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# A Dual-Purpose Real-Time Indicator and Transcriptional Integrator for Calcium Detection in Living Cells

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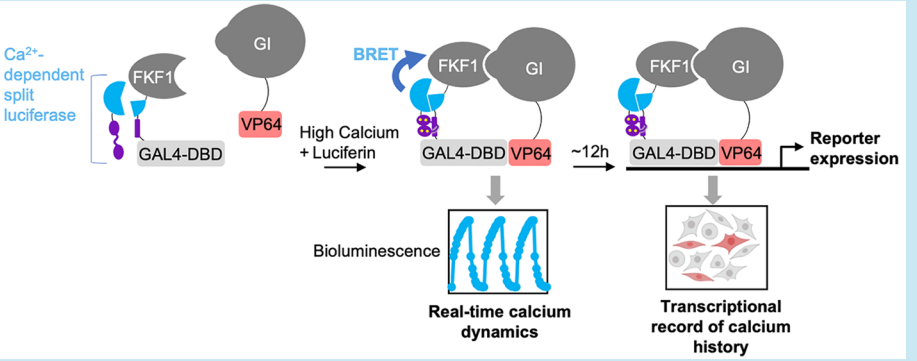


**ABSTRACT:** Calcium is a ubiquitous second messenger in eukaryotes, correlated with neuronal activity and T-cell activation among other processes. Real-time calcium indicators such as GCaMP have recently been complemented by newer calcium integrators that convert transient calcium activity into stable gene expression. Here we introduce LuCID, a dual-purpose real-time calcium indicator and transcriptional calcium integrator that combines the benefits of both calcium detection technologies. We show that the calcium-dependent split luciferase component of LuCID provides a real-time bioluminescence readout of calcium dynamics in cells, while the GI/FKF1 split GAL4 component of LuCID converts calcium-generated bioluminescence into stable gene expression. We also show that LuCID's modular design enables it to read out other cellular events such as protein−protein interactions. LuCID adds to the arsenal of tools for studying cells and cell populations that utilize calcium for signaling.

**KEYWORDS:** *NanoLuc, NanoBiT, BRET, bioluminescence, optogenetics, calcium sensor, biosensor, molecular recorder*



## INTRODUCTION

Calcium is a central player in a vast array of signaling processes, from neuron and T-cell activation[1] to mitochondria-ER coupling[2] and transcriptional regulation.[3] Consequently, real-time indicators for visualizing the spatiotemporal patterns of calcium signaling in living cells have been transformative for cell biology, neuroscience, immunology, and other fields.[4,5] Recently, these indicators have been complemented by a new class of calcium probes—calcium *integrators*—which convert transient calcium activity into stable signals that can be read out by large-scale microscopy, RNA-sequencing or, if desired, altered cellular behavior. Such integrators include the calcium- and UV-light dependent photoswitchable fluorescent protein CaMPARI[6−8] and the calcium- and blue light-gated transcription factors FLARE,[9] FLiCRE,[10] scFLARE,[11] and CaLight.[12]

Calcium indicators and calcium integrators each have unique and complementary strengths: indicators provide real-time, dynamic read-out over long experimental time windows with millisecond temporal resolution and high subcellular spatial resolution. Integrators record the cumulative calcium activity of cells over a single user-defined time window, but they are also highly scalable and versatile: the calcium record can be read out by microscopy, FACS, or single-cell RNA sequencing, and can also be used to drive the expression of functional proteins such as channelrhodopsin for reactivation of previously active cell subpopulations.[10] Given the complementary strengths of calcium indicators and integrators, we sought to design a dual-purpose probe that can function in either capacity, giving experimentalists maximal flexibility. In addition, we simplified our probe by removing the requirement for exogenous light. Instead, it is gated by a bio-orthogonal and nontoxic small molecule, which makes the probe potentially applicable to large and nontransparent tissue regions.

Our new probe, named LuCID, for Luciferin- and Calcium-Induced Dimerization, is a calcium and drug-gated tran-

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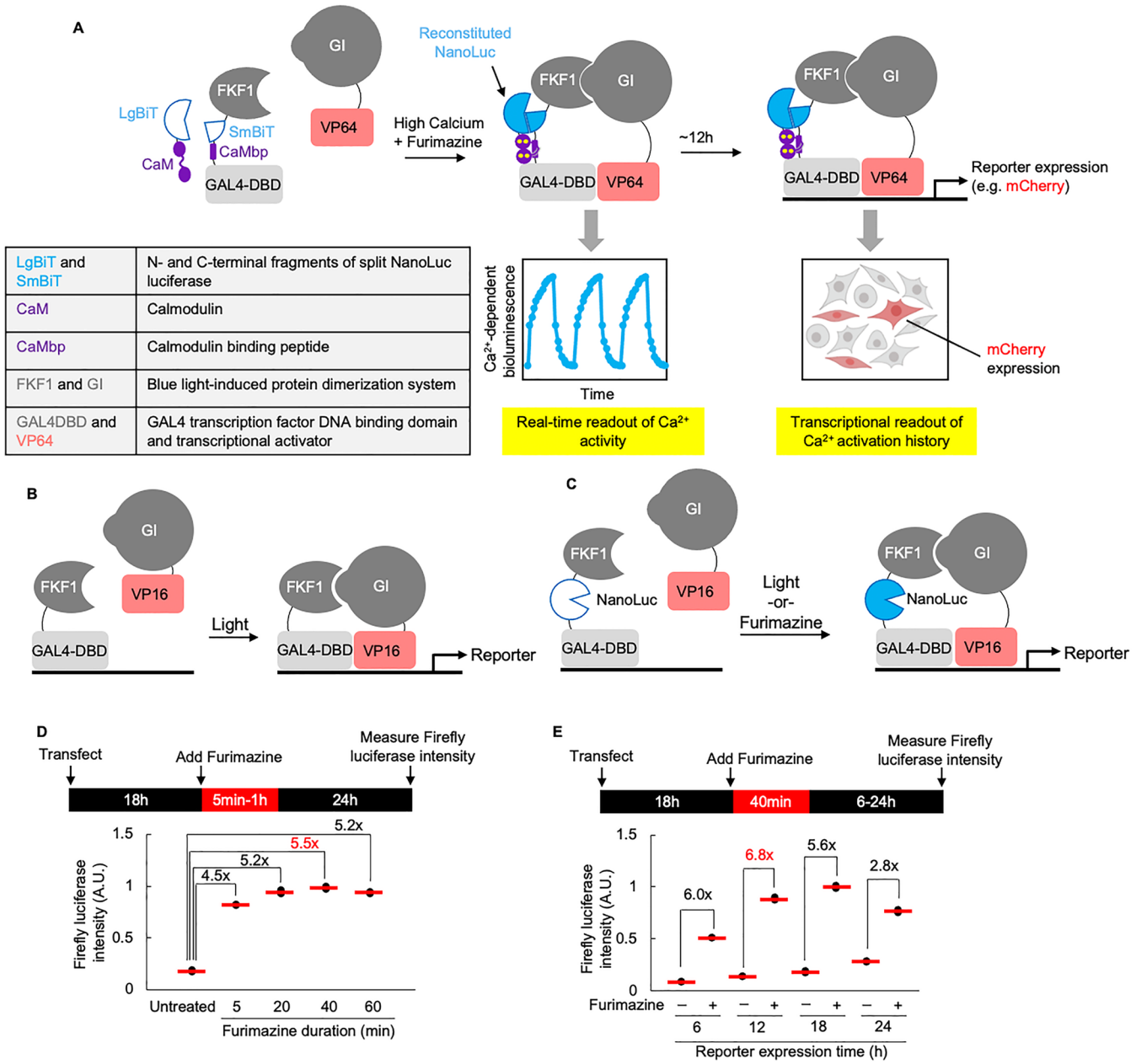


**Figure 1.** Schematic of LuCID and development of luciferin-gated gene expression. (A) LuCID is a dual calcium indicator and integrator. Furimazine is the substrate used by the luciferase enzyme NanoLuc to generate bioluminescence. (B) Light-gated split GAL4 system from Quejada et al., 2017 (C) Insertion of NanoLuc into constructs in B confers furimazine-gating. Bioluminescence from NanoLuc activates FKF1 via BRET, which recruits GI-VP16 to GAL4-DBD to drive reporter gene expression. (D) Results from constructs in C using firefly luciferase (FLuc) as the reporter gene. Furimazine was added to cells for 5 min to 1 h, and FLuc activity was recorded 24 h later. Two replicates per condition. Red line, mean. (E) Same as D but varying the wait time for FLuc reporter expression after furimazine addition. Two replicates per condition. Red line, mean.

scription factor that gives a bioluminescence readout of calcium activity as it is occurring in real-time in living cells, as well as a stable transcriptional record of that past calcium activity 12−18 h later.

## RESULTS AND DISCUSSION

The design of LuCID is shown in Figure 1A. Calcium is detected via reconstitution of a split luciferase, NanoBiT,[13] giving real-time bioluminescence readout of intracellular calcium levels. To convert NanoBiT bioluminescence to gene expression, we envisioned BRET from NanoBiT driving the activation of a light-regulated transcription factor. While our previous work showed that BRET between the luciferase NanoLuc and the photosensory domain LOV is possible,[14] it was unknown if luciferase-generated bioluminescence would be sufficient to drive the activity of existing light-dependent transcription factors.

To test this, we first selected NanoLuc as our BRET donor, because it is the brightest of all known luciferases, giving 150-fold more luminescence than either Firefly or Renilla luciferases in the presence of its small-molecule luciferin substrate, furimazine.[15] Furthermore, NanoLuc does not require ATP for light generation, and it has been shown to


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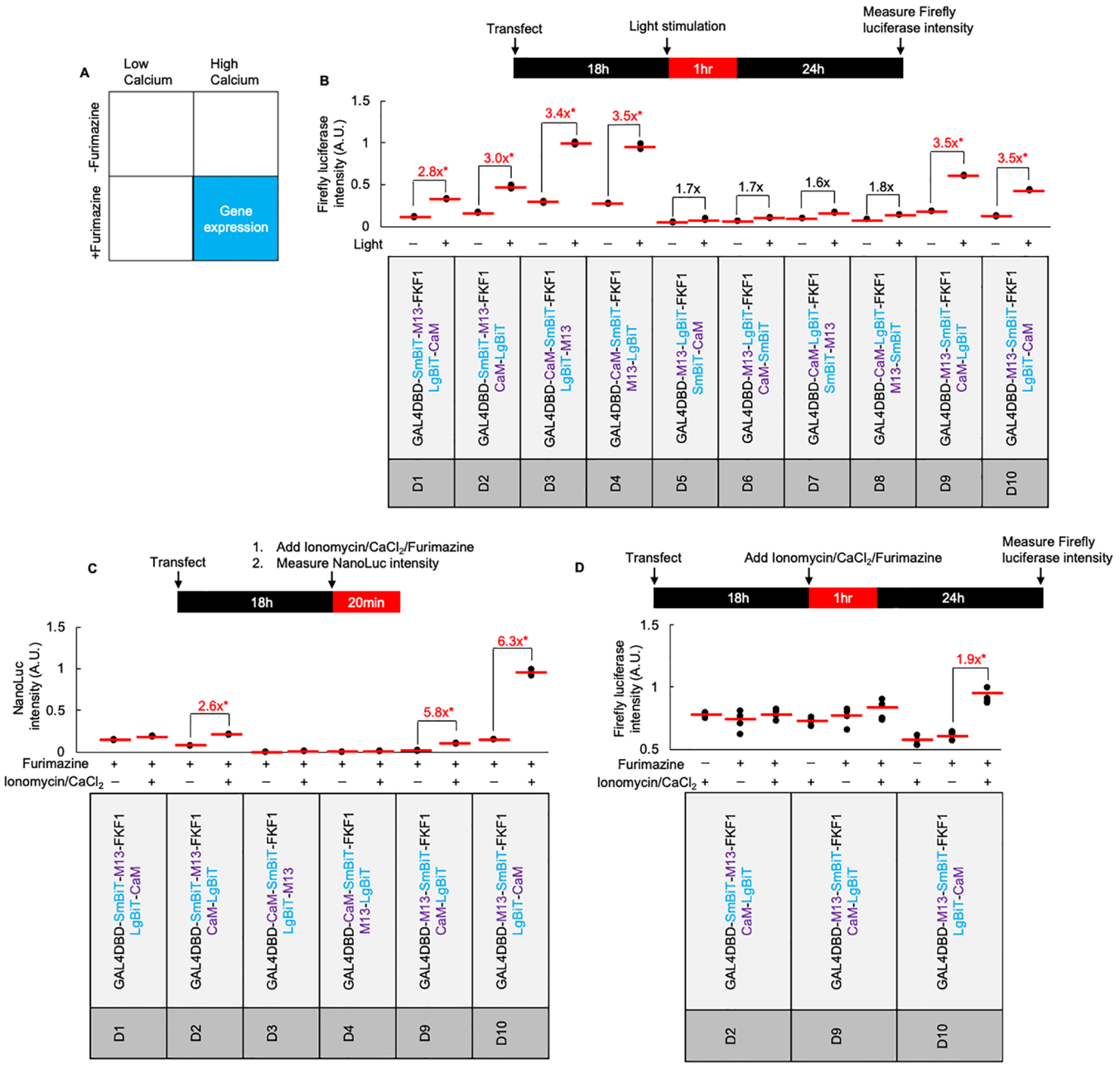


**Figure 2.** Introducing calcium gating into LuCID. (A) LuCID is an "AND" logic gate, requiring both elevated calcium and furimazine to produce gene expression. (B) Optimizing LuCID geometry. Construct pairs D1−D10 were tested in HEK293T cells with 1 h of light stimulation. Data for eight additional pairs (D11−18) shown in Figure S2C. The six best pairs are starred. Two replicates per condition. Red line, mean. (C) The six best pairs from B were tested with 20 min of furimazine ± calcium stimulation (2 $\mu$M ionomycin and 5 mM $CaCl_2$) in HEK293T cells. NanoLuc bioluminescence was recorded ∼4−6 min after calcium and furimazine addition. Two replicates per condition. The three best pairs are starred. (D) FLuc reporter readout 24 h poststimulation, for three best pairs (starred) from C. Four replicates per condition. Red line, mean.

work in a variety of *in vivo* settings including the mouse brain[16] and liver,[17] worms,[18] and zebrafish.[19] Because NanoLuc emits blue light with a peak of ∼450 nm, it is well matched to the activation spectrum of cryptochromes that use flavin as a cofactor. Several cryptochrome-based photoactivatable transcription factors have been reported, including LightOn,[20] LITEZ,[21] TULIP,[22] TAEL,[23] and GI/FKF1.[24,25] We selected the GI/FKF1 system for use in LuCID because its reconstitution upon light stimulation is slow to reverse (>2 h[26]), a desirable feature for an integrator (Figure 1B).

To test NanoLuc regulation of GI/FKF1, we prepared genetic fusions of NanoLuc to FKF1, the light-sensitive, flavin-containing component of the GI/FKF1 pair (Figures 1C, S1A). We expressed the components in HEK293T cells, along with a UAS-firefly luciferase reporter (FLuc), which is orthogonal to NanoLuc, does not use furimazine, and emits 560 nm green light instead of 460 nm light. Cells were treated with furimazine for 1 h and then cultured for 24 h to allow time for FLuc transcription and translation. Figure S1A shows that FLuc activity was detected in cells either treated with furimazine or exposed to light, but not in untreated cells left in the dark. Similarly, expression of a different reporter gene, mCherry, was 6-fold greater in HEK293T cells treated with furimazine compared to untreated cells (Figure S1B,C). Fusion


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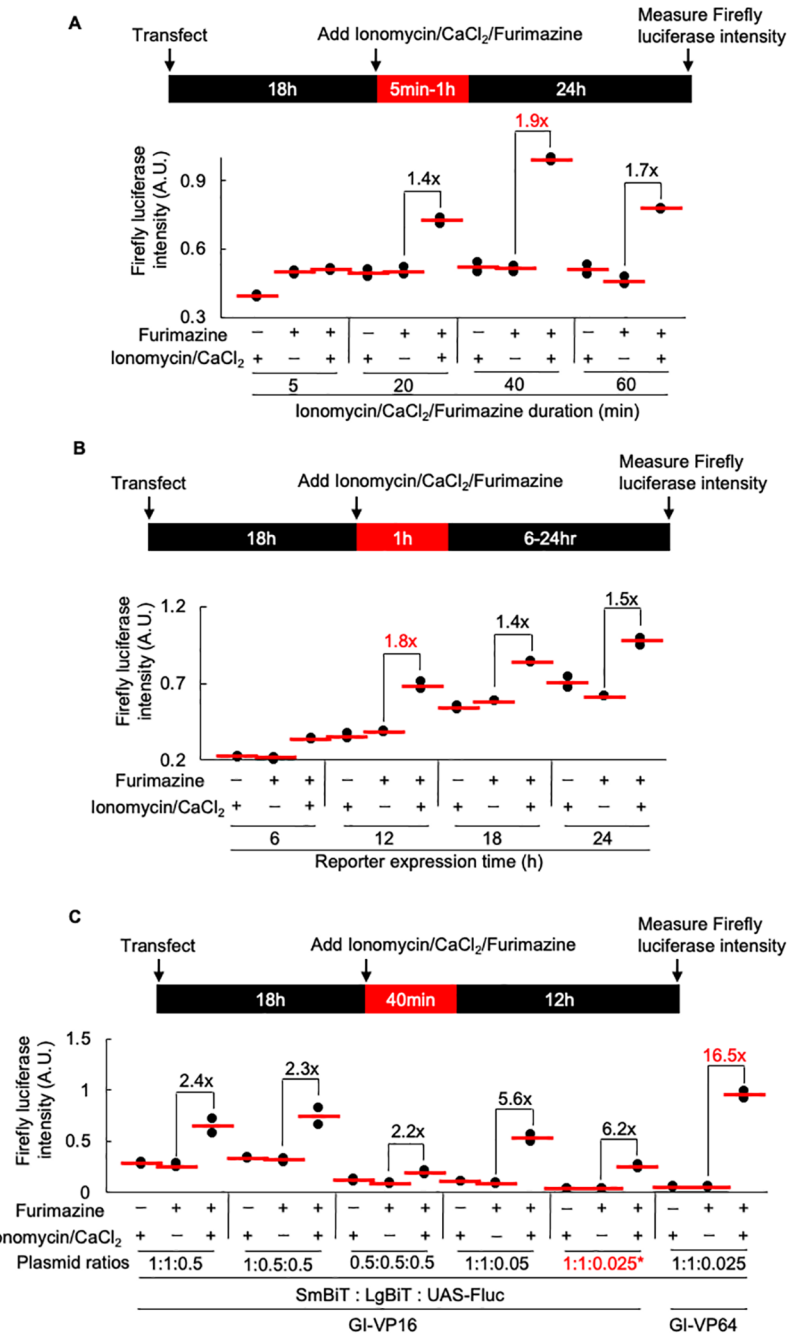


**Figure 3.** Optimization of LuCID dynamic range. (A) Optimizing stimulation time. FLuc expression in HEK293T cells measured 24 h poststimulation. Two replicates per condition. 40 min is best. (B) Optimizing FLuc reporter expression time. Two replicates per condition. Twelve hours is best. (C) Testing different plasmid ratios and transcriptional activator domains (VP16 vs VP64). SmBiT = GI-VP16-IRES-GAL4DBD-M13-SmBiT-FKF1. LgBiT = LgBiT-CaM. Stimulation time was 40 min, and FLuc activity was measured 12 h later. Two replicates per condition. Best plasmid ratio is at the right.

of NanoLuc to the N-terminus of FKF1 gave stronger turn-on than fusion to FKF1's C-terminus (Figure S1A).

Optimization showed that the highest signal-to-noise ratios were obtained with a furimazine treatment time of 40 min and FLuc reporter expression time of 12 h (Figure 1D,E). We performed a control in which NanoLuc was coexpressed with, but not directly fused to, GI/FKF1; no FLuc reporter expression was observed (Figures S1E). This is consistent with our previous observation that NanoLuc-LOV BRET is strongly proximity-dependent[14] but inconsistent with a previous report[27] showing BRET-based activation of GI/FKF1 via nonfused NanoLuc (Figure S1D). In Figure S1E, we are unable to observe furimazine-driven gene expression using this nonfused NanoLuc-GI/FKF1 system (BEACON)[27] despite detecting high bioluminescence from NanoLuc (Figure S1F) and correct nuclear localization of BEACON components (Figures S1G).

Next, we designed the calcium sensing component of LuCID. Previous studies have described a number of biosensors that detect calcium with bioluminescence readout.[28−30] We tested three single-component calcium biosensors: GeNL($Ca^{2+}$) based on calmodulin (CaM)/M13


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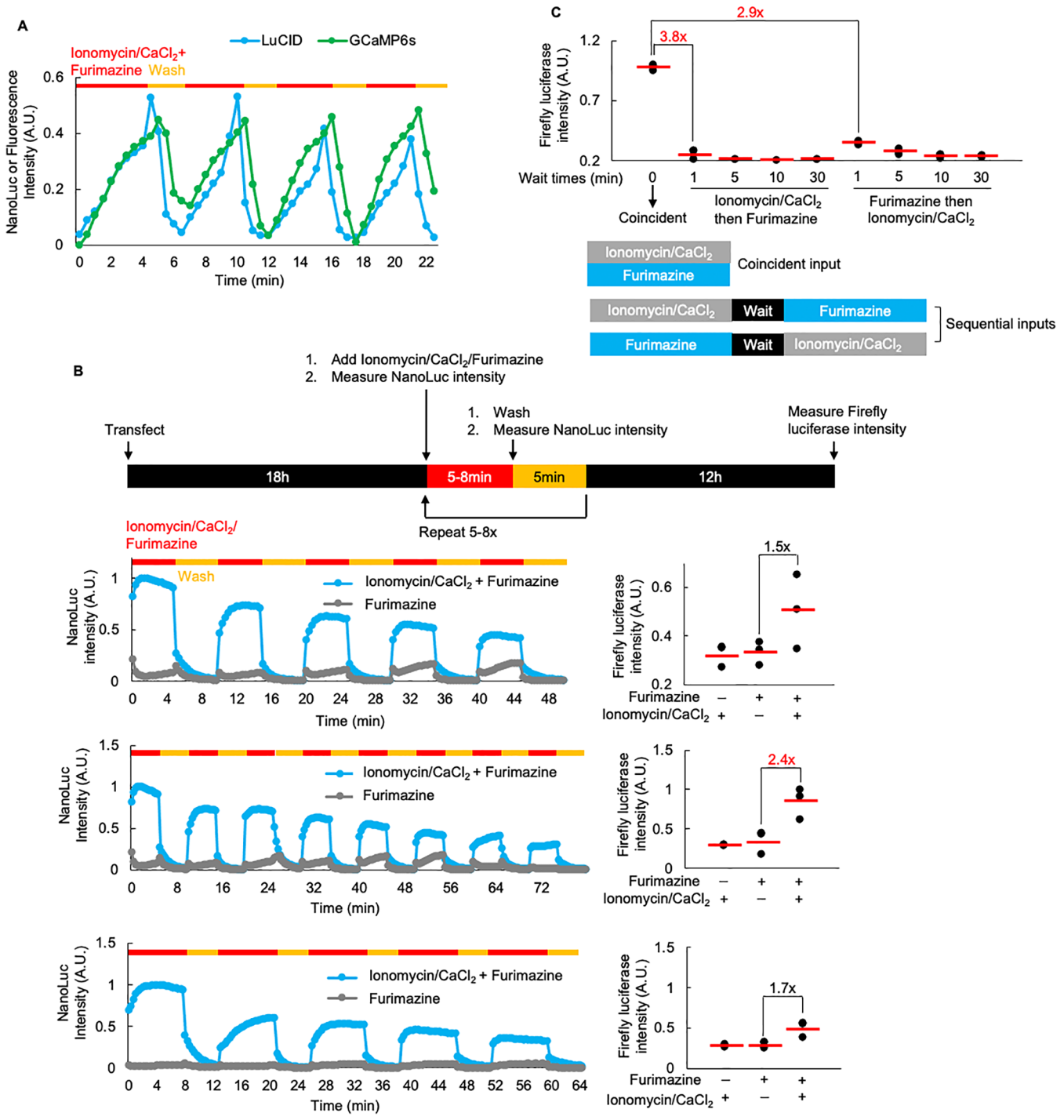


**Figure 4.** LuCID for bioluminescence measurement of real-time calcium dynamics. (A) Simultaneous measurement by LuCID (blue) and GCaMP6s (green) expressed together in HEK293T cells. Cells were stimulated repeatedly with 2 $\mu$M ionomycin, 10 mM $CaCl_2$, and 15 $\mu$M furimazine for 4 min (red bars) and washed for 2 min (yellow bars), while bioluminescence was recorded at 460 nm for LuCID and fluorescence was recorded at 512 nm (497 nm excitation) for GCaMP6s. This experiment was repeated three times. One representative trace shown. (B) Demonstration of the dual functionality of LuCID. Matched samples were stimulated repeatedly with calcium and furimazine as indicated. Bioluminescence was recorded from one sample in real-time (time traces at left), while the other sample was quantified for FLuc reporter expression 12 h later (graphs at right). Three replicates per condition. (C) LuCID requires coincident activation by calcium and furimazine. HEK293T cells expressing LuCID were stimulated sequentially with calcium (20 min) followed by furimazine (20 min) or the reverse. Intervening wait times ranged from 1 to 30 min. Three replicates per condition. Red line, mean.

insertion into enhanced NanoLantern,[31] CaMBI based on CaM/M13 insertion into Antares,[17] and an unpublished circularly permuted NanoLuc (personal communication[32]). In all cases, the dynamic ranges of these biosensors were insufficient in the context of LuCID, primarily due to high background in the absence of calcium (Figure S2A,B). We hypothesized that a two-component sensor in which calcium drives the reconstitution of a split luciferase, such as in Nguyen et al., 2020,[33] might have a greater dynamic range because of



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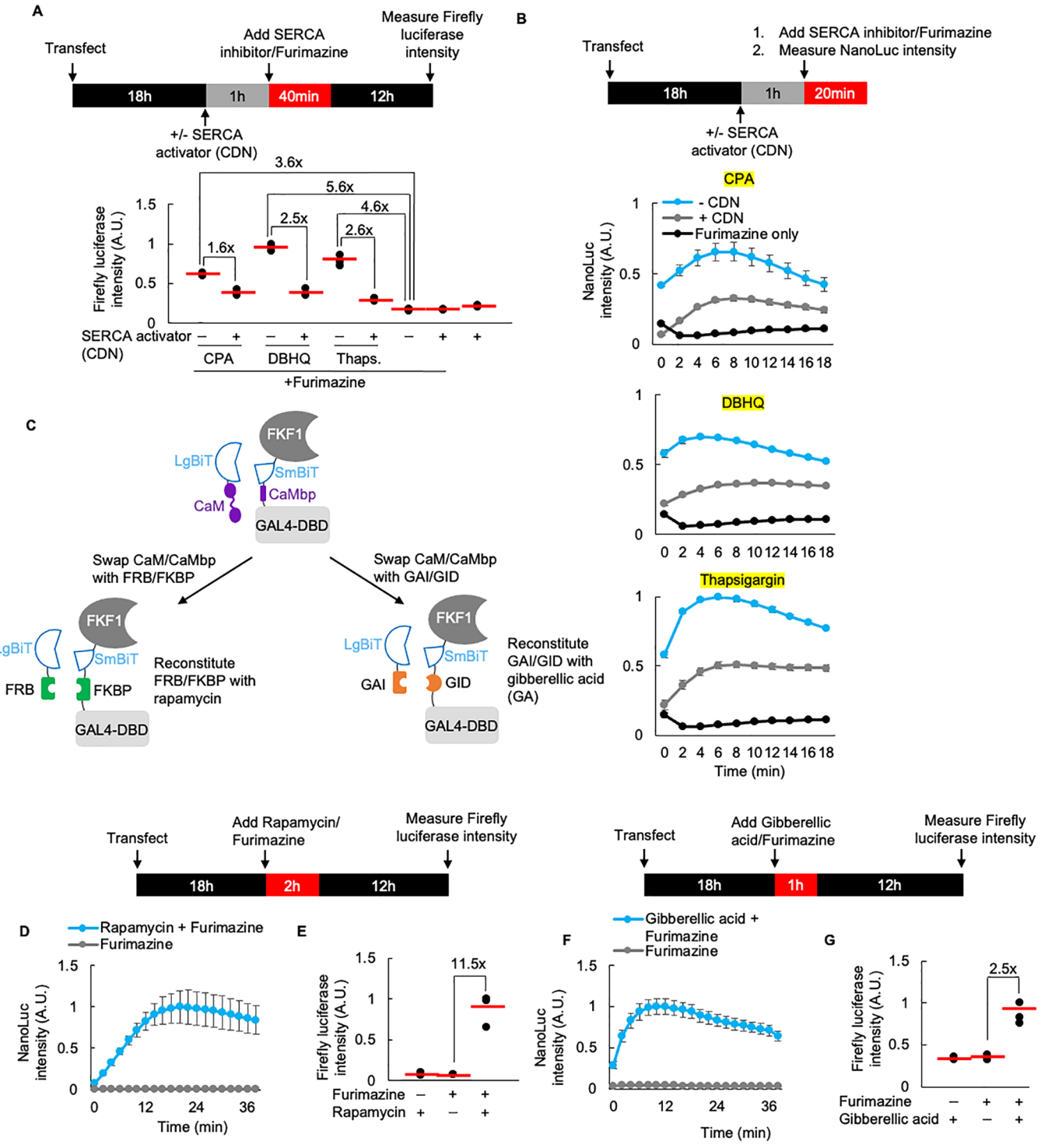


**Figure 5.** LuCID for detection of pharmacologically induced calcium transients and protein−protein interactions (A) HEK293T cells expressing LuCID were treated with the SERCA activator CDN1163 (CDN) for 1 h, then one of three SERCA inhibitors (CPA (cyclopiazonic acid), DBHQ (2, 5-di(*tert*-butyl) hydroquinone), or thapsigargin) for 40 min together with furimazine. FLuc expression was measured 12 h later. Three replicates per condition. Red line, mean. (B) Real-time calcium measurements from cells treated as in A. Error bars = ±1 SD. (C) Schematic showing replacement of calcium-responsive domains of LuCID with protein−protein interaction partners. (D) Real-time bioluminescence measurements of rapamycin-triggered FRB-FKBP interactions with LuCID in HEK293T cells. Three replicates per condition. Error bars = ± 1 SD. (E) Same as D, but FLuc reporter expression was measured 12 h later. Three replicates per condition. Red line, mean. (F) Real-time bioluminescence measurements of gibberellic acid-triggered GID/GAI interactions with LuCID in HEK293T cells. Three replicates per condition. Error bars = ± 1 SD. (G) Same as F, but FLuc activity was measured 12 h later. Three replicates per condition. Red line, mean.

lower background from nonassociated luciferase fragments in the low calcium state.

Thus, we tested the split NanoLuc system called NanoBiT in LuCID. NanoBiT's two fragments, LgBiT (18 kDa, amino acids 1−156) and SmBiT (1.3 kDa, amino acids 157−167)



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have low affinity for one another ($K_d$ = 190 $\mu$M) and must be driven together by a protein−protein interaction in order to reconstitute and give bioluminescence.[13] We fused the calcium-binding protein calmodulin (CaM) to LgBiT and its calcium-dependent binding peptide M13 to SmBiT, and measured bioluminescence in HEK293T cells treated with $CaCl_2$ and ionomycin or left untreated. We observed a ± calcium signal ratio of 48, in contrast to the single-component calcium biosensors which gave a maximum ± calcium signal ratio of 1.4 (Figure S2B).

Encouraged by this large dynamic range, we inserted the NanoBiT fusions into the GI/FKF1 system. We generated 18 distinct combinations of genetic fusions (D1−18) with varying geometries (Figures 2B, S2C), guided by the following principles: (1) LgBiT or SmBiT were placed close to FKF1 to allow efficient BRET between the luciferase and flavin; (2) smaller protein domains were sandwiched between GAL4DBD and FKF1 in order to reduce the distance between them and maintain ability of GI/FKF1 interaction to drive GAL4DBD-VP16 reconstitution; (3) we avoided fusions to the C-terminus of FKF1, which is deleterious based on our data with full-length NanoLuc in Figure S1A; and (4) the GI-VP16 component was held constant.

All 18 construct combinations were first tested for their ability to drive light-dependent gene expression (Figures 2B, S2C). The six pairs that gave 2.5-fold or greater ± light FLuc signal ratio were then tested for calcium-dependent bioluminescence (Figure 2C). The three best pairs had either C-terminal or N-terminal exposed CaM domains, which may facilitate intermolecular complexation with M13 fusions. These three pairs were then expressed in HEK293T cells along with UAS-FLuc reporter (Figure 2D). The pair with the best ± calcium FLuc expression ratio was used to optimize calcium/furimazine treatment times (Figure 3A) and concentrations (Figure S3C−F), reporter expression times (Figure 3B), and plasmid ratios (Figures 3C). Finally, we replaced the VP16 transcriptional activator domain with VP64, a stronger activator, to produce LuCID. Under optimized conditions, LuCID's ± calcium signal ratio was ∼16.5 in HEK293T cells (Figure 3C, S3G).

LuCID's design also enables it to read out real-time calcium dynamics in addition to integrating calcium pulses into a stable transcriptional signal. This is because LuCID's calcium sensor−the calcium-regulated split NanoLuc−gives an instantaneous bioluminescence readout and is fully and rapidly reversible. To test LuCID's function as a real-time calcium indicator, we simulated calcium pulses in HEK293T cells with ionomycin + $CaCl_2$ and measured LuCID bioluminescence concurrently with the fluorescence of a "gold standard" calcium indicator, GCaMP6s.[34] We found that the two readouts were well-matched (Figure 4A). Interestingly, the on-rate kinetics of LuCID were slower compared to GCaMP6s, while the off-rate kinetics were faster. This is likely because LuCID's design is intermolecular while GCaMP6s is intramolecular.

We also tested whether LuCID could detect noncontinuous calcium pulses in HEK293T cells. We stimulated HEK293T cells with three different pulse patterns (Figure 4B). We measured both real-time calcium activity from each of these pulse patterns (Figure 4B, left) and calcium integration 12 h later by checking for UAS-FLuc reporter expression (Figure 4B, right). S/N in reporter expression is much lower for these pulse-pattern treatments (highest S/N of 2.4 with 8 pulses of 5 min of calcium/furimazine) compared with the continuous calcium/furimazine treatment condition (Figure 3C; S/N = 16.5), which suggests that the M13-SmBiT/LgBiT-CaM component of LuCID is so quickly reversible after washout of calcium/furimazine that the GI/FKF1 cannot be sustainably engaged. This is consistent with previous research showing that the interaction between FKF1 and GI has a slow on-rate, on the time scale of many minutes.[24]

We further characterized LuCID's performance as a calcium integrator. If LuCID functions as an "AND" logic gate or coincidence detector, as we have designed, then the staggering of calcium and furimazine inputs should not produce reporter gene expression. Figure 4C shows that when furimazine precedes calcium by 1 min, or vice versa, LuCID's resulting signal output is significantly reduced. LuCID is a coincidence detector of calcium and furimazine due to the reversibility of its calcium sensing and the speed of furimazine washout from cells.

To highlight LuCID's versatility in terms of readout, we also used a UAS-mCherry reporter and microscopy to detect LuCID turn-on. 40 min of calcium and furimazine treatment induced significant mCherry reporter expression in HEK293T cells ∼18 h later (Figure S3A-B). Cells receiving either calcium-only or furimazine-only treatment exhibited minimal mCherry expression. The mean mCherry-to-HA fluorescence intensity ratio (mCherry/HA) for the double-positive +calcium/+furimazine condition was 12.7-fold higher than for the single-positive calcium-only or furimazine-only conditions. An HA tag fused to FKF1 allowed us to confirm equal expression levels across conditions using antibody staining (Figure S3A).

To test if LuCID could detect endogenous calcium, rather than exogenously supplied calcium, we treated HEK293T cells with one of three Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) inhibitors to elevate cytosolic calcium, by blocking the continuous uptake of cytosolic calcium into ER stores. Figure 5A shows that LuCID detected the action of all three inhibitors, giving 3.6-, 5.6-, and 4.6-fold increase in FLuc reporter expression in response to cyclopiazonic acid (CPA), 2,5-di(*tert*-butyl)-1,4-hydroquinone (DBHQ), and thapsigargin (Thaps.), respectively, compared with the furimazine-only treatment. We could also use this assay to detect the activity of a SERCA activator, CDN1163 (CDN), by pretreating the HEK293T cells with this drug before supplying SERCA inhibitors. In all cases, we observed a reduction in LuCID-driven FLuc-expression in the presence versus absence of CDN (Figure 5A). Finally, we measured the real-time effects of these drugs, using LuCID's bioluminescence readout (Figure 5B). All three SERCA inhibitors gave increases in LuCID bioluminescence, while pretreatment with SERCA activator CDN reduced but did not fully attenuate the signal, relative to furimazine-only control. These results suggest that LuCID could potentially be used to screen for novel drugs that modulate endogenous calcium signaling.

Last, due to the modularity of LuCID's design, we tested if it could be adapted to detect other biochemical events besides calcium rises, such as protein−protein interactions (PPIs). To test this, we replaced the CaM and CaMbp calcium-sensing domains of LuCID with the chemically induced protein dimerizing domains FRB and FKBP[35] or GAI and GID[36] (Figure 5C). Figure 5E shows that 2 h of rapamycin and furimazine treatment of HEK293T cells expressing the FKBP/FRB reporter induces 11.5-fold higher FLuc expression than rapamycin-only or furimazine-only treatments. Two hours of


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gibberellic acid (GA) and furimazine treatment of the GAI/GID set induces 2.5-fold higher FLuc expression than GA-only or furimazine-only treatments (Figure 5G). As with calcium-sensing LuCID, real-time PPI dynamics of both the rapamycin- and gibberellic-acid-based systems could also be measured (Figure 5D,F).

In summary, LuCID is a dual-purpose calcium probe that can be used both for real-time bioluminescence readout of calcium dynamics and stable recording of past calcium activity in the form of new gene expression. We perform extensive protein engineering and optimization to develop LuCID, characterize its performance in cell culture, use it in a small-scale screen of SERCA inhibitors, and show that the design can be extended to the detection of other cellular events such as protein−protein interactions.

Compared with other calcium integrators, such as FLiCRE developed by our lab,[9−11] and others,[6−8,12] LuCID has the advantage of being temporally gated by a small-molecule (furimazine) rather than external light. This makes it potentially easier to use *in vivo*, across large swaths of tissue and for high-throughput robotics-assisted screens. Previous work[16,17] has shown that furimazine can access the mouse brain and liver to produce bioluminescence. Compared with other real-time calcium indicators, LuCID's main advantages are that it does not require external excitation light, it does not photobleach, and it has higher dynamic range than single-chain calcium bioluminescence indicators such as CaMBI.[17] CaMPARI[6−8] is notable because, like LuCID, it can function as both a calcium integrator and a real-time calcium biosensor. However, as an integrator, CaMPARI is not as versatile as LuCID because it is a photoswitchable fluorescent protein that only provides a color readout rather than a transcription factor that can drive the expression of a wide array of reporter genes.

LuCID does have important limitations, however. Compared with FLiCRE, LuCID has much poorer temporal resolution, requiring 40 min of stimulation compared to 1 min.[10] This is partly because furimazine delivery/washout are slower than light delivery, but we believe that the primary reason is that GI/FKF1 requires several minutes of sustained NanoLuc bioluminescence to become activated. This is supported by the observation that short-lived increases in cytosolic calcium induced by ATP or histamine do not give detectable bioluminescence or transcriptional signal with LuCID (Figure S5A,B). Improved transcriptional activators and improved geometry that optimizes BRET between NanoLuc and transcription factor may improve sensitivity and temporal resolution in future designs. A second limitation is that LuCID has multiple components, one of which is large (GI is ∼129 kDa) and difficult to package even into a lentiviral vector. The tool could benefit from simplification, as we did to produce single-chain FLARE,[11] so that it is smaller, has fewer components, and exhibits less expression-level variation that affects performance across cells and experiments. Despite these limitations, LuCID does offer new capabilities among the constellation of calcium detection technologies and provides a new way to measure and study calcium signaling in biology.

## METHODS

**HEK293T Cell Culture and Transfection.** HEK 293T cells from ATCC were cultured as monolayers in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) and +1% (v/v) penicillin−streptomycin (PS) at 37 °C under 5% $CO_2$. Cells were grown in 24-well, 48-well, or 96-well plates pretreated with 50 μg/mL human fibronectin (Millipore) for at least 10 min. Cells were transfected at 60−90% confluence with 1 mg/mL PEI max solution (polyethylenimine HCl max pH 7.3). Plasmid DNA was mixed with PEI max in serum-free DMEM and incubated at room temperature for 25−30 min. For LuCID, the optimal plasmid ratio is 1:1:0.025 of GI-VP64-IRES-GAL4DBD-M13-SmBiT-FKF1: LgBiT-CaM: UAS-FLuc. For BEACON, a plasmid ratio of 2:2:1:1 of NLS-CeNLuc (Addgene #135952): GI-GAL4DBD (Addgene #42500): FKF1-VP16 (Addgene #42499): UAS-FLuc was used. After mixing DNA with DMEM, complete DMEM with 10% FBS + 1% PS was then added to the mixture, and the entire volume was added to a well of a multiwell plate. Exact amounts of PEI, serum-free DMEM, and complete DMEM used are summarized in Table 2 in Supplementary methods. The plate was wrapped in aluminum foil and incubated in 37 °C incubator for 12−24 h.

**Stimulation of HEK293T Cells with Light, Furimazine, and/or Calcium.** HEK293T cells cultured and transfected as described above were stimulated as follows. For experiments with full-length NanoLuc (Figures 1 and S1) and BEACON[27] (Figure S1), media in each well was replaced with 100 μL (for 96-well plate format), 200 μL (for 48-well plate format), or 400 μL (for 24-well plate format) complete DMEM with or without 10 μM furimazine (Promega). Cells were incubated at 37 °C under 5% $CO_2$ either in the dark, wrapped in aluminum foil, or stimulated with 467 nm blue light at 60 mW/cm$^2$ and 50% duty cycle (3 s of light every 6 s) by placing the entire plate directly above an LED light array for the indicated times. For experiments with calcium stimulation (Figures 2 and S2), media in each well was replaced with 100 μL (for 96-well plate format) or 200 μL (for 48-well plate format) complete DMEM in addition to 2 μM ionomycin (Sigma-Aldrich) and 5 mM $CaCl_2$ with or without 10 μM furimazine (unless indicated otherwise). For Figure S4B,C, 100 μM ATP or 100 μM histamine was used in place of ionomycin/$CaCl_2$. After stimulation, media was removed from the wells and replaced with the same volume of complete DMEM, and the cells were returned to the 37 °C incubator wrapped in aluminum foil for reporter expression. All manipulations were done under red light. For the pulsed stimulation experiment in Figure 4, 100 μL of complete DMEM containing 2 μM ionomycin and 10 mM $CaCl_2$ with or without 15 μM furimazine was added to each well for the indicated times and then replaced with complete DMEM without additional drugs during the indicated washes. Calcium/furimazine addition and wash steps were repeated 4−8 times to simulate calcium pulses. Repeated calcium/furimazine addition and wash steps, however, can lift cells off the plate, resulting in a decrease in measured bioluminescence intensity with each repeated pulse (observed in Figure 4B).

**Real-Time Bioluminescence Measurements with LuCID and NanoLuc.** Real-time bioluminescence measurements from either full-length NanoLuc or reconstituted NanoBiT in LuCID were taken on a Tecan Infinite M1000 Pro platereader immediately after adding complete DMEM with 2 μM ionomycin + 5 mM $CaCl_2$ with or without 10 μM furimazine (unless otherwise indicated) to each well. Measurements were taken using the blue filter (450 nm) with 1000 ms integration time every 2 min for 20 min. Bioluminescence from the pulsed experiment in Figure 4 was measured both immediately after adding complete DMEM with 2 μM


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ionomycin + 10 mM $CaCl_2$ with or without 15 $\mu$M furimazine and also after adding complete DMEM without additional drugs using the platereader's kinetic cycle function with the following settings: 10 cycles, 500 ms kinetic interval with 100 ms integration time with 450 nm blue filter. For real-time calcium measurements with GCaMP6s, fluorescence signal was measured immediately after adding complete DMEM with 2 $\mu$M ionomycin + 10 mM $CaCl_2$ with or without 15 $\mu$M furimazine when GCaMP6s was coexpressed with LuCID (Figure 4A) or after adding complete DMEM with 2 $\mu$M ionomycin + 10 mM $CaCl_2$ when GCaMP6s was expressed alone (Figure S4) using the same kinetic cycle as above. Measurements were recorded with 497 nm excitation and 512 nm emission wavelengths.

**Measurement of LuCID-Driven Gene Expression.** 12−24 h after calcium/furimazine stimulation as described above, firefly luciferase (FLuc) reporter gene expression was measured by luminescence on a platereader (Tecan Infinite M1000 Pro) using the Bright-Glo Luciferase Assay System (Promega). The Bright-Glo reagent was thawed at room temperature and diluted 2-fold with PBS. Media was aspirated from each well and 50 $\mu$L of the diluted Bright-Glo reagent was added to each well. Luminescence was immediately analyzed at 25 °C using a 1000 ms integration time, the Green-1 filter (520−570 nm), and linear shaking for 10 s.

**SERCA Inhibitor/Activator Experiments (Figure 5).** HEK293T cells were transfected as indicated in Table 2. After an 18-h incubation wrapped in foil in a 37 °C/5% $CO_2$ incubator, media was replaced with 100 $\mu$L complete DMEM ± 30 $\mu$M SERCA activator CDN1163 (Sigma-Aldrich) and incubated at 37 °C for 1 h. Then, media was replaced again with 100 $\mu$L DMEM + 10% FBS + 1% PS containing 10 $\mu$M furimazine and one of the following SERCA inhibitors: 10 $\mu$M cyclopiazonic acid (CPA) (Sigma-Aldrich), 10 $\mu$M 2,5-di(*tert*-butyl)-1,4-hydroquinone (DBHQ) (Sigma-Aldrich), or 10 $\mu$M Thapsigargin (EMD Millipore). Bioluminescence measurements were taken in real-time immediately after adding SERCA inhibitor + furimazine and compared with cells treated with just 10 $\mu$M furimazine. Firefly luciferase reporter expression was measured ∼12 h later, as described above.

**Rapamycin and Gibberellic Acid Experiments (Figure 5).** HEK293T cells were transfected as indicated in Table 2. After an 18-h incubation, media in each well was replaced with 100 $\mu$L complete DMEM with the following drug combinations: (1) 100 nM rapamycin only, (2) 10 $\mu$M furimazine only, and (3) 100 nM rapamycin + 10 $\mu$M furimazine combined for 2 h at 37 °C under 5% $CO_2$ or with (1) 10 $\mu$M gibberellic acid (GA) only, (2) 10 $\mu$M furimazine only, and (3) 10 $\mu$M GA + 10 $\mu$M furimazine combined for 2 h at 37 °C under 5% $CO_2$ prior to luminescence readout 12 h later, respectively, for each experiment. Real-time measurements were taken immediately after adding the above drug combinations on the platereader.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00597.

> Testing full-length NanoLuc designs, comparison to BEACON, using mCherry as reporter, testing alternative NanoLuc-based calcium sensors, and optimization of stimulation conditions; additional methods and information on cloning, cell fixation, immunostaining, and fluorescence microscopy; list of all plasmids used in Table 1 and plasmid amount used in each experiment in Table 2 (PDF)

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### Author Contributions

E.E. performed all experiments. E.E. and A.Y.T. designed the research, analyzed the data, wrote and edited the paper.

### Notes

The authors declare no competing financial interest.

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