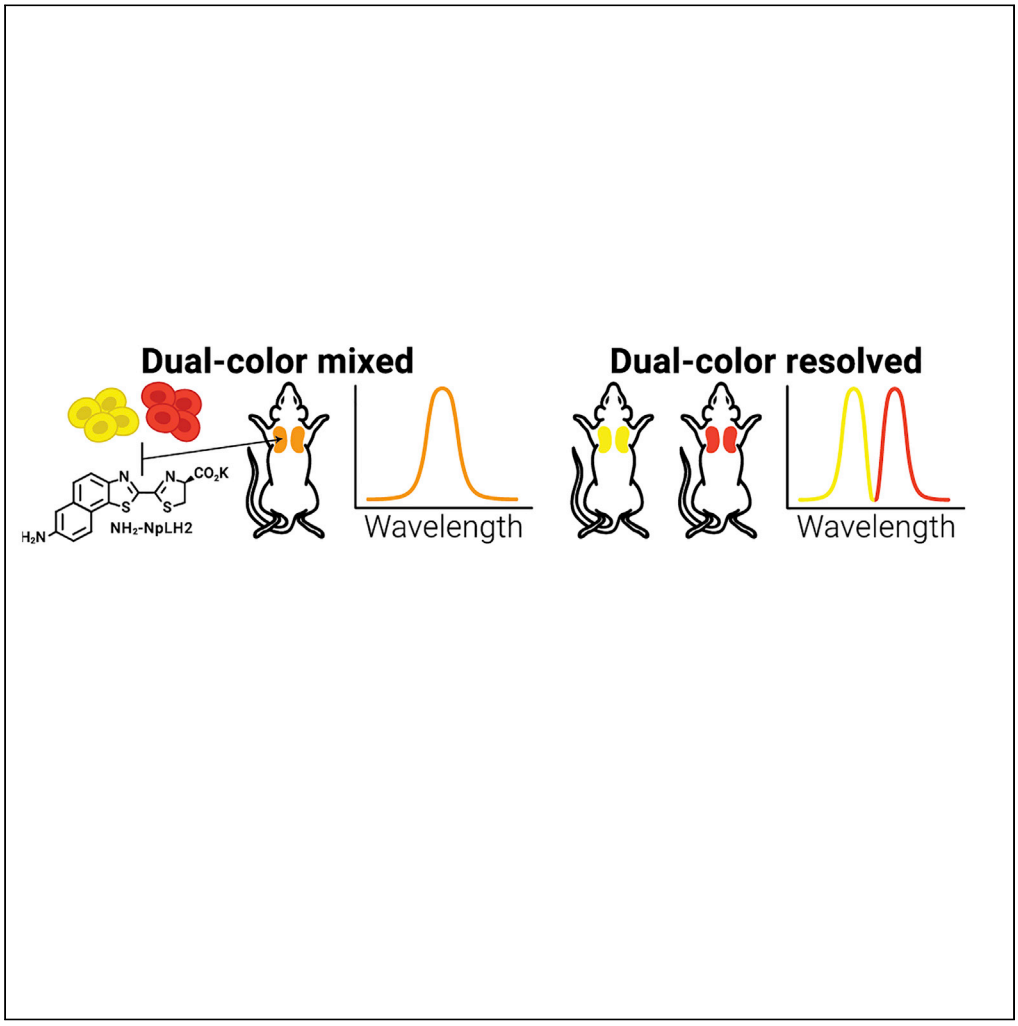
iScience

CellPress
OPEN ACCESS

Article

Red-shifted click beetle luciferase mutant expands the multicolor bioluminescent palette for deep tissue imaging



Giorgia Zambito, Mary P. Hall, Monika G. Wood, ..., Lance P. Encell, Clemens Löwik, Laura Mezzanotte

l.mezzanotte@erasmusmc.nl

HIGHLIGHTS

CBG2 is a new click beetle mutant derived from CBG99 luciferase

It has a far red-shifted bioluminescent emission with NH_2-NpLH2 luciferin (λ = 660 nm)

CBG2 with CBR2 luciferase and NH_2-NpLH2 allows fast acquisition of data in deep tissue

Zambito et al., iScience *24*, 101986
January 22, 2021
https://doi.org/10.1016/j.isci.2020.101986

iScience

CellPress
OPEN ACCESS

Article

Red-shifted click beetle luciferase mutant expands the multicolor bioluminescent palette for deep tissue imaging

Giorgia Zambito,[1,2,3] Mary P. Hall,[4] Monika G. Wood,[4] Natasa Gaspar,[1,2,5] Yanto Ridwan,[1,2] Fabio F. Stellari,[6] Ce Shi,[7] Thomas A. Kirkland,[7] Lance P. Encell,[4] Clemens Löwik,[1,2,8] and Laura Mezzanotte[1,2,9,*]

SUMMARY

For *in vivo* multicolor bioluminescence applications, red and near-infrared signals are desirable over shorter wavelength signals because they are not as susceptible to light attenuation by blood and tissue. Herein, we describe the development of a new click beetle luciferase mutant, CBG2, with a red-shifted color emission. When paired with NH_2-NpLH2 luciferin, CBG2 (λ = 660 nm) and CBR2 (λ = 730 nm) luciferases can be used for simultaneous dual-color bioluminescence imaging in deep tissue. Using a spectral unmixing algorithm tool it is possible to distinguish each spectral contribution. Ultimately, this enzyme pair can expand the near-infrared bioluminescent toolbox to enable rapid visualization of multiple biological processes in deep tissue using a single substrate.

INTRODUCTION

Bioluminescence imaging (BLI) has become a highly adopted technique for preclinical and non-invasive study of biological events *in vivo* (Kaskova et al., 2016; Mezzanotte et al., 2017). The production of bioluminescence depends on luciferase-enzyme-catalyzed oxidation of a luciferin substrate (Wilson and Hastings, 1998). The use of luciferases emitting photons in the "bio-optical window" (λ = 600 nm–800 nm) is highly recommended to limit light absorption by tissue components *in vivo* (Jathoul et al., 2014; Smith et al., 2009). Thus, red-shifted luciferase mutants improve the sensitivity of BLI and allow tracking of single cells over time in deep tissue (Branchini et al., 2010; Iwano et al., 2018). However, it is still challenging to visualize multiple biological processes over time in deep tissue because current BLI offerings are limited. In many of the systems currently used, sequential administration of multiple substrates is required, making interpretation of data challenging (Maguire et al., 2013; Taylor et al., 2018). Previously, we attempted dual-color BLI using green click beetle (CBG99) and red firefly (PpyRe8) luciferases with D-LH2. However, the signal for CBG99 was attenuated in deep tissue, resulting in acquisition of predominantly the red contribution (Mezzanotte et al., 2011).

An ideal approach for deep tissue multicolor BLI would be to utilize a single substrate with two luciferases emitting spectrally separated signals in the near-IR bio-optical window. Notably, the recent development of infra-luciferin (iLH2) proved to shift the FLuc mutants to the far-red and near-infrared region of spectrum (FLuc_green ~680 nm and FLuc_red ~720 nm) (Jathoul et al., 2014; Branchini et al., 2007). Stowe et al. demonstrated that engrafted red-CAR T cells can expand and reach the green-Raji B lymphoma when iLH2 is used *in vivo* (Stowe et al., 2019). Green and red signals were acquired with a sensitive CCD camera and quantified using a validated spectral unmixing algorithm as part of the instrument software (Aswendt et al., 2019; Gammon et al., 2006).

Herein, we introduce a novel click beetle mutant named CBG2. CBG2 paired with NH_2-NpLH2 substrate (λ = 660 nm) can be integrated with the near-infrared system CBR2/NH_2-NpLH2 (λ = 730 nm) (Hall et al., 2018) for dual-color near-infrared (NIR) BLI *in vivo*. We demonstrate that it is possible to spectrally resolve and quantify the bright emissions of CBG2 and CBR2 using a spectral unmixing algorithm. The high solubility and low toxicity associated with the salt form of NH_2-NpLH2 luciferin make the system amenable to *in vivo* injection, thus expanding the BLI toolbox for measuring multiple biological processes in a single imaging session using a single luciferase substrate.

[1]Erasmus Medical Center, Radiology and Nuclear Medicine, Rotterdam, 3015 CE, the Netherlands

[2]Erasmus Medical Center, Molecular Genetics, Rotterdam, 3015 CE, the Netherlands

[3]Medres Medical Research GmbH, Cologne 50931, Germany

[4]Promega Corporation, Madison, WI 53711, USA

[5]Percuros B.V., Leiden, 1333 CL, the Netherlands

[6]Chiesi Farmaceutici, Parma, 43122, Italy

[7]Promega Biosciences Incorporated, San Luis Obispo, CA 93401, USA

[8]CHUV Department of Oncology, University of Lausanne, 461011 Lausanne, Switzerland

[9]Lead contact

*Correspondence: l.mezzanotte@erasmusmc.nl

https://doi.org/10.1016/j.isci.2020.101986

Table 1. Relative light unit (RLU) of CBR2, CBG99, and CBG2 measured with D-LH2 and NH_2-NpLH2 in transiently transfected and lytic HEK293T cells

Purified Mutant Enzyme	D-LH2 lytic	Spectral Peak D-LH2 (nm)	NH_2-NpLH2 lytic	Spectral Peak NH_2-NpLH2 (nm)
CBR	0.96	620	26.72	660
CBR2	1.97	620	26.34	730
CBG99	0.83	540	0.58	545
CBG99opt	1.00	550	1.00	545
CBG2	4.38	585	14.72	660

Spectral peak data were acquired using purified enzymes.

RESULTS

Rational design of CBG2 luciferase and spectral characterization

Color variation in click beetle luciferases can be influenced at the protein level by a small number of amino acid positions. The best characterized mutants, CBG99, CBR, and CBR2, differ at only nine positions. To create a luciferase that can produce NIR emission suitable for multiplexing with CBR2 and efficiently utilize NH_2-NpLH2, we chose CBG99 as our starting point. CBG99 was preferred over CBR primarily because of its narrower spectrum (Figure S1A). We first codon-optimized CBG99 luciferase (CBG99opt) to improve gene expression and protein levels in mammalian cells. CBG99opt has identical codons to CBR2 except at sites where there are amino acid differences. We confirmed that CBG99opt produces a spectral peak at 540 nm when combined with D-LH2 (Xu et al., 2016; Miloud et al., 2007) and a peak at 545 nm when used with NH_2-NpLH2 (Table 1).

Next, we designed a panel of mutants based on the nine amino acid differences between CBG99 and CBR2. The set included amino acid substitutions in the active site known to red-shift emission of beetle luciferases (Viviani et al., 2016). The mutant of highest interest that emerged from this analysis, CBG2, differs by six residues compared with CBG99 and by three residues compared with CBR2. CBG2 was red-shifted by 75 nm with NH_2-NpLH2 (660 nm), when compared with the wild-type CBG99/NH_2-NpLH2 (545 nm) (Table 1). A summary of the spectral characterization and brightness for the purified luciferase mutants is presented in Table 1. Residues that differ between CBR2 and CBG2 are highlighted in the structure model shown in Figure 1A. These residues are mainly located in the luciferin binding pocket of the enzymes and contribute to substrate affinity and color-shift (Woodroofe et al., 2008). We employed D-LH2 and its analogs NH_2-NpLH2 and AkaLumine-HCl (depicted in Figure 1B) to evaluate the function of the novel mutant in this study.

Luminescence signals for CBR2 and CBG2 with D-LH2 and NH_2-NpLH2 (100 μM) were measured in live cells and lysates (Figure 1C). With D-LH2 as substrate, CBG2 was ~5-fold brighter and red-shifted ~40 nm (to 585 nm) relative to both CBG99 and CBG99opt. Interestingly, the specificity of CBG2 in live cells is significantly higher than both CBG99 and CBG99opt with NH_2-NpLH2, producing a 25-fold increase in light output with a ~115 nm red-shift (660 nm). CBR2 luciferase yielded the brightest photon emission when used with NH_2-NpLH2 and a near-infrared peak at 720 nm (Figure 1C). The CBR variant (also giving a peak at 660 nm) was considered for multiplex BLI, but further investigations in live cells revealed a broad, more intense spectral profile that significantly overlapped with the CBR2 spectrum (Figure S1A).

Kinetic properties for the mutant luciferases were tested in HEK293T live cells with 1.85 μM of NH_2-NpLH2 (Figure 1D). After an initial loss (~10-fold) in signal, CBG2 reached steady state after 10 min. The signal strength for CBR2 was higher compared with CBG2, but its signal duration was longer (Figure 1D). V_{max} and Km parameters of the enzymes with titrated D-LH2 or NH_2-NpLH2 can be found in Figure S1B. Luminescence photon fluxes of HEK cells stably expressing CBG2, Luc2, CBR2, or Akaluc luciferases and their respective brightness with D-LH2, NH_2-NpLH2, and Akalumine-HCl are highlighted in Figure S2A.

We attempted to unmix (i.e., resolve) the spectra of CBG2 and CBR2 with D-LH2 or NH_2-NpLH2 using transfected HEK293T cells. When treated with D-LH2, CBG2 cells produced nearly 2-fold higher photon flux compared with CBR2. Emission peaks for the two systems were separated by 35 nm. This modest separation, combined with the broad emission spectrum for CBR2, prevented efficient resolution of signals

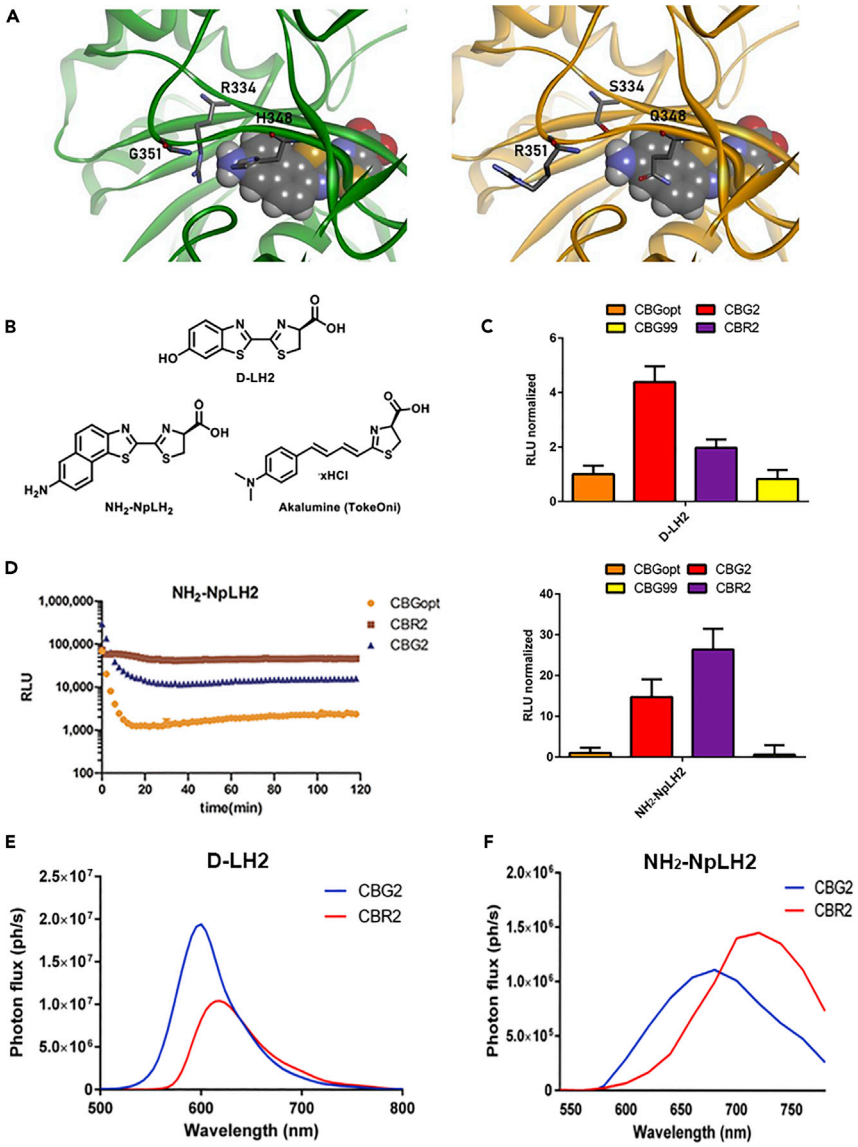


Figure 1. Rational design of CBG2 luciferase and spectral characterization

Figure360▷ For a Figure360 author presentation of this figure, see https://doi.org/10.1016/j.isci.2020.101986.

(A) Homology models of CBG2 (left) and CBR2 (right) luciferases with bound NH_2-NpLH2 substrate, based on firefly luciferase X-ray structure templates (PDB accession codes 2D1S, 4G36, and 5KYT). Residues that differ between CBG2 and CBR2 (334, 348, 351) are indicated. Modeling and rendering were performed using Discovery Studio software (BIOVIA).

(B) Chemical structures of D-LH2, NH_2-NpLH2, and AkaLumine-HCl substrates.

(C) Bioluminescence intensity (RLU_{max}) of CBG99, CBG99opt, CBR2, and CBG2 (cell lysates) combined with D-LH2 or NH_2-NpLH2 substrates (n= 3 samples; error bars represent ±SD). The spectra of CBG2/D-LH2 (top panel) and CBG2/NH_2-NpLH2 (bottom panel) are presented as reference points. A summary of spectral peaks of the different combinations is reported in Table 1.

(D) Kinetics of HEK293T CBG99opt, CBG2, and CBR2 cell lysets treated with NH_2-NpLH2.

Bioluminescence emission spectra for lysates containing CBG2 and CBR2 with D-LH2 (E) or NH_2-NpLH2 (F). Spectra were acquired using an IVIS Spectrum with the following settings: FOV C, f/stop=1, medium binning, 30 s exposure time, and a range of band pass filters (500 nm to 780 nm).

(Figure 1E). In contrast, CBG2 cells treated with NH_2-NpLH2 showed a consistent, red-shifted bioluminescent spectrum peaking at ~660 nm and with a photon emission of 1.2×10^6 ph s^{-1}. This allowed enough spectral separation from the 730 nm peak for CBR2 (Figure 1F). Moreover, we confirmed sufficient spectral

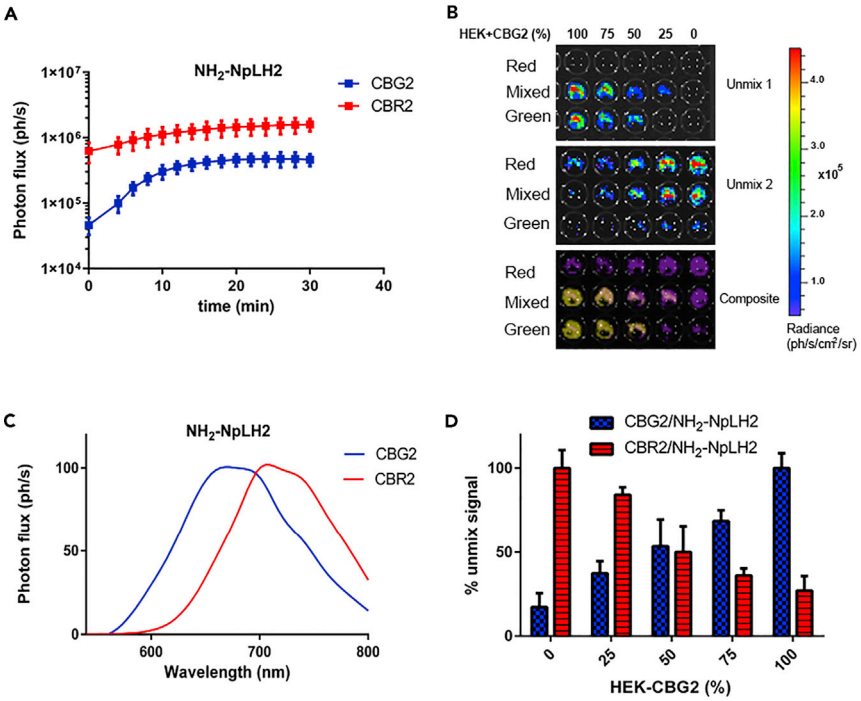


Figure 2. *In vitro* kinetics and spectral unmixing of CBG2 and CBR2 luciferases

(A) Live-cell bioluminescence kinetics reported for HEK-CBG2 and HEK-CBR2 with NH_2-NpLH2. Imaging was performed using an IVIS Spectrum with no filters and a 30 s exposure time. Imaging acquisitions were made every 2 min for a total of 15 acquisitions. This experiment was performed in triplicate; error bars represent ±SD.

(B) Spectral unmixing of HEK cell expressing CBG2 or CBR2 and mixed in various proportions ranging from 100% to 0% of the total population. Plate was spectrally imaged using IVIS system. Spectral unmix was produced building a specific library for each pure luciferase/luciferin BLI and then applied to the mixture.

(C) Normalized bioluminescence spectra generated by the spectral unmixing and revealing the feasibility to efficiently separate green and red spectra. Spectra were normalized to the peak emission for each Click beetle mutants with each substrate.

(D) Quantification of the percentage unmixed signals of HEK-CBG2 and HEK-CBR2 with NH_2-NpLH2. Unmixed signals were normalized to 100% cell ratios with $p < 0.0001$ and F-ratio 30.26 for mean values of HEK-CBG2 group and 31.82 for mean values of HEK-CBR2, calculated by one-way ANOVA. Error bars represent ±SD.

separation when CBR2 and CBG2 were co-transfected HEK cells. Use of the spectral unmixing tool allowed us to calculate the respective unmixed photon fluxes from cells expressing both luciferases (Figure S2B).

In vitro kinetics and spectral unmixing of CBG2 and CBR2 luciferases

Kinetic profiles for CBG2 and CBR2 were measured in stable luciferase-expressing HEK cells *in vitro* (Figure 2A). To validate the spectral unmixing of HEK-CBG2 and HEK-CBR2 signals, cells expressing either CBG2 or CBR2 were plated in various ratios (ranging from 100% to 0%) in 96-well black plates (Figure 2B). Spectral imaging and unmixing were performed by selecting 14 band pass filters ranging from 540 to 800 nm on the IVIS spectrum using NH_2-NpLH2 as substrate (1 mM). Interestingly, the algorithm was able to measure pure green signals (100% CBG2) and pure red signals (100% CBR2), making it possible to build a specific library for each luciferase contribution. The library was then applied to spectral unmixing. Figure 2C shows the successful unmixing of each spectrum, which then allowed us to plot the normalized and partially overlapped spectra of CBG2 (blue line) and CBR2 (red line). The same library was also used to quantify the photon flux of mixed green and red cell populations at different percentages between 100% and 0% (Figure 2D).

In vivo characterization of CBG2 and CBR2 mutants and spectral unmixing

To validate the potentiality of the dual-color BLI system in deep tissue, we first injected HEK-CBG2 or HEK-CBR2 to build a guided library for pure green or red signals. Images were captured using an IVIS imager with 15 band pass filters ranging from 540 to 800 nm. Pure HEK-CBG2 and HEK-CBR2 or a mixture of the

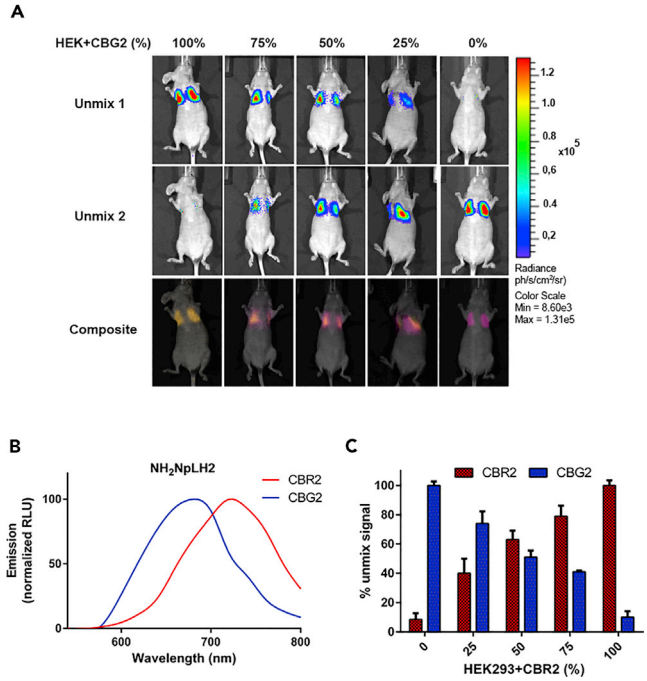


Figure 3. *In vivo* characterization of CBG2 and CBR2 mutants and spectral unmixing

(A) Representative unmixed bioluminescence images of CBG2 and CBR2 with NH_2-NpLH2 in deep tissue. Representative images of mice injected with different cell ratios of HEK-CBG2 and HEK-CBR2 with NH_2-NpLH2 in a lung model (n = 3 samples). HEK-CBG2 or HEK-CBR2 cells were injected (I)v. at different proportions and NH_2-NpLH2 substrate was injected (I)p. Images were acquired 15 min after substrate injection. Acquisition time for each filter was of 30 s. Band pass filters selected were between 540 nm and 800 nm. Filters selected for the green spectral unmixing is at 680 nm and for the red spectral unmixing is at 720 nm. Images were recorded 15 min after substrate injection considering the enzyme kinetics. Composite images indicate the linearity between the percentage of cells and the photons emitted.

(B) Spectral proprieties HEK-CBG2 and HEK-CBR2 with NH_2-NpLH2.

(C) Quantification of the photon fluxes of the different percentages of HEK-CBG2/NH_2-NpLH2 and HEK-CBR2/NH_2-NpLH2 ranged from 100% to 0% as shown in Figure 3A. Bioluminescent unmixed signals were normalized to 100% cell ratios (n = 3 samples) with $p < 0.05$, F-ratio 4.064 for mean values of HEK-CBG2 group, and 16.33 for mean values of HEK-CBR2, calculated by one-way ANOVA. Error bars represent ±SD.

two cell types was injected following the schema: 100%–0%; 75%–25%; and 50%–50% of green-red and then the same for red-green. NH_2-NpLH2 substrate was injected intraperitoneally and photons flux was recorded 10 min after substrate injection. The spectral unmixing algorithm efficiently extracted green or red contributions at the different percentages (Figure 3A). Notably, unmixing was also successful when 25% of the total population of the unmixed green was injected. Quantitative analysis for the unmixed green and the unmixed red photon fluxes for each cell percentage (0%–25%; 50%–75%; 100%) was performed using Living Image software.. Figures 3B and 3C reveals a linear correlation between the percentage of cells injected and photons recorded for both HEK-CBG2 and HEK-CBR2.

Versatility of CBG2 luciferase combined with AkaBLI system for dual color imaging

We further explored whether CBG2/NH_2-NpLH2 could be combined with Akaluc/AkaLumine-HCl (Iwano et al., 2018) for dual-color BLI. We selected two filters: 700 nm for CBG2/NH_2-NpLH2 and 660 nm for Akaluc/AkaLumine-HCl. Akaluc yielded the brightest photon emission with AkaLumine-HCl (~20-fold higher than CBG2/AkaLumine-HCl, Figure 4A). When the filter was set at 700 nm, CBG2/NH_2-NpLH2 was ~40-fold higher than Akaluc/NH_2-NpLH2. Interestingly, the pairings CBG2/Akalumine-HCl and Akaluc/NH_2-NpLH2 both recorded a dim signal, suggesting low enzyme activity for these combinations (Figure 4A). Thus, for dual-color BLI application the use of a single substrate where the enzymes have comparable expression was not feasible. Spectral curves and respective photon fluxes are depicted in Figure 4B. Next, we investigated whether a mixture of CBG2 or Akaluc cells could be measured using AkaLumine-HCl and NH_2-NpLH2 for dual-color BLI. First, the original spectrum libraries were efficiently built with

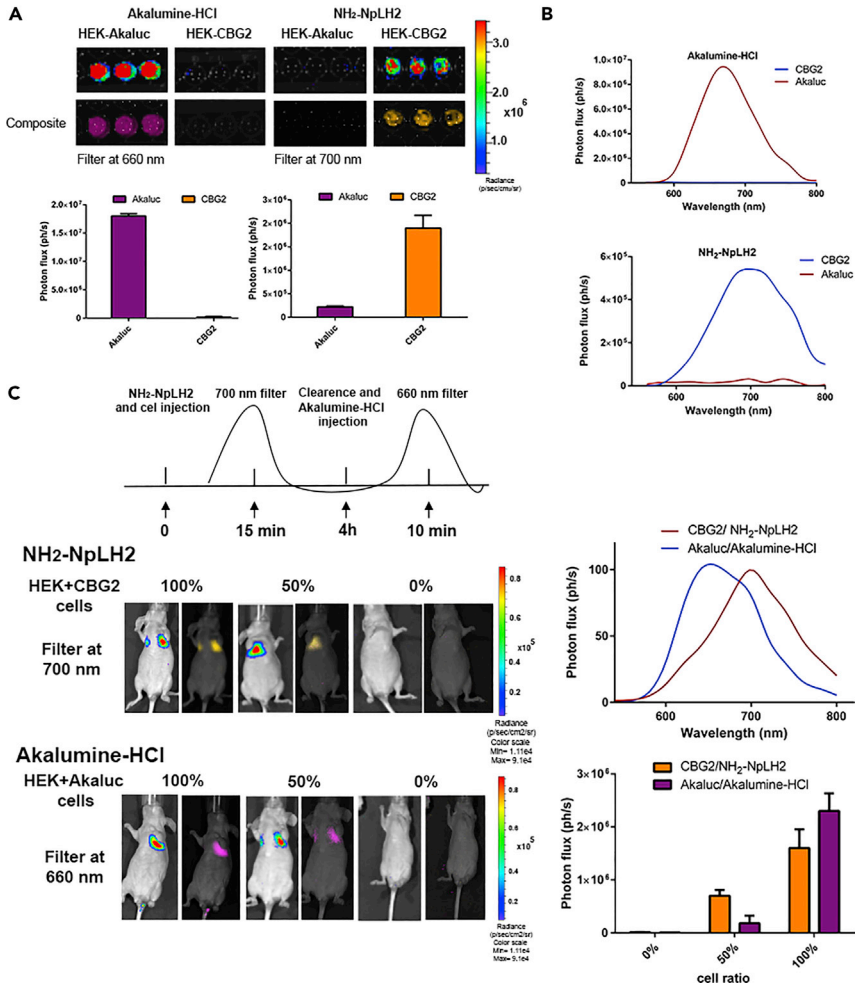


Figure 4. Versatility of CBG2 luciferase combined with AkaBLI system for dual color imaging

(A) Representative live cell images of HEK-cells expressing Akaluc and CBG2 and tested with AkaLumine-HCl and NH_2-NpLH2. Filters for the spectral unmixing were set at 660 nm for Akaluc/AkaLumine-HCl and at 700 nm for CBG2/NH_2-NpLH2. Plots indicate photon fluxes for *in vivo* measurements. The experiment was performed in triplicate; error bars represent ±SD.

(B) Spectral properties of HEK-Akaluc and HEK-CBG2 treated with AkaLumine-HCl and NH_2-NpLH2 (0.1 mM).

(C) Representative spectral unmixing *in vivo* (n = 3 samples) for 100%, 50%, or 0% of HEK-CBG2 or HEK-Akaluc with AkaLumine-HCl or NH_2-NpLH2. Cells were injected intravenously and substrates were injected intraperitoneally. Images were acquired first 15 min after NH_2-NpLH2 injection. Then, when pre-scan confirmed the clearance of NH_2-NpLH2, AkaLumine-HCl was injected and images acquired 5 min after substrate injection. Guided libraries were generated to identify each signal by Living Image software (PerkinElmer). Normalized spectra generated (top-right panel) confirmed the feasibility to separate CBG2 and Akaluc signals *in vivo* by selecting filters at 660 nm for Akaluc/AkaLumine-HCl and 700 nm for CBG2/NH_2-NpLH2. Acquisition time for each filter was 30 s. Quantification of the photon flux (bottom-right panel) at the different cell percentages (100%–50%–0%) for HEK-CBG2 or HEK-Akaluc with NH_2-NpLH2 and for HEK-CBG2 or HEK-Akaluc with AkaLumine-HCl are plotted (right-bottom), $p < 0.05$, n = 3 samples; error bars represent ±SD.

100% CBG2 cells or 100% Akaluc cells using Living Image software (PerkinElmer). Each luciferase contribution was effectively separated and quantified Figure 4C (left). Separation could be achieved when the luciferase contributions were equal (50% HEK-CBG2 and 50% HEK-Akaluc). The spectral curves and quantification of luminescence signals at different cell ratios are depicted in Figure 4C (right).

DISCUSSION

We report here on a novel dual NIR click beetle luciferase system that can record semi-quantitative data from deep-tissue and whole-body imaging by reducing light attenuation caused by hemoglobin, melanin,

and water. Importantly, the administration of NH_2-NpLH2 as single substrate provides high sensitivity, reduces the number of animals required, and minimizes animal discomfort during the study (Cool et al., 2013). In comparison to previously published method (Stowe et al., 2019), the brightness of our system allowed for a substantial reduction in imaging time to 6 min or less (acquisition time per filter of 30 s instead of 120 s). Similar to previous reports using green luciferases, we observed some attenuation of CBG2 light emission due to the partial absorbance of the emitted green photons and a shift of the spectral peak to ~680–700 nm in deep tissue (Rumyantsev et al., 2016). However, the spectra of CBG2 and CBR2 maintained adequate spectral separation in the lungs, which allowed us to distinguish each luciferase contribution.

We also compared the versatility of the new CBG2/NH_2-NpLH2 system with the recently developed AKA-BLI system (650 nm) (Iwano et al., 2018). This method can be exploited for multiplexed bioluminescence applications where Akaluc/AkaLumine-HCl and CBG2/NH_2-NpLH2 can give distinct signals. Indeed, this setup will effectively probe more than one cellular process, each producing specific BL signals upon sequential administration of each substrate *in vivo*. However, this multiplex application will be less specific with CBR2/NH_2-NpLH2 (730 nm) due to the bioluminescence recorded when AkaLumine-HCl is used (Hall et al., 2018; Zambito et al., 2020). Another limitation is that sequential substrate administration requires longer imaging sessions (Kleinovink et al., 2019; Yeh et al., 2019). Indeed, injection of two substrates requires the clearance of the first substrate but provides maximal light emission for each luciferase, thereby reducing spectral interference from each luciferase. Finally, we envision a highly sensitive triple color BLI application with CBG2/CBR2 with NH_2-NpLH2 (680 nm and 720 nm, respectively) coupled with the novel optimized NanoLuc/hydrofurimazine (460 nm) in the same animal model (Su et al., 2020). This system (i.e., tri-plex BLI) could be used to visualize localization, activation, and other functional states of immune cells. In conclusion, a novel dual-color BLI in the NIR window can be accessed using CBG2 and CBR2 cells treated with a single substrate, NH_2-NpLH2. This represents a promising approach for simultaneous visualization and quantification of two cell populations in deep tissue and in the same animal model. Collectively, this work will contribute to expand the toolset for *in vivo* multicolor bioluminescence imaging.

Limitations of the study

The size and the photon flux of the signal area in deep tissue represent a potential limitation in the ability to efficiently separate and quantify the contribution of each luciferase. Although we demonstrated feasibility for lung imaging, we expect the method to be more accurate for dual color imaging of small areas (e.g., lymph nodes and depots of injected cells in deep organs) as demonstrated previously (Branchini et al., 2007). The kinetics of light emission after i.p. administration of NH_2-NpLH2 is slow (BL signals peaking at ~20 min), so repeated administration of substrate and imaging must be delayed by approximately 3 h. When CBG2/NH_2-NpLH2 and Akaluc/AkaLumine-HCl are used for multiplex imaging, sequential administration of the two substrates is needed. This may require longer imaging sessions depending on substrate properties as solubility, enzyme affinity, different bio-distribution, and serum stability (Yeh et al., 2019).

Resource availability

Lead contact

Further information and requests should be directed to the Lead Contact, Dr. Laura Mezzanotte (l.mezzanotte@erasmusmc.nl)

Materials availability

Materials are available from the corresponding author on request.

Data and code availability

This study did not generate computer code. All data and analytical methods are available in the main text or in Supplemental information.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2020.101986.

ACKNOWLEDGMENTS

We acknowledge the funding for this work provided by the European Commission under the H2020-MSCA-RISE award grant number 777682 (CANCER) and under the H2020-MSCA-ITN award, grant number 675743 (ISPIC). This work was supported by the Applied Molecular Imaging Erasmus MC (AMIE) facility.

AUTHOR CONTRIBUTIONS

Conceived and Performed Experiments, G.Z., M.P.H., and F.S.; Writing–Original Draft, G.Z. and M.P.H.; Review & Editing, N.G., M.P.H., T.K., L.P.E., C.L., and L.M.; Provided Expertise and Feedback, Y.R., L.M., M.P.H., F.S., and M.W.; Funding Acquisition, L.M. and C.L.; Supervision, L.M.

DECLARATION OF INTERESTS

Authors have no financial interests/commercial Conflict of Interest.

Received: October 2, 2020
Revised: December 7, 2020
Accepted: December 21, 2020
Published: January 22, 2021

REFERENCES

Aswendt, M., Voge, I. S., Schäfer, C., Jathoul, A., Pule, M., and Hoehn, M. (2019). Quantitative in vivo dual-color bioluminescence imaging in the mouse brain. Neurophotonics *6*, 1–11.

Branchini, B.R., Ablamsky, D.M., Murtiashaw, M.H., Uzasci, L., Fraga, H., and Southworth, T.L. (2007). Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. Anal. Biochem. *361*, 2.

Branchini, B.R., Southworth, T.L., Fontaine, D.M., Kohrt, D., Florentine, C.M., and Grossel, M.J. (2010). Red-emitting luciferases for bioluminescence reporter and imaging applications. Anal. Biochem. *396*, 290–297.

Cool, S.K., Breyne, K., Meyer, E., De Smedt, S.C., and Sanders, N.N. (2013). Comparison of in vivo optical systems for bioluminescence and Fluorescence imaging. J. Fluoresc. *23*, 909–920.

Gammon, S.T., Leevy, W.M., Gross, S., Gokel, G.W., and Piwnica-Worms, D. (2006). Spectral unmixing of multicolored bioluminescence emitted from heterogeneous biological sources. Anal. Chem. *78*, 1520–1527.

Hall, M.P., Woodroofe, C.C., Wood, M.G., Que, I., van't Root, M., Ridwan, Y., Shi, C., Kirkland, T.A., Encell, L.P., Wood, K.V., et al. (2018). Click beetle luciferase mutant and near infrared naphthyl-luciferins for improved bioluminescence imaging. Nat. Commun. *9*, 132.

Iwano, S., Sugiyama, M., Hama, H., Watakabe, A., Hasegawa, N., Kuchimaru, T., Tanaka, K.Z., Takahashi, M., Ishida, Y., Hata, J., et al. (2018). A Single-cell bioluminescence imaging of deep tissue in freely moving animals. Science *359*, 935 LP–939.

Jathoul, A.P., Grounds, H., Anderson, J.C., and Pule, M.A. (2014). A dual-color far-red to near-infrared firefly luciferin analogue designed for multiparametric bioluminescence imaging. Angew. Chem. Int. Ed. *53*, 13059–13063.

Kaskova, Z.M., Tsarkova, A.S., and Yampolsky, I.V. (2016). 1001 lights: luciferins, luciferases, their mechanisms of and applications in chemical analysis, biology and medicine. Chem. Soc. Rev. *45*, 6048–6077.

Kleinovink, J.W., Mezzanotte, L., Zambito, G., Fransen, M.F., Cruz, L.J., Verbeek, J.S., Chan, A., Ossendorp, F., and Löwik, C. (2019). A dual-color bioluminescence reporter mouse for simultaneous in vivo imaging of T cell localization and function. Front. Immunol. *9*, 3097.

Maguire, C.A., Bovenberg, M.S., Crommentuijn, M.H., Niers, J.M., Kerami, M., Teng, J., Sena-Esteves, M., Badr, C.E., and Tannous, B.A. (2013). Triple bioluminescence imaging for in vivo monitoring of cellular processes. Mol. Ther. Nucleic Acids *2*, e99.

Mezzanotte, L., Que, I., Kaijzel, E., Branchini, B., Roda, A., and Löwik, C. (2011). Sensitive dual color in vivo bioluminescence imaging using a new red codon optimized firefly luciferase and a green click beetle luciferase. PLoS One *6*, e19277.

Mezzanotte, L., van 't Root, M., Karatas, H., Goun, E.A., and Löwik, C. (2017). In vivo molecular bioluminescence imaging: new tools and applications. Trends Biotechnol. *35*, 640–652.

Miloud, T., Henrich, C., and Hammerling, G.J. (2007). Quantitative comparison of click beetle and firefly luciferases for in vivo bioluminescence imaging. J. Biomed. Opt. *12*, 1–5.

Rumyantsev, K.A., Turoverov, K.K., and Verkhusha, V.V. (2016). Near-infrared bioluminescent proteins for two-color multimodal imaging. Sci. Rep. *6*, 36588.

Smith, A.M., Mancini, M.C., and Nie, S. (2009). Bioimaging: second window for in vivo imaging. Nat. Nanotechnol. *4*, 710–711.

Stowe, C.L., Burley, T.A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D.M., Parkinson, G.N., Southworth, T.L., Agliardi, G., Hotblack, A., et al. (2019). Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin. Elife *8*, e45801.

Su, Y., Walker, J.R., Park, Y., Smith, T.P., Liu, L.X., Hall, M.P., Labanieh, L., Hurst, R., Wang, D.C., Encell, L.P., et al. (2020). Novel NanoLuc substrates enable bright two-population bioluminescence imaging in animals. Nat. Methods *17*, 852–860.

Taylor, A., Sharkey, J., Plagge, A., Wilm, B., and Murray, P. (2018). Multicolour in vivo bioluminescence imaging using a nanoluc-based bret reporter in combination with firefly luciferase. Contrast Media Mol. Imaging *2018*, 2514796.

Viviani, V.R., Simões, A., Bevilaqua, V.R., Gabriel, G.V.M., Arnoldi, F.G.C., and Hirano, T. (2016). Glu311 and Arg337 stabilize a closed active-site conformation and provide a critical catalytic base and countercation for green bioluminescence in beetle luciferases. Biochemistry *55*, 4764–4776.

Wilson, T., and Hastings, J.W. (1998). Bioluminescence. Annu. Rev. Cell Dev. Biol. *14*, 197–230.

Woodroofe, C.C., Shultz, J.W., Wood, M.G., Osterman, J., Cali, J.J., Daily, W.J., Meisenheimer, P.L., and Klaubert, D.H. (2008). N-Alkylated 6′-aminoluciferins are bioluminescent substrates for Ultra-Glo and QuantiLum luciferase: new potential scaffolds for bioluminescent assays. Biochemistry *47*, 10383–10393.

Xu, T., Close, D., Handagama, W., Marr, E., Sayler, G., and Ripp, S. (2016). The expanding toolbox of in vivo bioluminescent imaging. Front. Oncol. *6*, 150.

Yeh, H.-W., Wu, T., Chen, M., and Ai, H.-W. (2019). Identification of Factors complicating bioluminescence imaging. Biochemistry *58*, 1689–1697.

Zambito, G., Natasa, G., Ridwan, Y., Hall, M.P., Shi, C., Kirkland, T.A., Encell, L.P., Löwik, C., and Mezzanotte, L. (2020). Evaluating brightness and spectral properties of click beetle and firefly luciferases using luciferin analogues: identification of preferred pairings of luciferase and substrate for in vivo bioluminescence imaging. Mol. Imaging Biol. *22*, 1523–1531.

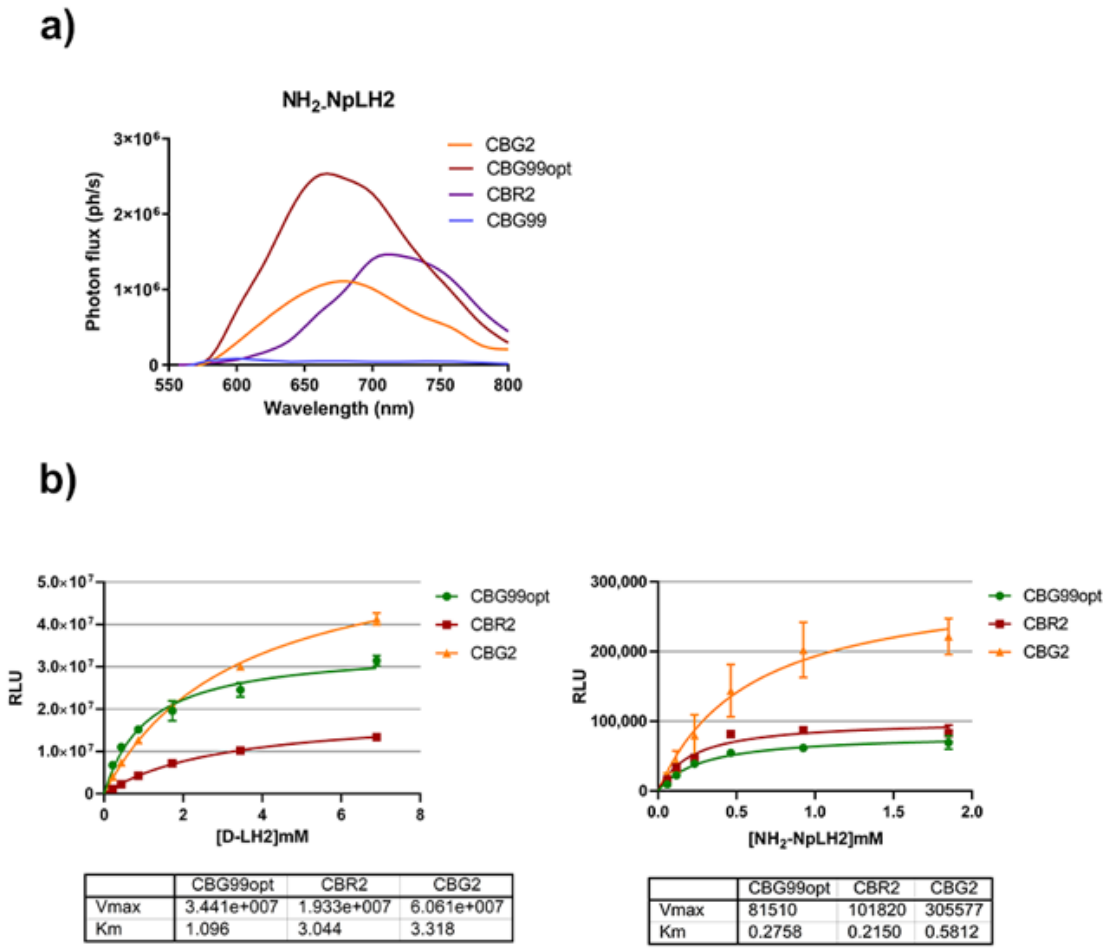
iScience, Volume *24*

Supplemental Information

Red-shifted click beetle luciferase mutant expands the multicolor bioluminescent palette for deep tissue imaging

Giorgia Zambito, Mary P. Hall, Monika G. Wood, Natasa Gaspar, Yanto Ridwan, Fabio F. Stellari, Ce Shi, Thomas A. Kirkland, Lance P. Encell, Clemens Löwik, and Laura Mezzanotte

Supplemental Information



	CBG99opt	CBR2	CBG2
Vmax	3.441e+007	1.933e+007	6.061e+007
Km	1.096	3.044	3.318

	CBG99opt	CBR2	CBG2
Vmax	81510	101820	305577
Km	0.2758	0.2150	0.5812

Figure S1. In vitro characterization of click beetle luciferase variants, Related to Figure 1. (a) Live HEK293T cell lines were transfected with plasmids expressing CBR2, CBG2, CBG99 or CBG99opt luciferase genes. Photon fluxes were quantified after treatment with NH_2-NpLH2 substrate (1mM) by the IVIS imager. Spectral curves were built by Living image software 4.5 (Perkin Elmer). **(b)** V_{max} and Km parameters of the CBG99opt, CBG2 and CBR2 enzymes with titrated D-LH2 or NH_2-NpLH2. Km and RLU_{max} values were calculated using GraphPad Prism (Michaelis–Menten regression).

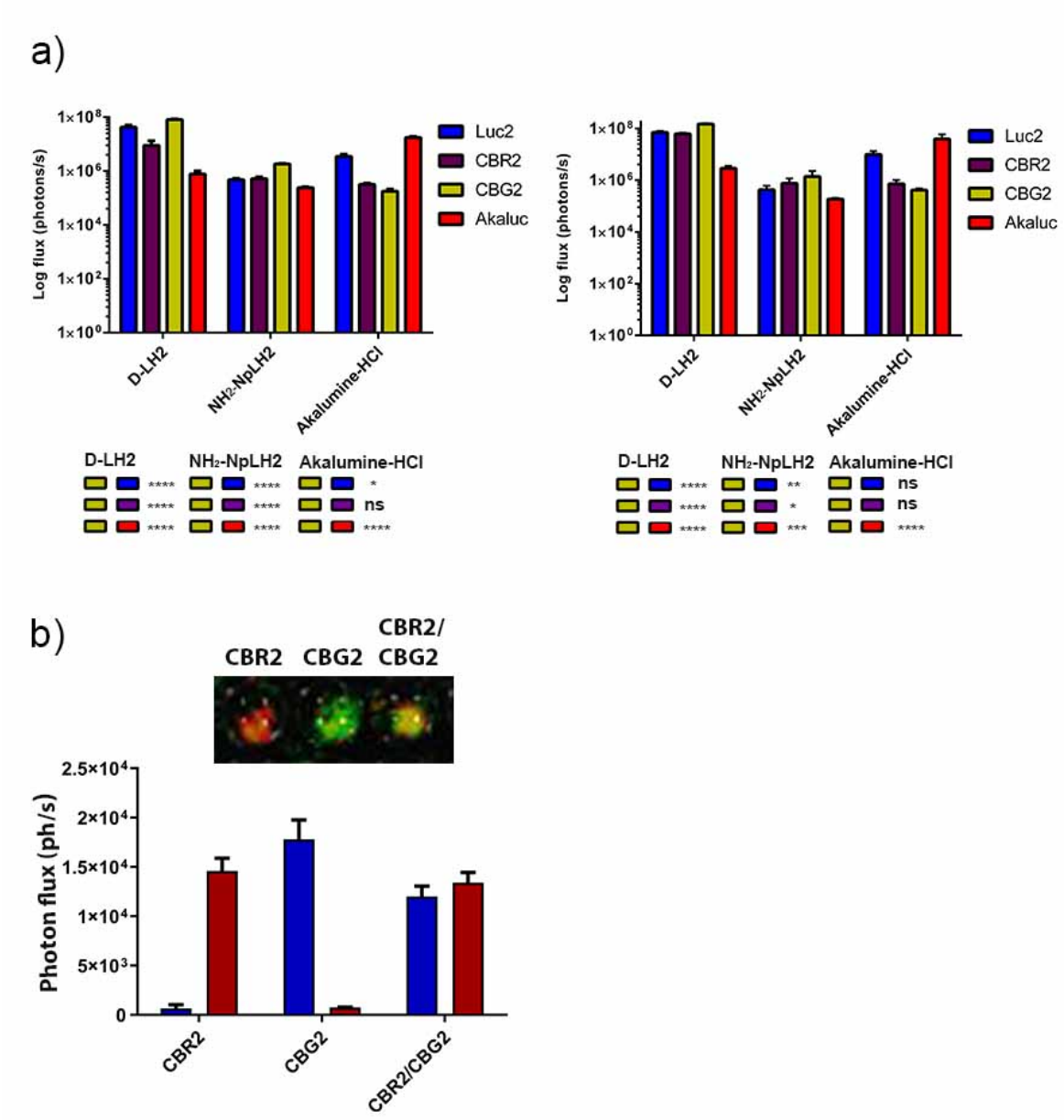


Figure S2. In vitro measurements of the photon fluxes and two-population luciferase brightness test. Related to Figure 2. (a) Live HEK293T cells stably expressing Luc2, CBR2, CBG2 and Akaluc luciferases, were tested for their brightness after addition of 0.1 mM (left panel) and 1 mM (right panel) of D-LH2 or analogues. Quantifications were performed 10 min after substrate addition. BL signals were compared to the brightness of CBG2 and statistical analysis was performed by One-way ANOVA, p<000.1. Experiment was performed in triplicate (n=3 samples). Error bars represent ±SD. **b)** In vitro two-population dual-color imaging of transfected HEK293T cells expressing CBR2 or/and CBG2 and respective quantification of the unmixed photon fluxes by spectral unmixing algorithm (n=3 samples).

Transparent Methods

Click beetle mutant construction. CBG99 and CBR wild type were codon optimized to match the codon usage in CBR2. Substitutions known to red shift CBG99 were added to the codon optimized CBG99 template, while substitutions known to blue shift CBR were added to the codon optimized CBR template. Substitutions of interest were combined on a common template to arrive at CBG2. Plasmids were constructed by Gene Dynamics, LLC.

Screening of luciferase mutants in live and lytic cell assay

Media was aspirated from a confluent flask of HEK293 cells (ATCC Hek293 cell (CRL1573). Cells were washed with DPBS (10 ml) (Life Technologies 14190) and then detached from the flask with TrypLE Express Trypsin (3 ml) (Life Technologies (12604). Cells were counted and resuspended to a concentration of 100,000 cell ml^{-1} in DMEM media supplemented with FBS (10%) and then diluted cells (100 µl) of was added to each well of a 96 well assay plate. Plates were grown for 24 h at 37 °C with 5% CO_2. The following day transfection complexes were prepared for each construct (CBGopt, CBR2, CBG2) which consisted of DNA (1 µg) diluted in a final volume of OptiMEM (50 µl) (11058) with Fugene transfection reagent (3.3 µl) (Promega E2311). Transfection complexes were incubated (15 min) and then each complex (5 µl) was added to wells of assay plates for each sample. Samples were then incubated for an additional (24 h). The following day both lytic and live cell assays were performed on each sample. For the live cell readings substrates were titrated as described below for Km and RLU_{max} determination. For the lytic reading BrightGlo assay buffer (Promega E264B) supplemented with ATP (1 mM) and D-Luciferin (100 µM) or NH_2-NpLH2 (100 µM) was added to 4 wells for each sample. Plates were placed on an orbital shaker at 600 RPM for 3 or 10 min and then luminescence was measured on a GMM+ GloMax® Multi+ for D-Luciferin or LAS 4000 CCD imager for NH_2-NpLH2.

Enzyme characterization: Km and RLU_{max} determination

The following substrate solutions were prepared in DPBS: D-LH2 (30 mM) (Promega), NH_2-NpLH2 (8 mM). Two-fold serial dilutions were prepared for each substrate (30 mM to 0.93 mM for D-LH2 or 8 mM to 0.25 mM for NH_2-NpLH2). Prior to assay, media from transfected wells was aspirated and replaced with CO_2 independent media supplemented with FBS (10%). Substrate dilutions (30 µl) were added to cells transfected with either CBGopt, CBG2 and CBR2. The plate was manually shaken, and immediately placed in a GloMax®-Multi+ luminometer (Promega) set to 37 °C. Kinetic reads were obtained for each substrate and sample combination. Initial timepoints were used to determine Km and RLU_{max} for each substrate. Km and RLU_{max} values were calculated using GraphPad Prism (Michaelis–Menten regression).

Cell culture and Preparation for cell assays

HEK293T cells were grown in DMEM medium (Sigma, St. Louis, Mo, USA) supplemented with FBS (10%) and of penicillin (1%) and streptomycin (1%) and incubated at 37 °C with 5% CO_2. When cell confluence reached around 80%, cells were washed with DPBS (life technologies 14190) and detached with 1 ml of Trypsin. Cells were centrifuged and re-suspended with fresh new medium (8 ml). Cell counting was performed using BioRad TC20 cell counter.

Cell transfection

HEK293T were plated at the density of 2 x 10^4 cells per well in a black 96 well plate (100 µl of complete DMEM). DNA plasmid of each luciferases (LUC2, CBG2, CBR2 or CBRopt) was diluted (at the concentration of 0.020 µg $µl^{-1}$ in 16 µl of sterile deionized water) to transfect 3 wells. Fugene® HD reagent (1 µl) was added to the complex and after mixing carefully, it was incubated for 10 min at room temperature. the preparation (5 µl) was added to each well of previously plated HEK293T. Plate was incubated at 37 ºC with 5% of CO_2 for 24 h.

Lentiviral production

Virus production and cell transduction were performed under appropriate biosafety level conditions (ML-II) in accordance with the National Biosafety Guidelines and Regulations for Research on Genetically Modified Organisms. Procedures and protocols were reviewed and approved by the EMC Biosafety Committee (GMO permit 99-163). The lentiviral plasmids pCDH-EF1-CBG2-T2A-copGFP, pCDH-EF1-CBR2-T2A-copGFP were used to transfect HEK-293 T cells with three packaging plasmids (pCMV-VSVG, pMDLg-RRE, pRSV-REV; Addgene, Cambridge, MA, USA) and the lentiviral vector plasmids using PEI transfection reagent (1mg/ml)/µg DNA) as previously described (Mezzanotte et al. 2011). The supernatant containing lentiviral particles were collected 48 h and 72 h after infection. Subsequent quantification of the virus was performed using a standard antigen-capture HIV p24 ELISA (ZeptoMetrix Corporation, NY, USA). Lentiviral plasmid of pCDH-EF1-Akaluc-T2A-copGFP was produced as previously described (Zambito et al. 2020).

Cell transduction

Cell transduction was performed by culturing HEK293T cells in complete DMEM at the density of 200,000 cells in a T25-flask with medium (5 ml). Expression in the lentiviral plasmid is driven by housekeeping elongation factor 1α (EF1) promoter. Cells were transduced with MOI 1 of either pCDH-EF1-CBG2-T2A-copGFP, pCDH-EF1-CBR2-T2A-copGFP with Polybrene (hexametride bromide, Sigma-Aldrich) at the final concentration (8 µg ml^{-1}). Cell were then expanded and sorted two times for copGFP (copepod Potenilla plumata) expression by FACS (BD-FACS AriaIII, BD Biosciences). HEK293T cells were transduced with pCDH-EF1-Akaluc-T2A-copGFP as previously described (Zambito et al. 2020).

***In vitro* bioluminescence imaging**

For in vitro BL imaging, HEK293T cells were plated at a density of 1 x 10^4 cells per well in a black 96-well plate (Greiner Cell Star®). Cells were transfected with DNA plasmids of CBG2, CBR2 or

simultaneously transfected with both plasmids. Prior imaging session, cells were washed and resuspended in PBS.

For the other tests with HEK-EF1-CBG2-T2A-copGFP, HEK-EF1-CBR2-T2A-copGFP and HEK-EF1-Akaluc-T2A-copGFP, cells were plated in triplicate in a black 96-well plate at the density of 1 x 10^4 cells per well and incubated at 37 °C for 24 h. Prior imaging session, cells were washed and resuspended in PBS. For kinetic study of HEK-EF1-CBG2-T2A-copGFP and HEK-EF1-CBR2-T2A-copGFP, imaging was performed after addition of NH_2-NpLH2 (final concentration 0.1 mM). Imaging settings were made at the IVIS spectrum system with open filter, 30 s exposure time, FOV C, f/stop=1, medium binning. Imaging acquisitions were made every 2 min for a total of 15 acquisitions. This experiment was performed in triplicate. For all other in vitro measurements, imaging was performed after addition of D-LH2, NH_2-NpLH2 or Akalumine-HCl (final concentration of 1 mM resuspended in PBS; 100 µl per well of a 96 black well plate). To accurately measure photon output while taking the kinetic profiles into account, images were acquired 10 min after addition of D-LH2 or NH_2-NpLH2 substrates and 5 min after addition of the Akalumine-HCl substrate. Images were acquired with the following settings: FOV C, f/stop=1, medium binning, 30 s exposure time and open filter. Images were acquired selecting a series of band pass filters ranged from 540 nm to 800 nm on the IVIS Spectrum (20 nm bandpass from 560 nm to 800 nm). ROI analysis of the spectral profiles and spectral unmixing were made on Living image software 4.5 (Perkin Elmer).

For spectral unmixing analysis, guided spectral unmixing of pure (100%) HEK-CBG2 cells, HEK-CBR2 or HEK-Akaluc cells was created. This allowed us to obtain a specific library of spectra for each luciferase and a distinct spectral signature for each luciferase/substrate. The specific library of spectra was then used to perform the spectral unmixing on a mixture of cell populations. Therefore, the relevant library of spectra allowed us to quantify the photons derived from the mixed cell population and to distinguish contributions from HEK-CBG2, HEK CBR2 or HEK-Akaluc. The

spectral unmixing allowed also to draw respective spectral profiles for HEK-CBG2, HEK-CBR2 and HEK-Akaluc. Experiments were repeated three times with three replicates.

In vivo animal model

Animal studies were approved by the Ethical Committee of Erasmus Medical Center, Rotterdam, The Netherlands. Animal care and handling was in accordance with the guidelines and regulations as stipulated by the Dutch Experiments on animal act (WoD) and The European Directive in the protection on animal used for scientific purposes (2010/63/EU). BALB/C nude (females) were purchased from Charles River Laboratory (The Netherlands). All mice aged 6–10 weeks were provided access to food and water *ad libitum* and were hosted in the animal facility at the Erasmus MC, Rotterdam, The Netherlands.

In vivo bioluminescence imaging: dual-color imaging, spectral unmixing and sequential imaging

Animal experiments were performed with mice anesthetized using isoflurane (1.5%). Groups of 3 mice were used for each condition. For the deep tissue model, HEK-EF1-CBG2-T2A-copGFP and HEK-EF1-CBR2-T2A-copGFP, HEK-EF1-Akaluc-T2A-copGFP were injected intravenously (i.v.) at a density of 1 million cells in 100 µl DPBS. NH_2-NpLH2 (220 mg Kg^{-1}) or Akalumine-HCl (50 mg Kg^{-1}) substrates were injected intraperitoneally (i.p.) (200 µl). In the case where animals received 50/50, 75/25 or 25/75 ratio of HEK-EF1-CBG2-T2A-copGFP and HEK-EF1-CBR2-T2A-copGFP, $5x10^5/5x10^5$, $7.5x10^5/2.5x10^5$ and $2.5x10^5/7.5x10^5$ cells were i.v. injected.

The dose of NH_2-NpLH2 or Akalumine-HCl substrates injected intraperitoneally were calculated based on the maximum solubility (especially for Akalumine-HCl), tolerability in mice and maximum attainable signal on previous findings (Branchini et al. 2010; Gammon et al. 2006). Images were acquired by IVIS spectrum (FOV C, binning medium, f/stop=1, 20 nm). Band pass filters were ranged between 540 nm to 800 nm and exposure time of 30 s was optimal for quantification of

bioluminescent signals required by spectral unmixing.). The series of images acquired with filters were taken 10, 16, and 21 min after i.p. injection of the substrates to determine the timing for maximal signal output. The stage was heated to 37 ºC. Open filters were used prior to the spectral recordings to assess stability of the substrate.

Spectral analysis and spectral unmixing of the in vivo images were performed by drawing ROIs with Living image software (Perkin Elmer). For guided spectral unmixing pure CBG2, CBR2 and Akaluc bioluminescent signals were recorded when combined with appropriate substrate. Once pure libraries were built, the relevant library spectra were then used to distinguish each luciferase contributions by spectral unmixing in the lung model. The spectral properties for each luciferase were drawn and quantified using the spectral unmixing algorithm.

For sequential imaging of HEK-CBG2 and HEK-Akaluc, mice were first injected with HEK-CBG2 cells ($1x10^6$) i.v. To quantify the bioluminescent signal required by spectral unmixing, the imaging session started 15 min after i.p. injection of NH2-NpLH2 (220 mg Kg^{-1}) using band pass filters that ranged between 540 nm to 800 nm with an exposure time of 30 s. Once the imaging session for HEK-CBG2/ NH2-NpLH2 was completed, clearance of the substrate was monitored after approximately 4 h by performing a pre-scan to access the absence of the signal. Then, HEK-Akaluc cells were injected i.v. ($1x10^6$) and Akalumine-HCl i.p. (50 mg Kg^{-1}). Images were acquired 5 min after substrate addition using band pass filters that ranged between 540 nm to 800 nm with exposure time of 30 s. Bioluminescent signals were quantified by spectral unmixing. All mice were culled at the end of the in vivo imaging sessions.

Statistical analysis

Km and RLU_{max} values were calculated using GraphPad Prism (Michaelis–Menten regression). The other in vitro and in vivo tests were performed using Graphpad 7 software and T test and ONE-way ANOVA. Results reported as mean ±SD and significance attributed when $p< 0.001$ (*) for in vitro tests or $p< 0.05$ (*) for in vivo tests.

Supplemental References

Mezzanotte, L., Que, I., Kaijzel, E., Branchini, B., Roda, A., Löwik, C. (2011) Sensitive dual color in vivo bioluminescence imaging using a new red codon optimized firefly luciferase and a green click beetle luciferase. PLoS One 6(4).

Zambito, G., Natasa, G., Ridwan, Y., Hall M.P., Shi, C., Kirkland, T.A., Encell, L. P., Löwik, C., Mezzanotte, L. (2020). Evaluating brightness and spectral properties of click beetle and firefly luciferases using luciferin analogues: identification of preferred pairings of luciferase and substrate for in vivo bioluminescence imaging. Molecular Imaging and Biology volume 22, 1523–1531.

Branchini, B.R., Southworth, T.L., Fontaine, D.M., Kohrt, D., Florentine, C.M., Grossel, M.J. (2010). Red-emitting luciferases for bioluminescence reporter and imaging applications. Anal Biochem. 396(2):290-297.

Gammon, S. T., Leevy, W. M., Gross, S., Gokel, G. W. & Piwnica-Worms, D. (2006). Spectral unmixing of multicolored bioluminescence emitted from heterogeneous biological sources. *Anal. Chem.* 78, 1520–1527.