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A "Caged" Luciferin for Imaging Cell–Cell Contacts

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Supporting Information

ABSTRACT: Cell–cell interactions underlie fundamental biological processes but remain difficult to visualize over long times and large distances in tissues and live organisms. Bioluminescence imaging with luciferase–luciferin pairs is sufficiently sensitive to image cells *in vivo* but lacks the spatial resolution to identify cellular locations and interactions. To repurpose this technology for visualizing cellular networks, we developed a "caged" luciferin that produces light only when cells are in close contact. This molecule comprises a nitroaromatic core that can be selectively reduced ("uncaged") by one cell type, liberating a luciferin that can be selectively consumed by neighboring, luciferase-expressing cells. When the two cell types are in contact, robust light emission is observed. This imaging strategy will enable the noninvasive visualization of cell–cell interactions relevant to organismal biology.

Cell–cell contacts govern numerous biological processes, including cell growth, motility, and immune function.[1] Our understanding of these interactions is critically dependent on our ability to "see" them, and several fluorescence imaging techniques have been developed for this purpose.[1,2] While powerful, these strategies require intense excitation sources and are thus largely limited to visualizing interactions on a microscopic scale.[2−4] Bioluminescence imaging (BLI), a complementary optical technique, is more suitable for capturing macroscopic cell movements in whole tissues and organisms, as it does not require excitation light. However, in its current form, BLI is incapable of reporting on direct cell–cell interactions owing to its low spatial resolution.[3,5,6] This leaves a gap in our ability to investigate dynamic cellular interactions across large length and time scales without knowing when and where to look.

To address this void, we are engineering bioluminescent probes that can report on cell–cell contacts. Bioluminescence exploits enzymes (luciferases) that catalyze light production via the oxidation of small molecule substrates (luciferins). The most widely used enzyme–substrate pair comprises the luciferase from the firefly (Fluc) and its small molecule substrate, D-luciferin.[3,7] When introduced into nonluminescent cells, these components produce photons that can be captured by sensitive cameras. The Fluc/D-luciferin pair has been widely used for tracking cells and gene expression patterns in mouse models of human biology.[3,7−9] Synthetic analogs of D-luciferin are also gaining traction in imaging studies, owing to their unique light emission profiles and cell permeabilities.[10−15]

We recently exploited a modified luciferin—known as a "caged" probe—to visualize cell–cell proximity in rodent models.[5] "Caged" luciferins typically comprise appendages (i.e., "cages") at the 6′-position of the scaffold, rendering the molecules incapable of interacting with luciferase to produce light.[16] However, upon removal of the cage, a functional luciferin (with an electron-donating group at the 6′-position) is generated and available for the light-emitting reaction. "Caged" probes have been previously employed to measure enzyme activities[17,18] and track bioactive small molecules[19,20] *in vitro* and *in vivo*. We utilized Lugal, a galactose-caged luciferin that is selectively cleaved by the uncaging enzyme, β-galactosidase (β-gal).[5] If β-gal is expressed in one cell (i.e., an "activator" cell) and Fluc is expressed in another (i.e., a "reporter" cell), administration of Lugal can report on the proximity of the two cells (Figure 1).[21] In this scenario, luciferin released by activator

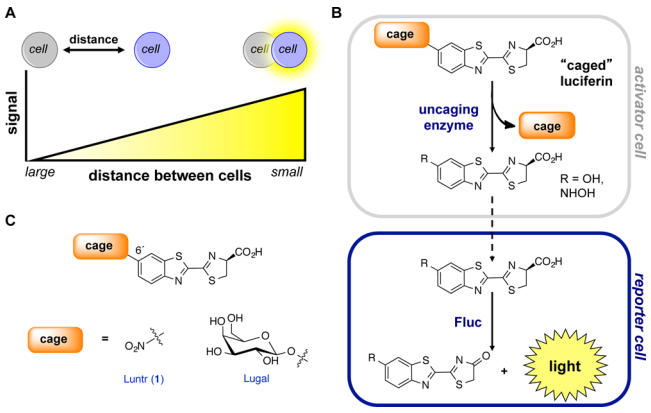

Figure 1. General strategy for visualizing cell–cell interactions. (A) Light is produced only when cells are in close contact. (B) "Caged" luciferins enter activator cells, where an uncaging enzyme (e.g., β-gal or NTR) liberates an active luciferin. This molecule diffuses into nearby reporter cells (expressing Fluc), and light is produced. (C) Structures of "caged" luciferins discussed in this work.

cells enters neighboring reporter cells and is used by Fluc to produce light (Figure 1B).[5] While Lugal is able to report on relative distances between cell populations, the molecule is prone to nonspecific uncaging in biological media.[5] Premature uncaging results in luciferin release and bioluminescent light emission in regions devoid of activator cells. Thus, sensitive imaging of cellular interactions with Lugal remains challenging.

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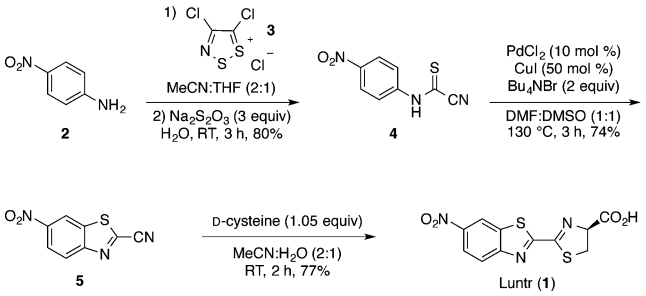
We aimed to develop an alternative caged luciferin with improved robustness and specificity. The ideal molecule would be readily accessible from common synthetic procedures, produce little to no light with Fluc, and, importantly, be resistant to nonspecific uncaging. Based on these considerations, we were drawn to the nitroaromatic luciferin Luntr (**1**) that could potentially be uncaged by bacterial nitroreductase (NTR) (Figure 1B–C). NTR and related orthologs have been widely used to activate nitroaromatic prodrugs[22−27] and imaging agents[28,29] *in vivo*. Mammalian cells also lack endogenous NTR activity, suggesting that heterologous expression of the enzyme in these cells would enable selective uncaging in preclinical models.[5] We further reasoned that the nitro group would serve as an effective luciferin cage. Active luciferins harbor electron-donating substituents at the 6′ ring position;[30] thus, the electron-withdrawing nitro group in Luntr would likely preclude robust light emission. Nitro group reduction by NTR, though, should restore electron density and, thus, provide a viable luciferin.

Luntr was readily synthesized using chemistries previously developed in our group (Scheme 1).[11,12] In brief, commercially

Scheme 1. Synthesis of the Caged Luciferin Luntr (1)



available 4-nitroaniline (**2**) was condensed with dithiazolium chloride **3** (Appel's salt). The product was fragmented *in situ* with sodium thiosulfate to generate cyanothioformamide **4** in good yield. This intermediate was subsequently cyclized[31] to provide cyanobenzothiazole **5**. A final condensation step with D-cysteine afforded the desired caged probe (Luntr, **1**). Importantly, Luntr was found to be stable in both aqueous solution and media (Figure S1). Chemically robust cages, as noted earlier, are critical for our approach to imaging cell contacts.

With the caged compound in hand, we evaluated its responsiveness to NTR. Luntr was incubated with purified NTR and NADH,[22,32] and substrate uncaging was monitored via fluorescence spectroscopy. As shown in Figures 2A and S2, uncaging was both selective and rapid, with product being detected after only 15 min of incubation. We hypothesized that the reduced compound was hydroxylamine **6**, as this molecule is capable of robust light emission with Fluc (Figure S3). However, we were unable to directly isolate and characterize **6** from the enzymatic reaction. Thus, we turned to ^{1}H NMR to monitor the enzymatic reduction *in situ*. Luntr was incubated with NTR and NADH in deuterated PBS, and aliquots of the reaction mixture were compared to synthetic standards of hydroxylamine **6** and amine **S1** (Scheme S1 and Figures S4–S5). NTR is known to reduce aryl nitro groups to hydroxylamines,[22−25,33,34] although nitroso and amine products have been detected in a few cases.[26,28,35] Diagnostic proton resonances for **6** were observed during the first few hours of incubation (Figure 2B), prior to compound degradation (Figure S6). Formation of the fully reduced 6-amino luciferin (**S1**) was not observed (Figure S5) during the course of the reaction. In the absence of NTR, no changes in the NMR spectra for Luntr or NADH were noted. Importantly, when the reaction mixtures (containing **6**) were added to purified Fluc, bioluminescent light was produced (Figure S7). These data indicate that hydroxylamine **6** is the uncaged form of Luntr.

Uncaged Luntr was also sufficiently cell permeant to induce bioluminescent light production in luciferase-expressing (Fluc$^+$) reporter cells. In these experiments, Luntr (**1**) was incubated in the presence or absence of recombinant NTR and NADH for 0–90 min. Aliquots of these reaction mixtures were added to Fluc$^+$ reporter cells, and bioluminescent light production was measured over time. As shown in Figure 2C, an ∼120-fold increase in bioluminescent signal was observed when Luntr was incubated with NTR and NADH for 30 min. Reduced light outputs were observed at longer incubation times, likely due to

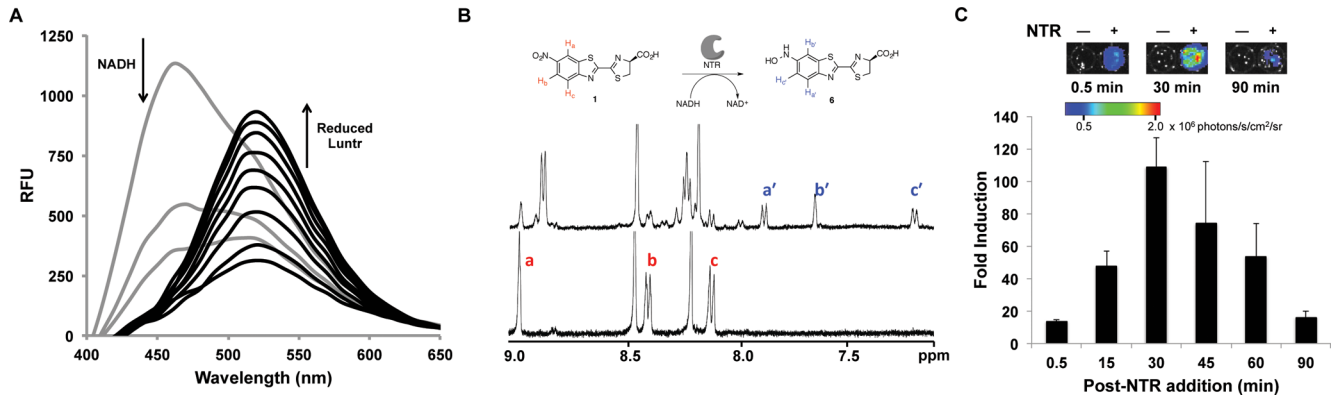


Figure 2. NTR-mediated reduction of Luntr produces a light-emitting luciferin. (A) Fluorescence emission spectra of Luntr (100 μM) incubated with NTR (2 μg/mL). The reaction was monitored over 170 min using an excitation wavelength of 350 nm. (B) Luntr (**1**, 5 mM) was combined with NTR (2 μg/mL) and NADH (7.5 mM) in deuterated PBS, and the reduction was monitored by ^{1}H NMR spectroscopy. Sample spectra (2 h post-NTR addition) are shown, and diagnostic resonances are labeled. (C) Reduced Luntr is cell permeant. Luntr (2.75 mM) was incubated with NADH in the presence (+) or absence (—) of NTR for 0–90 min. Aliquots were collected over 90 min, transferred to wells containing Fluc$^+$ HEK293 (reporter) or control cells, and imaged. Sample images are shown, and the fold induction in bioluminescent signal (from reporter vs control cells) is plotted. Error bars represent the standard deviation of the mean for n = 3 experiments.

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product inhibition or degradation of hydroxylamine **6** (Figure S6).

We hypothesized that Luntr would be beneficial for imaging cell contacts, as the uncaged luciferin would remain localized near the NTR source. To examine this possibility, we first monitored Luntr uncaging in NTR-expressing (NTR^+) activator cells.[22] Activator cells were seeded together with $Fluc^+$ reporter cells in a 96-well plate. When Luntr (250 μM) was added, only a modest increase in bioluminescent signal was observed in the mixed cultures compared to reporter cells incubated with control (NTR^-) cells (Figure 3A). Similarly weak signals were observed when Luntr was first incubated with NTR^+ cells, followed by media transfer to reporter cells (Figure S8). Previous experiments indicated that reduced Luntr **6** was sufficiently cell permeant to enter $Fluc^+$ cells (Figure 2C), and

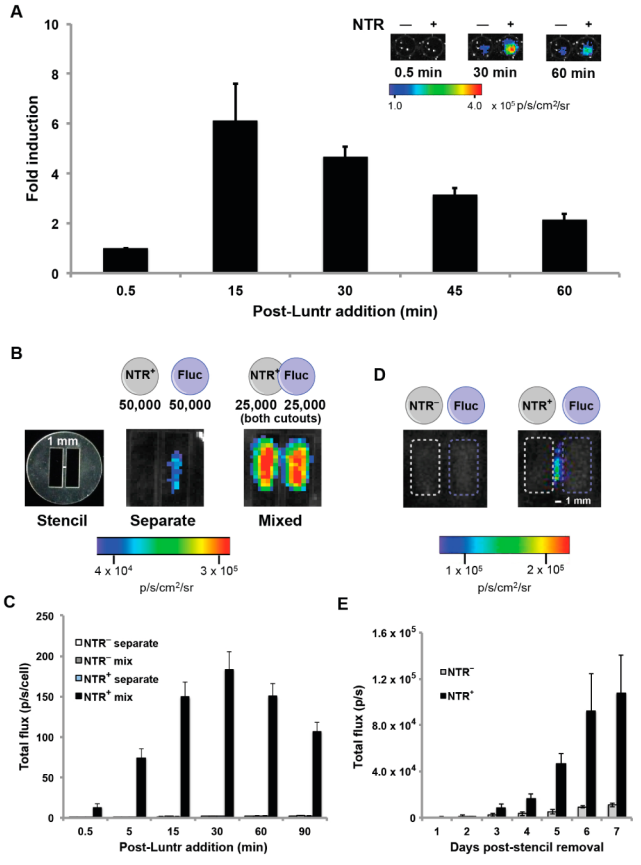

Figure 3. Luntr can be selectively uncaged and locally consumed. (A) Activator (NTR^+) or control (NTR^-) (50,000) cells were plated with 50,000 reporter ($Fluc^+$) cells. Luntr (250 μM) was added, and bioluminescence images were recorded over 60 min. Sample images are provided, and the fold induction in bioluminescent signal from NTR^+ vs NTR^- cells is plotted. (B and C) Close proximity is necessary for signal induction. Fabricated stencils (left) were used to separate (blue bars) or mix (black bars) activator and reporter cells. Luntr (250 μM) was added, and images were recorded from 0 to 90 min. NTR^- cells were also mixed (gray bars) or separated (white bars) with reporter cells and imaged. For (A) and (C), error bars represent the standard deviation of the mean for $n = 3$ experiments. (D and E) NTR^- or NTR^+ cells were plated 1 mm apart from reporter cells (dotted lines indicate initial plating areas). The cells were allowed to grow into contact over 7 d. Images were acquired daily (25 min post-Luntr addition). Those shown are from day 6. Error bars in (E) represent the standard deviation of the mean for $n = 3$ samples. Data are representative of three replicate experiments.

NTR enzyme assays suggested that uncaging activity was present in the activator cells (Figure S9). Thus, the low signals were likely due to limited release of **6** out of the NTR^+ activator cells. To test this hypothesis, we generated cells that stably expressed Fluc directly fused to NTR (Fluc-NTR). In these cells, reduced Luntr can be immediately processed by Fluc, without having to diffuse out of one cell and into another. Fluc-NTR^+ cells were plated and incubated with Luntr (250 μM) for 0–60 min prior to imaging. Control experiments included mixtures of NTR^+ and $Fluc^+$ cells, along with $Fluc^+$ cells plated with NTR^- cells. Comparable levels of NTR activity were observed in the Fluc-NTR^+ and NTR^+ cells (Figure S9). However, the Fluc-NTR^+ cells provided a nearly 40-fold increase in bioluminescent signal compared to $Fluc^+$ cells mixed with NTR^+ cells (Figure S10). These data suggest that once Luntr is uncaged, the molecule does not readily escape activator cells for use by neighboring reporter cells.

Limited release of uncaged Luntr is advantageous for imaging direct cell–cell interactions, as active luciferin should be completely consumed by the most proximal reporter cells. $Fluc^+$ cells located farther away should remain dark, owing to insufficient quantities of luciferin reaching distant areas. Indeed, when $Fluc^+$ and NTR^+ cells were plated in direct contact, light emission was readily observed following Luntr addition (Figures 3 and S11–S12). When separated by just 1 mm, though, $Fluc^+$ and NTR^+ cells produced little light in the presence of Luntr (Figures 3B–C and S13). Similar trends were observed using another cell type (Figure S14). Robust light emission was also observed when $Fluc^+$ and NTR^+ cells were allowed to grow into contact (Figures 3D–E and S15–S16). In all cases, no cell death was observed upon trypan blue staining. The stringent dependence of light emission on cellular distance is ideal for use in tissues and other complex environments, as only cells in close contact should produce light.

The development of imaging tools to visualize cell contacts addresses a void in our ability to "see" microscopic events at the macroscopic level. In this work, we designed and synthesized a novel caged luciferin, Luntr, which is stable in biological media. Additionally, we verified that Luntr can be selectively uncaged by bacterial nitroreductase *in vitro* for bioluminescent photon production. NTR was expressed in various cell lines and used to activate Luntr in mixed cell cultures. The limited release and lifetime of uncaged Luntr proved advantageous for visualizing cells in close contact. We anticipate that the technology will be most useful for imaging cell–cell interactions where the largest numbers of interacting cells are expressing NTR. We are currently evaluating Luntr for visualizing host–pathogen interactions *in vivo*, but the caged luciferin technology will be broadly applicable to imaging cellular interactions relevant to immune function, cancer progression, and numerous other biological processes.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, full spectroscopic data for all new compounds, and additional images. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b02774.

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Notes

The authors declare no competing financial interest.

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