED50, dequench limit (equivalent in each case to FRET efficiency, assuming adduct has no effect) and sensitivity are derived. The C450A optoJNKi construct, which cannot form an adduct and therefore for which no activation or relaxation values could be derived, is also listed in the table. Source data are provided as a Source Data file.

Re-programming and quantifying signalling pathway dynamics. A slower-relaxing optoJNKi would allow simpler and more efficient experimentation than in Fig. 7. mTq2-optoJNKi-sr (slow relaxation), incorporating the V416L mutation, has a relaxation time of ~2 min (Supplementary Fig. 14), 7 times slower than the original optoJNKi (Table 1a) and is the most sensitive construct tested (lowest ED50). We compared the applicability of optoJNKi variants to regulate neuronal JNK pathway activation by pacitaxel and vincristine, clinically used chemotherapeutic agents associated with central neurotoxicity.

In a traditional through-the-objective actuation configuration—where imaging and actuation light use a common epifluorescence path—optoJNKi requires five flashes per min to suppress JNK activity (Fig. 9a–c), precluding parallel sample imaging. Response modulation is gradual (tau ~4–16 min), demonstrating the requirement for continued inhibition to modify the response. In contrast, 0.5 flashes per min is sufficient for optoJNKi-sr to rapidly suppress the JNK pathway (Fig. 9d, e). This permits multi-sample imaging, >tenfold increased throughput, and JNK inhibition with tenfold less blue light. Under these conditions, neurons tolerate repeated long-term modulation of the pathway (Fig. 9e).

Selection of a LOV2 mutant with in situ response times matching target kinetics (JNK regulation) simplifies optogenetic modulation of the JNK pathway. Although JNKktr requires nuclear-cytoplasmic translocation to report a change, measured translocation rates (tau 1.8–2.8 min) are considerably faster than response rates seen here (Fig. 9f). Light-evoked inhibition of reporter is threefold faster than recovery in darkness 20 min later (Fig. 9d, f). This suggests phosphatases act more rapidly than kinases on the c-Jun derived reporter in this condition, and reporter translocation cannot explain this discrepancy. In Fig. 9e, JNK is inhibited considerably longer and the difference is greatly diminished (Fig. 9f). This reveals significant differences in re-
activation rates between the two conditions but no difference between inactivation rates (Fig. 9f). This could reflect negative feedback on the JNK pathway, whereby persistent inhibition of substrate phosphorylation by optoJNKi-sr in Fig. 9d results in higher activity, leading to more rapid recovery of the reporter response in Fig. 9d. This data alone does not discriminate between changes in kinase or phosphatase activities. Subsequent recovery in all cases to about the same level (as prior to inhibition) argues either for balanced changes in both or a rapid recovery in all cases to about the same level (as prior to spontaneous relaxation of actuators to the inactive dark-state. Relaxation rate has been measured in cell-free conditions but not in intact biological systems. Optogenetic inhibitors of endogenous logical tools can be straightforward to implement for proof-of-concept in delocalised activities—optogenetic equivalent of pharmacological tools—are particularly rare because indirect methods are generally required to validate their actions. Optogenetic activators can be straightforward to implement for proof-of-concept in

Discussion
Optogenetic approaches have revolutionised behavioural neuroscience. Implementation in cell biology and related fields has also been impressive but limited to relatively few labs. It remains challenging to develop and use optical regulators of biochemical processes. One difficulty is the inability to directly monitor the switched state of optogenetic actuators. The vast majority of cellular optogenetic actuator studies generate easily visualised end points, such as protein translocation and cell motility, where cellular outcomes can be directly verified. This is a powerful approach when activation of signalling is achieved by changes spatially tethered to a membrane compartment, for example. However, many targets are not constrained to easily visualised loci. This problem is compounded by invisible spontaneous relaxation of actuators to the inactive dark-state. Relaxation rate has been measured in cell-free conditions but not in intact biological systems. Optogenetic inhibitors of endogenous delocalised activities—optogenetic equivalent of pharmacological tools—are particularly rare because indirect methods are generally required to validate their actions. Optogenetic activators can be straightforward to implement for proof-of-concept in

Fig. 6 Activation rates and dark state relaxation rates of LOV2-based actuators fused to Ypet. a Fluorescence emission of Ypet-optoNES, detected through a 542/26 nm filter, on excitation pulses with 14–140 μmol m$^{-2}$ of 438 nm excitation light (as Fig. 3) shows reducing intensity or quenching at a rate depending on the photon dose. This is consistent with a FRET from LOV2* to Ypet that is diminished as LOV2-390 is formed. Note 5x more light is used here than for mTq2 fusions. Similar results are obtained with b Ypet-spacer-optoNES and c Ypet-optoJNKi. Means (n = 3) are shown. d shows the best-fit maximal quench increases (±S.E., n = 3 wells) with illumination intensity increases, allowing a quench limit to be estimated by fitting to a single site model (dotted line). Quench limits, activation rate constants and relaxation time constants during illumination for the Ypet constructs, and corresponding data from mScarlet constructs, are shown in Supplementary Fig. 13, indicating FRET from LOV2 to the fluorescent tags in all cases and to a reduced extent in the spacer construct (the Ypet-spacer construct with limited energy transfer was too dim for parameter estimation). e The constructs (means of n = 3 wells shown) were subjected to cycles of activation and darkness as in Fig. 4 and fluorescence (438 nm excitation, 500 nm emission, corrected to control Ypet-C1 signal shown as dotted line) measured and f fitted to repeated exponential decays with a common time constants for each construct in each experiment. g The best-fit decquench ± S.E. from f is plotted against the preceding dark time and fitted to an exponential to derive a relaxation time in darkness. The best-fit parameters—max. quench at 140 μmol m$^{-2}$ photons, apparent activation during concurrent relaxation and relaxation time during darkness—estimated from activation-relaxation cycles are listed for both Ypet and mScarlet fusions, together with derived parameters, in Table 1A. The quench limits and sensitivities are in general consistent with and dark relaxation times are slightly slower than lit relaxation times, suggesting a contribution of photochemical back reaction in d. Source data are provided as a Source Data file.
model cell lines. However, inhibitors of endogenous signalling processes can be more valuable tools to investigate physiological mechanisms than imposition of overexpressed synthetic activity. Moreover, biological end-points of interest may require maintenance of actuation for prolonged periods of time, unlike the milliseconds of activation in channelrhodopsin applications. For popular blue-light photosensors, using endogenous flavins rather than exogenous chromophores like phytochromobilin, this is an advantage because even low doses of blue light can perturb biological systems in the long term.12

Building on the easily visualised responsivity of LEXY9, the light-regulated NES, we found sensitivity could be doubled by engineering RET from a cyan fluorescent donor (Fig. 1e). Conversely red and yellow proteins act as RET acceptors, reducing sensitivity ~3.5-fold (Fig. 1 and Supplementary Table 1). Sensitivity can be dynamically tuned with a photo-switchable protein. This potentially opens the door to multiplexing, whereby orthogonal inputs (non-blue light) determine which actuator present will respond to blue light. Linker optimisation increases energy transfer from the cyan protein (Fig. 4 and Table 1a), potentially further sensitising actuation (Fig. 4e). The finding that fluorescent tags modulate activation under linear illumination complements previously described mutational tuning of LOV2 relaxation time.33,35 Combining control of both activation and relaxation rates offers precise blue-light activated cellular optogenetic design. Long-term experiments, with considerably more optogenetic manipulations, become possible before blue-light phototoxicity becomes limiting. If fewer photons are required to activate LOV2, improving temporal resolution with faster relaxing LOV2 mutants becomes possible.

Sensitisation of flavin-based optogenetic switches under non-linear illumination (two-photon microscopy) was recently described36 using mtagBFP and mTFP1 for CRY2 and LOV2 respectively. Sensitisation was not seen in wide-field mode, perhaps because mtagBFP2 and mTFP1 are less suitable than mTurquoise2 for FRET. We predicted limited overall RET between mTFP1, with characteristics mid-way between mTurquoise2 and Ypet, and LOV2 (Supplementary Fig. 15A, B). In practice, mTFP1 performs poorly as an indicator of AsLOV2 state compared with mTurquoise2 or Ypet (Supplementary Fig. 15C–E). Under two-photon microscopy, two photons are absorbed within a short time, and the higher absorbance of mTFP1 than AsLOV2 becomes the dominant factor under non-linear conditions. Under more commonly used linear excitation, we found mTurquoise2 offers at least twofold increased optogenetic sensitivity.

A second consequence of RET in LOV2-based tools is that the photocycle, i.e. optogenetic actuator switching, can be generically monitored in situ because this influences fluorescent tag quenching. Note this does not report the contextual sensitivity, by
Reduction the possible level of dequench at limiting photon flux, because a slower relaxation allows a more complete activation during the illumination phases, while faster relaxation counteracts apparent activation.

The extents of activation of the fast relaxing mutants are low, because of the considerable concurrent relaxation taking place during the activation phases. Relaxation is faster at 37 °C in cell-free spectroscopic studies, but the actuator sequence, both within the LOV2-JNK system and the N and C-terminal flanking sequences, each contribute to define the relaxation time. It is important to quantify relaxation rates of optogenetic actuators used, but these are easily determined by LOV2FRET, either in situ or in cell lysates. The former may have greater accuracy and experimental relevance.

In situ activation and relaxation parameters predict illumination requirements for actuation. The availability of in situ JNK reporters allowed us to demonstrate this in practice with an optoJNKi panel fused to different fluorophores (Fig. 7). However, the straightforward calculation algorithm used (Supplementary file) guides design of effective illumination sequences even when in situ reporters are unavailable and on-line validation not feasible.

Fast relaxation complicates multi-point objective-based imaging and increases blue-light dosage. The AsLOV2-V416I mutant slows relaxation approximately tenfold and has occasionally been implemented in optogenetic actuators. Used in optoJNKi, the in situ relaxation rate is still sufficient for investigating the slow kinetics of the JNK pathway, while providing tenfold reduced light exposure and increased experimental throughput without loss of temporal precision. This allowed us to show that transient suppression of a pathway offers repeatable and relatively non-invasive in situ quantification of pathway dynamics, during both inhibition and re-activation not achievable by pharmacological approaches.

In summary, RET can sensitise an optogenetic switch >twofold (sevenfold when replacing a red or yellow protein). RET also reports real-time in situ activation state, revealing activation sensitivity and functional consequences of RET with LOV2-bound FMN quenching by elevated temperature, low extinction coefficient and broad spectra overlapping with multiple channels (Supplementary Fig. 1) make LOV2 fluorescence inadequate for single-cell imaging in practice. LOV2 is theoretically ~50-fold dimmer than Ypet, making it sensitive to bleed-through errors in multichannel experiments. Second, the conformational change in AsLOV2Ja and optogenetic CaMKII inhibitor paAIP2 modified by fusion to GFP and dark RET-acceptor ShadowY at N and C-termini generate detectable FLIM change. However, this approach may not be generally applicable; C-terminal fusion may inactivate inhibitors requiring free C-termini (e.g. PDZ domain ligands) or when docking of the C-terminal residue of the inhibitor with a LOV2 pocket is important for photo-regulation. Moreover, functionality of the modified construct used to demonstrate photoactivation was unclear as it was not used for inhibition.

Third, Murakoshi et al. (ref. 7) co-expressed Cherry-paAIP2 with a GFP-fused version of the inhibitor target, CaMKII in this case, to achieve a FLIM readout. This may not be routinely applicable as (i) successful two-component RET is dependent on distance and angle parameters not easily controlled, (ii) target overexpression is usually undesirable in inhibitor experiments, and (iii) the targets of optogenetic inhibitors are usually endogenous signalling components that are not fluorescent. However, our results suggest that both EGFP and ShadowY-fused constructs might, like Ypet fusions, exhibit RET and reduced sensitivity. Our more generically applicable and simpler dequenching method, requiring only a conventional microscope, could be a routine alternative to these more technically challenging approaches.

A third consequence of RET with LOV2-bound FMN is the in situ determination of relaxation time. Relaxation is typically assumed a fixed LOV2 domain property albeit altered by mutation. Relaxation is faster at 37 °C in cell-free spectroscopic studies, but actuators’ relaxation measured in situ appeared even faster. Ja modification has significant effects, whereas the photoregulated peptide has a modest impact. Here we demonstrated that the fluorescent tag usually has minimal impact but the linker between tag and LOV2 has a strong effect. We conclude that both temperature and the actuator sequence, both within the LOV2-JNK system and the N and C-terminal flanking sequences, each contribute to define the relaxation time. It is important to quantify relaxation rates of optogenetic actuators used, but these are easily determined by LOV2FRET, either in situ or in cell lysates. The former may have greater accuracy and experimental relevance.

The cases we compared showed no evidence for an impact of the intracellular environment per se on relaxation time.

In situ activation and relaxation parameters predict illumination requirements for actuation. The availability of in situ JNK reporters allowed us to demonstrate this in practice with an optoJNKi panel fused to different fluorophores (Fig. 7). However, the straightforward calculation algorithm used (Supplementary file) guides design of effective illumination sequences even when in situ reporters are unavailable and on-line validation not feasible.

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In summary, RET can sensitise an optogenetic switch >twofold (sevenfold when replacing a red or yellow protein). RET also reports real-time in situ activation state, revealing activation sensitivity and
relaxation rates. These parameters guide selection of optical conditions and implementation of mutants, reducing photon dose up to tenfold while increasing experimental throughput tenfold. Finally, this improved experimental throughput allows quantification of changes in bidirectional pathway kinetics in intact living cells over time.

Methods
Reagents and plasmids. Anisomycin was purchased from Sigma-Aldrich, and Vincristine and Paclitaxel from Adooq. Dulbecco’s Modified Eagle Medium, Foetal Bovine Serum, NeurobasalTM-A medium and B-27TM supplement were from ThermoFisher Scientific. Other reagents were obtained from Worthington, Sigma, Applichem, Apollo Scientific, NEB, ThermoFisher Scientific, Takara and VWR. PCR templates for mScarlet, miRFP670 and Dronpa were generous gifts of...
Fig. 9 Optimisation of relaxation in optoJNK-sr increases sensitivity and experimental throughput tenfold, facilitating investigation of bidirectional pathway dynamics in intact living cells. a Neurons expressing mTq2-optoJNK and miRF6760-JNKotr were exposed to chemotherapeutics (Vinristine and Paclitaxel, 100 nM each). The JNK pathway was measured during alternating periods of blue light flashes and darkness, b After 6 h, JNK reporter activity increases steadily. Using the original optoJNK, blue light (420 µmol m−2 438 nm every 2 min, 8.3 mHz, vertical blue lines) has no affect. Predicted adduct levels decay rapidly after each flash (inset); c 11 h after addition of chemotherapeutics, the reporter response has stabilised, and tenfold increased flash rate (every 12 s, 83 mHz) achieves detectable slow reduction of JNK reporter response, consistent with adduct prediction (inset). The reporter response gradually recovers in darkness; d Experiments in c are repeated using optoJNK-sr, harbouring AsLOV2-V416I in as in Fig. 8, slowing relaxation tenfold (Supplementary Fig. 14). The lower flash rate (8.3 mHz) is now sufficient to maintain substantial adduct state (inset) and inhibit the pathway, as judged by the reporter readout, while reducing tenfold both photon dose and time spent on an imaging field. The latter permits the imaging of multiple samples in parallel (12 here compared with only 1 in b, c, d). Samples, 11 h after chemotherapeutic addition, were imaged as in d with an extended actuation period (60 min every second hour) repeated five times. Inhibition and recovery reaches equilibrium in lit and dark phases. e The averaged JNK pathway reporter response to light in d were fitted to exponentials with best-fit time constants shown (tau) as estimates of the reduction and recovery rates of reporter activity. Comparison of time-constants by F-test indicates significant two- to threefold differences between rates of inhibition and reactivation 20’ later (F(1,15) = 13.46) and of reactivation after 20’ vs. after 60’ inhibition (F(15, 55) = 9.611), suggesting different reactivation mechanisms depending on the duration of inhibition. The small difference between inhibition and reactivation rate at 60’ is also seen (F(1,55) = 39.61). ***P < 0.0001, **P = 0.0023, n.s. not significant. Source data are provided as a Source Data file. Means ± SEM (n = 4 wells) are shown in b–e.

Guillaume Jacquemet42 and Stefan Jacobs43. VinculinTS, a gift from Martin Covert (Addgene plasmid # 59148; http://n2t.net/addgene:59148; RRID: Addgene_59148). The AAV plasmid backbone was derived from pAAV-Covert (Addgene plasmid # 59148; http://n2t.net/addgene:59148; RRID: Addgene_59148; ref. 25). The AAV plasmid backbone was derived from pAAV-hSyn-3xFLAG-WPRE (RRID:Addgene_127862), generated previously45. The addgene.org.

Cell culture. Hippocampal and cortical neuron cultures were prepared from P0 rats of either sex (mixed)46. Briefly, rat hippocampi and cortices were dissected separately in dissociation medium (81.8 mM Na2SO4, 30 mM K2SO4, 15.8 mM MgCl2, 0.25 mM CaCl2, 1.5 mM Hepes, 20 mM Glucose, 1 mM Kynurenic acid, MgCl2, 0.25 mM CaCl2, 1.5 mM Hepes, 20 mM Glucose, 1 mM Kynurenic acid, pH 7.4) and digested with 50U papain, followed by 50U DNaseI treatment. Tissue samples, 11 h after chemotherapeutic addition, were imaged as in d with an extended actuation period (60 min every second hour) repeated five times. Inhibition and recovery reaches equilibrium in lit and dark phases. The averaged JNK pathway reporter response to light in d were fitted to exponentials with best-fit time constants shown (tau) as estimates of the reduction and recovery rates of reporter activity. Comparison of time-constants by F-test indicates significant two- to threefold differences between rates of inhibition and reactivation 20’ later (F(1,15) = 13.46) and of reactivation after 20’ vs. after 60’ inhibition (F(15, 55) = 9.611), suggesting different reactivation mechanisms depending on the duration of inhibition. The small difference between inhibition and reactivation rate at 60’ is also seen (F(1,55) = 39.61). ***P < 0.0001, **P = 0.0023, n.s. not significant. Source data are provided as a Source Data file. Means ± SEM (n = 4 wells) are shown in b–e.

Transfection and AAV addition. For experiments in HEK293 cells, cells were transfected with corresponding plasmids using the calcium phosphate co-precipitation method, a procedure for which has been included in the step-by-step protocol for RET-enhanced measurement of relaxation time constants of LOV2-based actuators.13 Briefly, for one 35 mm dish of cells, a total 2.5 µg DNA was mixed with H2O and 6.25 µl 2.5 M CaCl2 at a total volume of 62.5 µl. DNA and CaCl2, were added to the same volume of 2XHebs buffer (274 mM NaCl, 10 mM KCl, 1.4 mM NaHPO4, 15 mM t-glucose, 42 mM Hepes, pH 7.10) while swirling. After 30 min incubation under ambient conditions, the suspension of precipitate was distributed evenly over the cultures dropwise. For experiments in neurons, corresponding AAV supernatants were added in the culture and experiments were carried out 7 days after AAV addition.

Spectral comparisons. All FP spectra were obtained from FPbase48, except for Dronpa off-state absorption spectra that were taken from ref. 11. LOV2-bound FMN spectra were obtained from ref. 38.

Image acquisition. For imaging, neurons were plated in 384 well carrier plates (Perkin Elmer cat 6057302) and HEK293 cells were plated on 96-well microwell plates (Greiner cat 655090). All image data were acquired with a BD pathway 855 High-Content Analyzer (Beckton Dickinson) running Attovision V1.6, equipped with high power LED sources at 405, 445 and 623 nm, as well a white light LED source (SOLIS-405C, 453C, 623C and 3C, respectively, Thorlabs). mTurquoise2, Ypet, mScarlet and miRF6760 were excited through 438/24 nm, 500/20 nm, 555/28 nm, and 635/20 nm excitation filters, and dichroics at 458, 515, 595 nm, and a multiplex dichroic for the miRF6760 channel (Chroma #480007). Images were acquired through emission filters 483/32 nm, 542/27 nm; 645/75 nm and 745/75 nm. Filters were from Semrock and Chroma. The LEDs were electronically shut-tered through an optocoupler/double-inverter TTL circuit for precise exposure times and synchronisation with camera acquisition. The rest of the imaging system was distributed evenly over the cultures dropwise. For experiments in neurons, corresponding AAV supernatants were added in the culture and experiments were carried out 7 days after AAV addition.

Nuclear translocation imaging. In Fig. 1, multiple miRF6760 images were captured to localise nuclear regions, followed by ten images each of mTurquoise2, Ypet and mScarlet at a rate of one every 10 s. The next well was then imaged and any one well was not visited again for at least 10 min to allow recovery of nuclear translocation. Exposures for miRF6760 (1 s), Ypet (0.5 s) and mScarlet (1 s) were kept constant in all cases and 436 nm exposure for mTurquoise2 and LOV2 activation was varied over the range indicated in the figures (10, 30, 100 and 300 ms, corresponding to 14–420 µmol photons.m−2) to determine the sensitivity of the response. To ensure consistent exposure conditions in all cases, all wells were exposed to the same series of excitations regardless of whether the well expressed only one fluorophore or all three. Each well was revisited for each exposure setting (10–300 ms) and this was repeated multiple times. The data shown for mTurq2, Ypet and mScarlet fusions of optoNES were obtained from an average of four visits at each setting using hippocampal neurons at 8 days in vitro (n = 6 replicates). In the case of Dronpa-optoNES, the experimental comparison in Fig. 1 (and Supplementary Fig. 9) was between Dronpa-off and Dronpa-on state at distinct illumination intensities. We established the sensitivity of Dronpa to photoswitching in our system in pilot experiments. Dronpa is known to be switched to fluorescent state with exposure to 405 nm light, and to non-fluorescent 400 nm-absorbing state.
(Supplementary Fig. 9, <1% maximal fluorescence1,14,15) with 488 nm light. The spontaneous relaxation is from dark-state back fluorescent state with a reported half-life before there will be no significant relaxation occurring during our experiments. We found 438 nm light was able to cause Dronpa on-switching, but 470 nm, which is similarly absorbed by LOV2 (Supplementary Fig. 1), could not. Therefore 470 nm was selected for LOV2 switching in place of 438 nm so as to avoid spontaneously affecting Dronpa state. With our illumination system, we were able to switch Dronpa off most effectively with 500 nm light (~80% drop in fluorescence with 30 s exposure) so this was selected for the Dronpa off-switching phase. At 405 nm, we achieved ~20% reactivation in 0.1 s. Therefore in the experiments. We found 438 nm light was able to cause Dronpa on-switching, but these images, the ROIs were cropped from the final nuclear ROIs for calculation, and expanded as a band to define a cytoplasmic ROI. For simplicity, and considering that it is neither practical nor necessary to accurately capture the entire cytoplasm of a neuronal cell under our conditions, cytoplasmic ROIs were not further processed and therefore contained some background area. This means the cytoplasmic ROIs are not consistent under-estimation of nuclear distribution (e.g. Fig. 1a, first frame) generated a nuclear:cytoplasmic ratio >1 (e.g. average 2.37 for mTq2-optoNES, Supplementary Table 1). This systematic nuclear bias as described was applied blindly to all samples and should not affect the relative changes. Most importantly it will not affect the rank photon-sensitivity of the different constructs which is orthogonal to the actual ratio values. Similarly, although spacer constructs had a more effective NLS sequence (Supplementary Fig. 8 and Supplementary movies 4–6), evidenced by over twofold higher baseline nuclear:cytoplasmic ratios (Supplementary Table 1), this should not affect the photon-sensitivity of the constructs. Mean intensities in nuclear and cytoplasmic ROIs were reported as thresholded values for thresholding of the final nuclear ROIs and remaining ROIs were used to determine nuclear:cytoplasmic ratios at each time point. At each time point ratios from all cells in a field were averaged to generate a single ratio per time point for each 438 nm exposure and each well revisit. Individual time point values for each well revisit were averaged to improve signal-to-noise. All images were normalized to the dark-field as shown in Fig. 1c. Only one field was captured from each replicate. Linear regression yielded a gradient and offset value from data from each well for each 438 nm photon dose, the gradient representing the rate of change of nuclear:cytoplasmic ratio per flash of light at the given dose. Means ± SEM of these values were determined from six replicates for each construct shown in Fig. 1 and these were plotted against photon dose as shown in Fig. 1e. Each gradient-dose curve was fitted to a single-site model (Eq. 1) using GraphPad Prism software to derive best-fit photon sensitivity and max response values and their standard errors, as shown in Supplementary Table 1. All scripts and data are available.

Calculation of fluorescent protein dequenching and quenching in situ

Quench and dequench experiments were carried out on HEK293 cells transfected with a single fluorescent construct only, as indicated. Images were acquired by repeated imaging, and for relaxation time calculations, defined periods without imaging as indicated in the figures. The pattern shown was repeated to allow data averaging and reduction of signal to noise. Image time-stacks were background-subtracted (rolling ball 200 pixels). ROIs were generated by segmentation of maximum projections of the entire image time-stacks in each case. ROIs from all ROIs segmented in each field were averaged. Only one field was captured per well, but repeated data captured from each field was averaged, for each time point. This provided one quench/dequench curve per replicate for each condition and averages of replicates are shown. SEMs are not shown in all the main figures for clarity but are available in the source data for each figure.

Acquired dequenching curves in response to 438 nm illumination of mTq2-LOV2 constructs (Figs. 2–5, 8) were fitted to exponentials and the corresponding quench curves (for Ypet and mScarlet fusions) were fitted similarly (Fig. 6 and Supplementary Fig. 15), providing time constants and max. apparent dequench/quench (at the given intensity; lower intensity results in reduced max. apparent dequench/quench because of concurrent relaxation—see Fig. 2e). Repeated dequench/quench-relaxation curves (Figs. 3–6, 8) were fitted to a common time constant but freely fitted max. apparent dequench/quench. In the relaxation experiments, the apparent dequench or quench was plotted against the preceding dark time and this curve fitted to an exponential to derive relaxation time constants and a better estimate of apparent dequench/quench (given the concurrent relaxation). The derived constants are shown together in Table 1.

Calculation of JNK pathway activity

Activation of the JNK pathway reporter JNK2r was quantified as the ratio of reporter signal detected in nuclear and cytoplasmic compartments. These were obtained with ImageJ scripts similar to that used for the analysis described in our previous Calculation of Nuclear Translocation. Multi-channel image time-stacks were background-subtracted (50 pixel rolling ball subtraction) and aligned. The locations of cell nuclei were determined by segmentation of maximum projection images of NLS-fused fluorescent proteins imaged, as described in the section JNK pathway Imaging. Nuclear and cytoplasmic ROIs were generated for each nucleus as described Calculation of Nuclear Translocation. Intensity ratios between cytoplasmic-nuclear ROI pairs were calculated, after thresholding each ROI for minimal and maximal intensities to remove very dim
Light calibration. Intensities at the sample plane were determined with a power metre with microscope-stage attachment (Ex-Cite XR2100 with X-Cite XP750, LDGII), set for the relevant central wavelength.

Measurement of dequench recovery in cell lysates. A step-by-step protocol for RET-enhanced measurement of the relaxation time constants of LOV2-based actuators has been deposited at protocol exchange. HEK293 cells were transiently transfected with CMV-driven optoNES, mTq2dC7-408LOV2-NS2, mTq2dC10-408LOV2-NS2, mTq2dC11-408LOV2-NS2 constructs using calcium phosphate precipitation. Cells were lysed 48 h post-transfection with low stringency buffer (LSB) supplemented with protease inhibitors and detergent 0.5% Igepal. Lysates were cleared by centrifugation at 20,000 × g, 4°C for 10 min. In total, 20 µl of cell lysate was transferred to a half-area 96 well µClear plate (Greiner cat 675090) and 5 µl of mineral oil (Sigma-Aldrich) was added to prevent evaporation. Before each measurement, the plate was equilibrated to either 25 or 37°C in a plate reader (POLARstar OPTIMA, BMG) for 5–10 min. The stability of the dark state was determined by measurement after temperature equilibration. The adduct state was determined by measurement after temperature equilibration. The adduct state was monitored (arbitrary units) at reading

\[ F_t = F_{\text{lim}} - A_t |x| \cdot e^{-t/\tau} \]

where \( x \) is the reading number (i.e. flash number), \( F_t \) is the fluorescence measured (arbitrary units) at reading \( x \), \( F_{\text{lim}} \) is the fitted fluorescence limit as exposure (8 flashes) \( \sim \infty, A_t = \) the fitted recovery from (de)quenching after the defined dark period \( t \), and \( \tau \) is the shared activation rate constant in units of flashes, \( \sim k_{\text{app}}/\text{photons per flash} \).

In Prism, each of the 12 cycles occupies a separate data column with unique \( A \) but shared \( T \) (see source data). In Excel the data can be fitted as a single column with row-specific \( A \) values but this does not directly provide standard errors of the fitted constants. In Figs. 3c, 6c, 7, the apparent activation rate or forward rate constant is shown as \( k_{\text{app}} \) in µmol m⁻² at the photon dose per flash used.

The \( A \) values obtained above for each dark time were plotted against dark time \( t \) and fitted to the exponential

\[ A(t) = A_0 \left( 1 - e^{-t/\tau} \right) \]

where \( \tau \) is the dark time, \( A(t) = \) the best-fit values of the recovery from dequenching or quenching after the dark time \( t \), from the curve fit above. \( A_0 \) is the fitted maximal increase in fluorescence as dark time \( \to \infty \), and \( \tau \) is the relaxation time constant, shared across cycles with the same construct.

This curve is shown in Figs. 3d, 4d, 5c, 6d, g, and 8c, where the dequench or quench maxima at the photon doses used were calculated from the maximal change in fluorescence as a fraction of the maximal fluorescence. Thus max. dequench max. at the photon dose per flash specified in the tables = \( A_0/(1 + A_0) \) as a %.

Quantification of dequench limit, ED50 and sensitivity. The above fit was carried out to help visualise the quench and dequench recovery under the conditions used. To acquire more fundamental properties the equation below was fitted to all datasets. One exception was the mTq2-optoNES v mTq2-spactor-optoNES dataset (Fig. 3). In this case the response of the spacer construct was low and the fits to individual datasets generated a poor fit. Therefore the averages \((n=5–6)\) were used instead for fitting to the equation below, which produced a much better fit.

Dequench limit, sensitivity and ED50 were obtained by fitting all datasets to the following formula:

\[ Y = \text{Offset} \times \left( 1 - D_{\text{lim}} \right) \times \left( D_{\text{max}} \cdot |dose| \cdot \left( 1 - \frac{1}{C_0 \cdot C_1} \right) \right) \]

where \( D_{\text{max}}/|dose| = D_{\text{max}}/(1 + k_{\text{app}}/k_{\text{app}}) \), \( k_{\text{app}} \) = the apparent forward rate constant \( k_1 + k_2, k_2 \) = the forward rate constant = dose/sensitivity, 56/sensitivity in µmol m⁻² in this case, \( k_2 \) = the reverse rate constant = 1/\( \tau \), the relaxation time constant.

In Prism this equation was expressed as:

\[ Y = \text{Offset} \times \left( 1 - D_{\text{lim}} \right) \times \left( D_{\text{max}} \cdot |dose| \cdot \left( 1 - \frac{1}{C_0 \cdot C_1} \right) \right) \]

where the fluorophore acted as an acceptor and was quenched, which was the case for the above equation, the solution limit, sensitivity and ED50 were obtained by fitting all datasets to the following formula:

\[ Y = \text{Offset} \times \left( 1 - Q_{\text{max}} \cdot |dose| \cdot \left( 1 - \exp(-d_{\text{lim}}/|dose|) \right) \times \left( 1 - \frac{1}{C_0 \cdot C_1} \right) \right) \]

and ED50 was calculated as sens/r.

Significant differences in best-fit parameters were determined in Table 1 with PRISM based on the manufacturer’s instructions (https://www.graphpad.com/prism/.guides/prism/7/curve-fitting/index.htm?reg_comparing_fits_with_anova.htm) using the standard errors of the best-fit parameters and degrees of freedom for each value by one-way ANOVA followed by Bonferroni’s multiple comparisons test.
Dark periods were 1–180 s as shown. The first dequequench was set arbitrarily as 600 s because a minimum of 10 min was left between well revisits and this is far above all time constants measured.

Analysis of JNKtr reductions and recoveries. Data from all replicates (n = 4) and all reduction phases or recovery phases (4–5 repeats per replicate), during blue-light illumination or blue-light darkness were averaged. The averaged curves were fitted to exponentials. The impact of replacing the dark-phase recovery time constant with an exponential decay, corresponding to the slow relaxation of the optoNIKs+ inhibitor, was considered. Thus in the parameter $e^{-t/\tau}$, the uninhibited forward rate constant $k_f$ was replaced with $(1-Ae^{-t/\tau})k_f$ where $A$ represents the maximal fractional reduction of $k_f$ by the optogenetic inhibitor, and $r$ the rate constant at which optogenetic inhibitor relaxes, ~2 min in this case. This had minimal effect (~10%) on fitted time constants, even on the fastest kinetics.

Therefore the slowed relaxation of optoNIKs+ cannot explain the slower recovery of JNKtr signal after 20' inhibition compared with after 60' inhibition (Fig. 9d, e). This decaying time constant was not routinely included, to limit the number of unknowns in the curve-fit formulae. To compare $JNKtr$ reduction and increase in the same curve fit for each experiment, spans were set as a shared variable, and the fitted constants were compared by F-test, the results of which are cited in the figure and in the results text.

Simulation of adduct state fraction. It is possible to simulate the expected proportion of actuator in adduct fraction over time, on the basis of the optogenetic actuator sensitivities and relaxation times, together with specific input of blue light over time. Sensitivities used were based on measurements with 438 nm light, because AsLOV2 absorbance at 438 nm (used in microscopy) and 465 nm (from the parallel LED trans-illuminator device) are very similar. An excel sheet generating the simulation with the desired time step resolution is provided as a Supplementary file.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data are provided with this paper. Figure data are in source data files. Raw imaging data, together with associated metadata files, for Figs. 1–9 are available at https://doi.org/10.5281/zenodo.3882572. Scripts used for controlling the image acquisition are within this metadata. All data in this manuscript are available from the corresponding author upon request. All plasmids used in this manuscript were new plasmids and deposited in addgene under IDs 159941–159976. They are described in detail in Supplementary Table 3. These plasmids were generated in part with the help of previously generated plasmids (including addgene IDs 89744, 89749, 127862) and plasmids provided by other labs (including addgene IDs 26019, 59148). The FBbase database was also used for this work (https://www.fpbase.org/). Any other relevant data are available from the authors upon reasonable request. Source data are provided with this paper.

Code availability
Scripts for analysing the optoNES translocation and in situ photocycling, outputting the source data, are in Supplementary files. The excel calculator for predicting adduct state over time is in the Supplementary.

Received: 25 February 2020; Accepted: 8 September 2020; Published online: 9 October 2020

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Acknowledgements
This work was supported by the Magnus Ehrnrooths Foundation (M.J.C.), the National Cancer Institute Grant R01CA200417 (M.J.C. and L.L.L.), the European Union Erasmus + programme (F.M.K.), and 7th Framework Programme Initial Training Networks FP7-PEOPLE-2013-ITN Project Number 608346 Project ‘Brain Imaging Return To Health’ r’Birth (A.P., L.L.L.), the Academy of Finland (mobility projects 309736 and 324581 to M.J.C.), the DAAD with funds from the German Federal Ministry of Education and Research (57348387 and 57498932 to F.F.), and by access to the facilities of the Turku Screening Unit, a member of the Biocentre Finland Drug Discovery and Chemical Biology Network. We thank David Kelly (Beckton Dickinson) for advice on Pathway 855 instrument maintenance, Michiyuki Matsuda (Kyoto University), Stefan Jakobs (Max Planck Institute for Biophysical Chemistry, Göttingen) and Guillaume Jacquemet (Åbo Academy University) for generously providing fluorescent protein templates, Peter Martinsson (University of Eastern Finland) for generating the Dronpa coding sequence, Elena Tcarenkova (University of Turku) for helpful discussion and James Conway (University of Turku) for critical reading of the manuscript.

Author contributions
L.L.L. prepared samples and reagents, and designed and carried out the experiments, analysed the data and performed statistical analyses. F.M.K. prepared samples and reagents, and performed experiments and analysed the data. L.L.G. participated in the preparation of initial optoNES constructs. A.P. participated in the preparation of AAV vector systems. F.F. assisted and advised on application of the AAV system. M.J.C. designed the project and experiments, prepared reagents, carried out experiments, analysed data, and prepared the manuscript. All authors assisted in drafting the manuscript.

Competing interests
The authors have no competing interest

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-18816-8.

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Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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