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Genetically targeted chromophore-assisted light inactivation

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Studies of protein function would be facilitated by a general method to inactivate selected proteins in living cells noninvasively with high spatial and temporal precision. Chromophore-assisted light inactivation (CALI)[1] uses photochemically generated, reactive oxygen species to inactivate proteins acutely, but its use has been limited by the need to microinject dye-labeled nonfunction-blocking antibodies. We now demonstrate CALI of connexin43 (Cx43) and α_{1C} L-type calcium channels, each tagged with one or two small tetracysteine (TC) motifs[2] that specifically bind the membrane-permeant, red biarsenical dye, ReAsH[3,4]. ReAsH-based CALI is genetically targeted, requires no antibodies or microinjection, and inactivates each protein by ~90% in <30 s of widefield illumination. Similar light doses applied to Cx43 or α_{1C} tagged with green fluorescent protein (GFP) had negligible to slight effects with or without ReAsH exposure, showing the expected molecular specificity. ReAsH-mediated CALI acts largely via singlet oxygen because quenchers or enhancers of singlet oxygen respectively inhibit or enhance CALI.

Approaches based on nucleic acids such as gene knockouts, antisense and RNA interference to eliminate specific proteins intrinsically have limited temporal and spatial resolution, because they only shut off the supply of mRNA and do not eliminate pre-existing copies of protein. Long-term slow ablations give ample opportunity for phenotypes either to be obscured by substitution of related proteins for the missing one, or to be amplified by cascades of abnormalities[5]. Small molecule inhibitors can act quickly, but finding potent and selective inhibitors for arbitrary proteins usually requires luck and the resources of a pharmaceutical company.

The most promising general approach for inactivating specific proteins with high spatial and temporal precision is chromophore-assisted light inactivation (CALI)[1,6]. Strong illumination of the chromophore generates short-lived reactive oxygen species that can inactivate nearly any protein in the immediate vicinity of the chromophore. CALI of selected antigens was originally done with malachite green–labeled antibodies that had to be microinjected into cells and excited with a pulsed laser. The reactive oxygen species was proposed to be a hydroxyl radical, and the radius of half-maximal CALI was estimated to be about 1.5 nm[7]. More recently, it has been found that fluorescein is a more efficient photosensitizer than malachite green and may work through singlet oxygen, giving a slightly longer inactivation range of 3–4 nm[8,9].

The major limitations of CALI in living cells have been the need to i) raise nonblocking antibodies to the protein of interest, ii) conjugate malachite green or fluorescein to the antibody and iii) microinject the labeled antibody into the cells at a concentration high enough to saturate the antigens but not high enough to promote nonspecific CALI. We present an alternative form of CALI that avoids the need to raise, label and microinject antibodies. Instead, the target protein, genetically tagged (as illustrated in **Fig. 1a**) with a small TC motif, is introduced by transfection[2]. The TC motif can be specifically labeled *in situ* with a red biarsenical fluorophore, ReAsH-EDT_2 (refs. 3,4). ReAsH is membrane permeant, so microinjection is not required, and excess dye can be washed away before illumination. ReAsH offers the optimum balance so far between ease of TC labeling, efficient generation of reactive oxygen species and resistance to photobleaching. An earlier biarsenical fluorophore based on fluorescein, FlAsH-EDT_2 (refs. 2,4) has been used to photoinactivate TC-tagged synaptotagmin I, which replaced native synaptotagmin *in vivo* in *Drosophila* neuromuscular junctions[10]. We therefore compared ReAsH and FlAsH and analyzed the mechanism of ReAsH-mediated CALI.

We first tested ReAsH-mediated CALI on Cx43, a building block of gap junctions[11], genetically tagged with a C-terminal TC domain[4] (Cx43-TC). **Figure 1b** shows a ReAsH-stained gap junction between two cells that were each patch-clamped to continuously monitor their electrical coupling (see **Supplementary Fig. 1** online for raw data traces). A typical Cx43-TC CALI experiment is shown in **Figure 1c**. Coupling was stable before illumination, decreased slightly during a 25-s illumination with 10% of maximal excitation, then was 95% eliminated by a 25-s excitation at full intensity (17 W/cm^2). Here the gap junction was stained with 600 nM ReAsH-EDT_2. Both fluorescence intensity and CALI rate approximately doubled at saturated staining conditions of 2.5 μM dye (**Fig. 1d**). Each 5-s illumination period inactivated about half the remaining channels. **Figures 1c and 1d** demonstrate the dependence of CALI on cumulative illumination and ReAsH loading, as well as the rapidity and completeness of ablation under optimal conditions.

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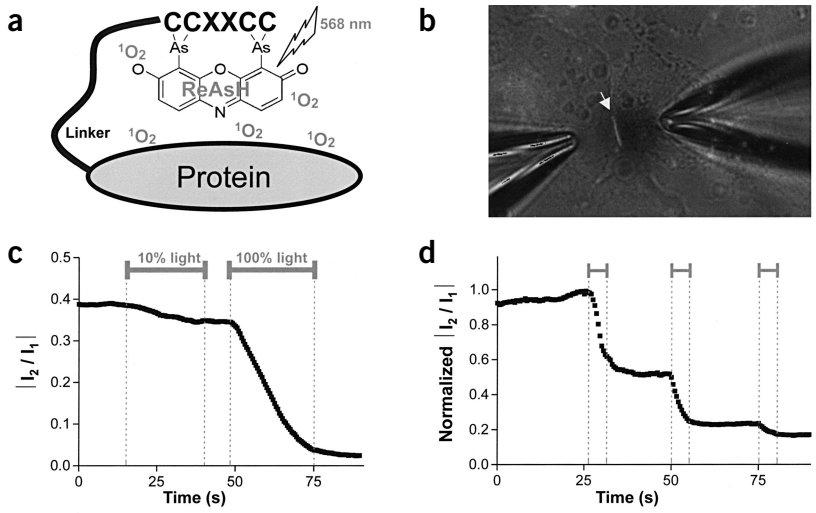

Figure 1 Staining and photoinactivation of TC-tagged gap junctions. (**a**) Illumination of ReAsH-labeled TC-tagged protein generates localized reactive oxygen species, especially singlet oxygen $^1O_2$, inactivating the protein. (**b**) A ReAsH stained gap junction (indicated by the arrow) is excited through a 568/55 filter; the emission was collected using a 653/95 filter. The two patch electrodes are shown using white light illumination. (**c**) Coupling ratio $|I_2/I_1|$ during a typical Cx43-TC CALI experiment. Twenty-five second exposure ('100% light', 17 W/cm^2 at 568/55 nm) inactivated 95% of the coupling. See **Supplementary Figure 1** online for raw data traces. (**d**) Higher levels of ReAsH staining increase the rate of CALI. Three exposures of 5 s during the periods indicated cumulatively inactivated 85% of the coupling.

To rule out the possibility that the observed gap junction inactivation was due to illumination alone, we monitored gap junctions composed of Cx43 fused at its C terminus to either GFP[12] (Cx43-GFP) or monomeric red fluorescent protein 1 (mRFP1)[13] (Cx43-mRFP). No decrease in cell coupling was ever observed when cells were exposed to either blue or green light without ReAsH present (**Fig. 2a,b**). When mock ReAsH staining was applied to these constructs lacking TCs, only 6–14% CALI resulted (**Fig. 2b**). As expected, higher concentrations of ReAsH led to undesired increases in nonspecific CALI.

FlAsH, a fluorescein analog, was the first successful biarsenical dye[2]. **Figure 2a** also shows CALI on FlAsH-labeled Cx43-TC. Full-power illumination for 30 s decreased cell coupling by 40%, demonstrating that FlAsH is substantially less effective than ReAsH for CALI.

For a second model system, we chose the α_{1C} L-type cardiac calcium channel, whose N terminus was fused to a tandem repeat of two improved TCs (2TC-α_{1C}), each containing a more recent, higher-affinity motif[3], CCPGCC. Peak current decreased stepwise after each of three light exposures (10 s each), accumulating to an 86% loss (**Fig. 3a**). There was no change in consecutive peak currents whenever the light was shut off (see superposition of currents in the insert, **Fig. 3a**), showing that CALI progressed only during actual illumination. After partial CALI, the residual currents were essentially identical in time course and voltage dependence to the full currents before CALI (**Supplementary Figs. 2** and **3** online). Therefore the channels seemed to undergo simple all-or-none photoinactivation. When α_{1C} channels without TCs were treated identically with ReAsH, the average CALI was only 21 ± 6% (n = 11; **Fig. 3b**, second column); when the channels were tagged with 2TC, CALI was 88 ± 5% (n = 17; **Fig. 3b**, first column), a 67% higher specific CALI than without TCs. As with Cx43-TC, FlAsH was less effective than ReAsH for CALI of 2TC-α_{1C}, and no detectable CALI was observed for GFP-α_{1C} (**Fig. 3c**).

An additional specificity control tested whether ReAsH bound to 2TC-α_{1C} mediates CALI of bystander, untagged membrane proteins not known to be complexed with Ca^{2+} channels. We chose to monitor a native chloride conductance present in HEK293 cells. An example of these currents before the end of the depolarizing step to +40 mV, after most of the Ba^{2+} currents have been inactivated, can be seen in **Supplementary Figure 3a,b** online. The chloride currents can be isolated by blocking the Ba^{2+} currents with 10 μM nifedipine (data not shown). In ten cells expressing 2TC-α_{1C} channels and stained according to the standard ReAsH protocol, we observed a residual chloride current of 96 ± 7% and 98 ± 7% after 10 s and 30 s, respectively, of high illumination (17 W/cm^2). Thus bystander inactivation of chloride channels is negligible during CALI of 2TC-α_{1C}. Such specificity is consistent with previous results[10], which showed that photoinactivation of electrically evoked transmitter release left hyperosmolarity-induced exocytosis largely intact.

We probed the mechanism of ReAsH-mediated CALI by adding singlet oxygen quenchers to the intracellular and external solutions (**Fig. 3d**). Sodium azide (NaN_3) at concentrations of 0.5–10 mM inhibited 25–90% of CALI in a dose-dependent manner. At 10 mM NaN_3, CALI of 2TC-α_{1C} was less than CALI of untagged α_{1C} without azide, showing the ability of NaN_3 to block nonspecific CALI as well. Although azide is a favorite singlet oxygen quencher in physical

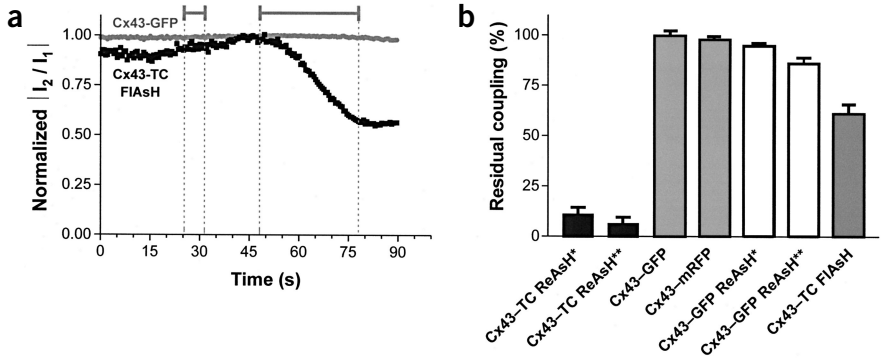

Figure 2 Comparing CALI using ReAsH, FlAsH and fluorescent proteins. (**a**) FlAsH (black) induced modest CALI. A 5-s exposure to 15 W/cm^2 at 480/35 nm gave no detectable inhibition of coupling, but a subsequent 30-s exposure gave moderate CALI. GFP (gray) does not induce any CALI under similar conditions. (**b**) A statistical summary of the extent of CALI induced by 30-s illumination (unless otherwise noted) of a variety of recombinant Cx43 fusions and staining conditions. Cx43-TC ReAsH*, stained with 600 nM ReAsH, washed with 500 μM EDT; Cx43-TC ReAsH**, 2.5 μM ReAsH, 500 μM EDT wash, 15-s exposure; Cx43-GFP and Cx43-mRFP, no dye added; Cx43-GFP ReAsH*, 600 nM ReAsH, 500 μM EDT wash; Cx43-GFP ReAsH**, 2.5 μM ReAsH, 500 μM EDT wash; Cx43-TC FlAsH, 1 μM FlAsH, 500 μM EDT wash. There were four to seven observations for each condition.

chemistry, it is a potent mitochondrial inhibitor. Imidazole lacks obvious toxicities and was also highly effective at limiting CALI, though 30 mM or higher concentrations were required (**Fig. 3d**).

Can CALI be enhanced? Substitution of D_2O for H_2O greatly extends the lifetime of singlet oxygen in aqueous solutions[14], and brief superfusion with 100% O_2-saturated medium increased oxygen partial pressure (pO_2) almost fivefold. These procedures caused a 1.68-fold and a 2.06-fold increase in CALI, respectively, after 10 s illumination, supporting the role of singlet oxygen (**Fig. 3d**).

In conclusion, the biarsenical-tetracysteine *in situ* labeling system was previously shown to be useful for pulse-chase differentiation of new versus old copies of a given protein species and for correlating live-cell fluorescence microscopy with higher resolution electron microscopy[4]. We have now demonstrated a third application: photochemical inactivation of genetically targeted proteins within seconds using widefield illumination rather than focused lasers. Such CALI provides a general strategy to inhibit proteins with high temporal and spatial resolution, which will be especially valuable when no specific pharmacological blockers are available or when one needs to confine the perturbation to a local zone.

FlAsH is an effective CALI sensitizer on synaptotagmin I, inducing maximal inactivation in less than 1 min[10]. In our hands, ReAsH was modestly superior to FlAsH at photoinactivating 2TC-α_{1C} channels and substantially more effective than FlAsH for CALI of Cx43-TC. The latter difference is most striking if we compare submaximal inhibitions, that is, short illumination periods. Exciting ReAsH (**Fig. 1d**) or FlAsH (**Fig. 2a**) for 5 s decreased Cx43-TC-mediated cell coupling by ~50% and ~2%, respectively. In our hands GFP was completely ineffective (**Figs. 2a,b** and **3c**) as a CALI inducer. Thus ReAsH seems the best current mediator of genetically encoded CALI; in a similar way, intense illumination of ReAsH, but not FlAsH or GFP, produces reactive oxygen species in fixed tissues for efficient photopolymerization of diaminobenzidine and high-resolution EM detection[4]. Intense, focused laser illumination of GFP-fused actinin causes CALI as shown by retraction of stress fibers[15], but the local photon doses used (105 kJ/cm^2) were ~200 times higher than the largest dose used here for ReAsH CALI.

We foresee several ways to increase specific CALI of the target protein and its local partners while minimizing nonspecific damage to the rest of the cell. In 2TC-α_{1C}, the CCRECC TC tag was replaced by a tandem repeat of two CCPGCC motifs, each of which has a ~20-fold higher affinity for biarsenicals[3] showing that CALI of proteins labeled on only one subunit is possible. On the other hand, CALI of Cx43-TC may have been facilitated by the presence of 12 TC sites per dodecamer channel[11] and tags on closely packed adjacent channels. Further combinatorial optimization of the three amino acids just before and the three just after the CCPGCC has produced substantial further increases in affinity and quantum yield (B. Martin, W.C. Jackson & R.Y.T., unpublished data). Another promising tactic is to use TCs on GFP as composite tags. Before ReAsH staining, the GFP is detectable as usual with very low background. After staining, excitation of GFP causes efficient fluorescence resonance energy transfer to ReAsH, which then functions photochemically as if directly excited. Nonspecifically bound ReAsH is discriminated against because it lacks GFP to serve as an antenna. Finally, yet better analogs of ReAsH may be invented.

The ability of FlAsH and ReAsH to photoinactivate proteins to which they are attached does suggest concerns for their use in live-cell imaging, especially over long observation periods. Fortunately, there is a considerable gap between the exposures required for fluorescence imaging (*e.g.*, 1.7 W/cm^2 for 0.25 s to obtain the fluorescence component of **Fig. 1b**) versus CALI (ten times the intensity for 100 times longer). An additional safety margin might come from control of the pO_2. Most mammalian cells *in vivo* normally live at pO_2 less than venous blood, or about 35 torr, far less than the 160 torr of air-saturated media. Reducing the pO_2 to that of venous blood would not only minimize CALI and other photodynamic damage but also be more hospitable to cells[16]. When CALI is the goal, brief superfusion with 100% O_2-saturated medium (pO_2 = 760 mm) is helpful (**Fig. 3d**).

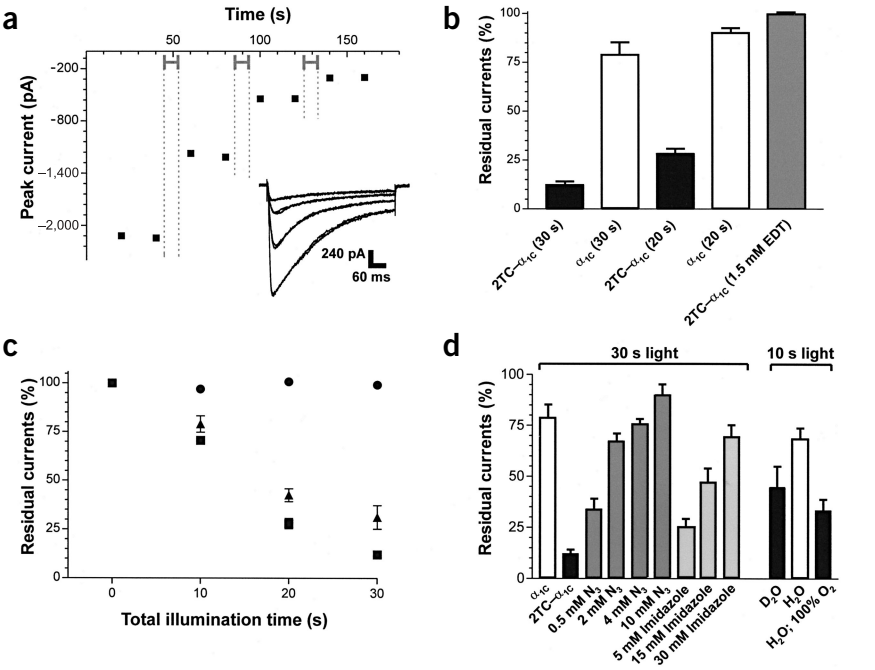

Figure 3 CALI of α_{1C} Ca^{2+} channels. (**a**) The time course of a typical 2TC-α_{1C} CALI experiment. After whole-cell patch clamping was established, 400 ms depolarizing pulses from –80 mV to 0 mV were applied at 20-s intervals. A total of 30 s of illumination applied in three 10-s exposures as indicated (17 W/cm^2 at 568/55 nm) caused an 86% decrease in peak currents. The inset shows these Ba^{2+} current traces superimposed. (**b**) 2TC-α_{1C} (n = 17) or α_{1C} (control; n = 7) CALI after 30 s (columns one and two) or 20 s (columns three and four) of illumination. CALI was almost completely prevented by increasing the EDT to 1.5 mM (3%; fifth column). (**c**) Comparison of efficiency of FlAsH (filled triangle), ReAsH (filled square) and GFP (filled circle) in inducing CALI of 2TC-α_{1C}. FlAsH induced somewhat less CALI than ReAsH (69% versus 88% inactivation after 30 s of illumination) whereas GFP fused to the N terminus of α_{1C} exposed to the same illumination showed no CALI. (**d**) ReAsH CALI is substantially mediated by singlet oxygen. Photoinactivation can be prevented by the addition of the singlet oxygen quenchers azide and imidazole to the external and internal solutions. Photoinactivation can be enhanced by replacing H_2O by D_2O in the external and internal solutions (increased from 31% to 52%; P = 0.008, unpaired, one-tailed t-test) or by bubbling the external solution with 100% O_2 (increased from 31% to 69%; P = 0.0003, unpaired, one-tailed t-test).

That singlet oxygen mediation is a major and possibly the dominant mechanism for ReAsH CALI has important consequences. Current estimates for the range of diffusion of singlet oxygen are ~3–50 nm[9,17,18]. The possibility of inflicting collateral damage on molecules within such immediate distances of the tagged protein may be desirable or undesirable depending on the specific experiment. It should be possible to decrease the radius of singlet oxygen action using singlet oxygen quenchers or to increase it by using D_2O, which should allow some control over the extent to which CALI affects bystander proteins.

The most intrinsic limitation of CALI mediated by biarsenical staining of TCs is that only exogenous tagged proteins are the direct targets of inactivation. Endogenous untagged copies should be affected only if they form multimeric complexes containing at least one tagged copy. To inactivate all copies, ideally one would genetically replace the endogenous protein by a TC-tagged version[10], most elegantly by homologous recombination in transgenic knock-in animals. Alternatively, RNA interference might be used to reduce the endogenous mRNA while one simultaneously introduced the tagged version by transfection of a gene with enough codon substitutions to avoid the RNA interference. CALI could then eliminate the tagged proteins with much higher temporal and spatial precision than that of any nucleic acid–based strategy. Thus nucleic acid–based and protein-based inactivation strategies would complement each other well.

METHODS

Gene construction. Cx43-TC, Cx43-GFP, and Cx43-mRFP were previously described[4,12,13]. DNA encoding the peptide AEAAAREACCPGCARARSA-EAAAREACCPGCARA (2TC with two PG cores) was fused to the 5′ end of the α_{1C} gene by polymerase chain reaction (PCR). The cloned product was sequenced to ensure fidelity, amplified in DH5α′ *Escherichia coli* and subcloned into the mammalian expression vector pCDNA3 (Invitrogen).

Cell culture. Intercellular communication-deficient, Cx43-negative HeLa cells for Cx43 experiments or HEK293 cells stably expressing β_1 and $\alpha_2\delta$ (stable HEK293, courtesy of R.W.T.[19], maintained with 600 ng/ml of G418; Invitrogen) for α_{1C} experiments were seeded onto 35-mm Petri dishes with glass windows, in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (enriched DMEM). The cells were grown for 24 h to about 40% confluence, transiently transfected using FuGENE 6 (Roche Diagnostics) and examined 24 to 48 h after transfection. HeLa cells were transfected with 1 μg of the various Cx43 constructs. Stable HEK293 cells were transiently cotransfected with 2 μg of the various α_{1C} constructs together with 1 μg of β_4 constructs.

ReAsH and FlAsH labeling. To stain Cx43 constructs, ReAsH-EDT_2 (2.5 μM) or FlAsH-EDT_2 (1 μM) was applied for 2 h at 22–25 °C in Hank's Balanced Salt Solution (HBSS) with 10 μM 1,2-ethanedithiol (EDT) and 5.5 mM D-glucose. Excess dye was removed by a 30 min wash with 500 μM EDT in HBSS with 5.5 mM D-glucose. An alternative protocol in a more nurturing environment was 600 nM ReAsH for 6 h followed by a 500 μM EDT wash (30 min) all in enriched DMEM in a 37 °C incubator. Staining of α_{1C} constructs: ReAsH-EDT_2 (2.5 μM) or FlAsH -EDT_2 (1 μM) was applied with EDT (10 μM) in the incubator for 2 h, followed by a 250 μM EDT wash (30 min), all in enriched DMEM in a 37 °C incubator.

Electrophysiology. To monitor Cx43-mediated junctional conductance, each cell of a junctional pair was independently voltage clamped with a separate patch clamp amplifier (Axopatch-200A and B, Axon Instruments). The bath solution contained 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM glucose, 2 mM pyruvate and pH adjusted to 7.4 with NaOH. The pipet contained 140 mM KCl, 7 mM $NaCl_2$, 4 mM $MgCl_2$, 5 mM HEPES, 2 mM EGTA, 0.2 mM $CaCl_2$, 3 mM disodium-ATP and pH adjusted to 7.3 with KOH. The function of α_{1C} channels was monitored by whole-cell recordings of Ba^{2+} currents with a bath solution of 160 mM tetraethylammonium chloride, 10 mM $BaCl_2$, 10 mM HEPES and CsOH to give a pH of 7.3, whereas the pipet contained 108 mM $CsMeSO_4$, 4.5 mM $MgCl_2$, 9 mM EGTA, 4.5 mM disodium-ATP, 0.3 mM Na_3GTP, 24 mM HEPES and CsOH to give a pH of 7.3. Currents were digitized and analyzed with Digidata 1200B and pCLAMP8 software (Axon Instruments).

Microscopy and CALI. Dishes were imaged on a Nikon Diaphot microscope with a Nikon 40× 1.3 NA objective. Illumination was provided by a 150 W xenon arc lamp (Optiquip M1600) through interference filters from Chroma. Excitation power at the specimen plane was measured using a field stop to define an illuminated area, a hemispherical lens to allow steeply diverging rays to escape into air, and an ILC1700w integrating sphere detector (International Light) to collect those rays. With no additional attenuation, the irradiances were 17 W/cm^2 at 568 nm (55 nm bandwidth) and 15 W/cm^2 at 480 nm (35 nm bandwidth).

Data are presented as mean ± s.e.m.

URL. Preliminary results of Cx43-TC CALI were previously given in a lecture available on the Internet (http://www.nobel.se/medicine/symposia/ncs2001-3/medvideo/tsien.ram).

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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