SUPPORTING INFORMATION

Rapid multicomponent bioluminescence imaging via substrate unmixing

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MATERIALS AND METHODS

Reagents

All reagents purchased from commercial suppliers were of analytical grade and used without further purification. 4'-BrLuc, 4'-MorphoLuc, 7'-MorPipLuc, and 7'-DMAMeLuc were prepared and used as previously described.[1-3]

General bioluminescence imaging

Assays were performed in black 96-well plates (Greiner Bio One). Plates were imaged in a light-proof chamber (IVIS Lumina, Xenogen) equipped with a CCD camera (chilled to –90 °C). The stage was kept at 37 °C during imaging experiments, and the camera was controlled using standard Living Image software. Exposure times ranged from 1 s to 5 min, and data binning levels were set to small or medium. Post-acquisition, regions of interest were selected for quantification. Total flux and radiance values were analyzed using Living Image software or ImageJ (NIH).

Bacterial lysate analysis of luciferase mutants

Bacterial cell stocks (stored in glycerol) expressing the mutants of interest were streaked on agar plates containing kanamycin. After overnight growth, colonies were picked and expanded overnight. Portions of the cultures (100 μL) were added to 5 mL of LB (kan) and luciferase expression was induced as described previously in Jones, *et al*.[1] For experiments involving gradients of bacterial lysate, luciferase-expressing bacteria were pelleted and resuspended with 600 μL of lysis buffer (50 mM Tris•HCl, 500 mM NaCl, 0.5% v/v Tween, 5 mM $MgCl_2$, pH = 7.4). In some cases, samples were diluted prior to plating to remain in the linear range of detection.

Substrate unmixing

Substrate unmixing experiments were designed such that a “positive” sample for each enzyme was present in the image to be acquired. For *in vitro* experiments, “positive” wells comprised one enzyme type only.

In mouse experiments, “positive” cells were also included as a reference. Images were acquired as a series following each substrate addition. Thus, an image was generated for each enzyme/substrate pair. Linear unmixing was conducted using ImageJ (installed under the FIJI package). Luminescence images containing the raw CCD counts (as TIFF files) were loaded into FIJI and subjected to a 2-pixel median filter to remove any cosmic noise. Next, the signal at each pixel was min-max scaled to lie between 0 and 65535 (the maximum value that can be stored in a 16-bit image). As a result, the brightest pixel in each image had a value of 65535, and the dimmest had a value of 0. Images were then stacked, and an additional image containing the maximum value of each of the stacked images was computed (as a Z projection). This new image was added to the stack, and signals were unmixed using the ImageJ plugin developed by Gammon, *et al*.[4] In the plugin, regions of interest (ROIs) for each luciferase were drawn around the “pure” areas of the image described above. Each ROI was drawn individually and added to the list by clicking “add.” Once all enzymes were added, “Unmix” was used to unmix the images. Pseudocolors were assigned in FIJI through the “Merge Channels” tool.

Mammalian plasmid construction

Luciferase-expressing DB7 cells were prepared via CRISPR gene insertion. The relevant luciferase genes (luciferase-G4SX2-FP-T2A-Puro) were amplified and inserted into CRISPR AAVS1 donor plasmids (courtesy of Drs. Theresa Loveless and Chang Liu, UCI). Cashew and Pecan inserts were amplified from pET vectors using the following primers:

5’- TGGCTAGCGCTACCGGTCGCCACCTCTAGAATGGAAGACGCCAAAAACATAAAGAAAGG -3’ and

5’- GCGGAAAGATCGCCGTGGGCGGAGGCGGGTCTGGGGGCGGAGGCTCT -3’

Antares inserts were amplified with the following primers:

5’- GCTAGCGCTACCGGTCGCCACCTCTAGAATGCGGGGTTCTCATCATCATCATC -3’ and

5’- TGCCTCTGCCCTCGCCGCTGCCCTCGAGCTTGTACAGCTCGTCCATGCCTCCG -3

Akaluc inserts were amplified with the following primers:

5'- ATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGC -3' and

5'- CACGGCGATCTTGCCGTCCTTCTTGGCCTTAGTGA -3'

Linearized vectors were generated via digestion with restriction enzymes *Xba*I and *Xho*I (New England BioLabs). The linearized vectors were combined with the appropriate luciferase insert by Gibson assembly. A portion of the reactions (3.0 µL) was directly transformed into XL1 competent *E. coli* cells. Sequencing analysis confirmed successful plasmid generation.

Mammalian cell culture and imaging

DB7 cells (courtesy of the Contag laboratory, Stanford) were cultured in DMEM (Corning) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Life Technologies), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were maintained in a 5% CO_2 water-saturated incubator at 37 °C. To create stable lines expressing mutant luciferases, DB7 cells were transfected with the AAVS1 mutant luciferase donor plasmid, Cas9 (Addgene #41815), and AAVS1 sgRNA (Addgene #53370) using lipofectamine. The mutant luciferases were integrated into the first locus of AAVS1 through homologous recombination. Transfected cells were then treated with puromycin (2 µg/mL) and FACS sorted at the Institute for Immunology Flow Cytometry Core (UCI).

DB7 cells stably expressing luciferases were added to black 96-well plates (1 x 10^5 cells per well). Stock solutions of 4'-BrLuc, D-luc, and AkaLumine (10 mM in PBS) were added to each well (100-500 µM final concentration). A solution of furimazine (1:40-1:100 dilution of the commercial stock, Promega) was then added. Sequential imaging was performed as described in the General bioluminescence imaging section (above).

In vivo imaging of orthogonal luciferase-luciferin pairs

Mouse experiments were approved by the UC Irvine Animal Care and Use Committee. FVB/NJ mice (The Jackson Laboratory) received subcutaneous dorsal injections of $1x10^6$ DB7 mutant luciferase expressing cells. After 24 h, animals were anesthetized (1-2% isoflurane) and placed on a warmed (37 °C) stage for imaging. Each mouse received an i.p. injection of luciferin (65 mM, 100 μL per mouse). Images were acquired with 5 min exposure times for 35 min using the General bioluminescence procedure. For sequential imaging, mice were immediately injected with the second substrate and imaged for an additional 35 min. Bioluminescent output was quantified as above.

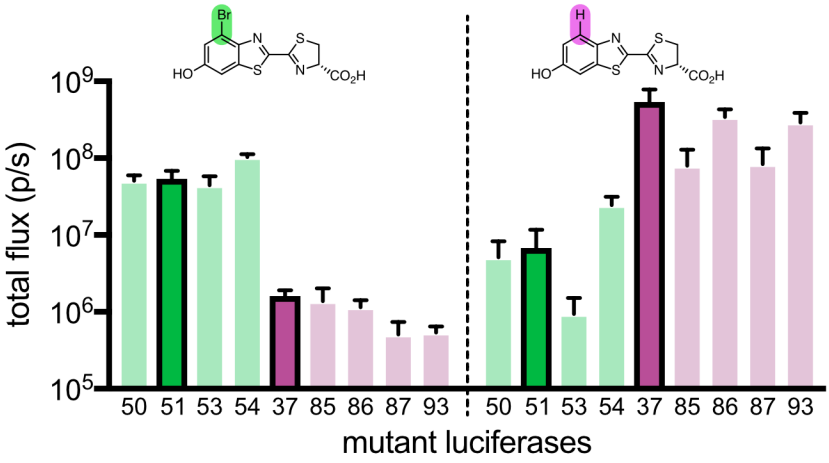

Figure S1. Identifying intensity-resolved orthogonal pairs. Candidate luciferases were expressed in bacteria and screened with 100 μM 4'-BrLuc or D-luc. Mutants exhibited orthogonal substrate use, with >10-fold substrate preference observed in most cases. The "winning combination" – mutants 51 and 37 – were also intensity resolved. Error bars represent the standard error of the mean for *n* = 3 experiments.

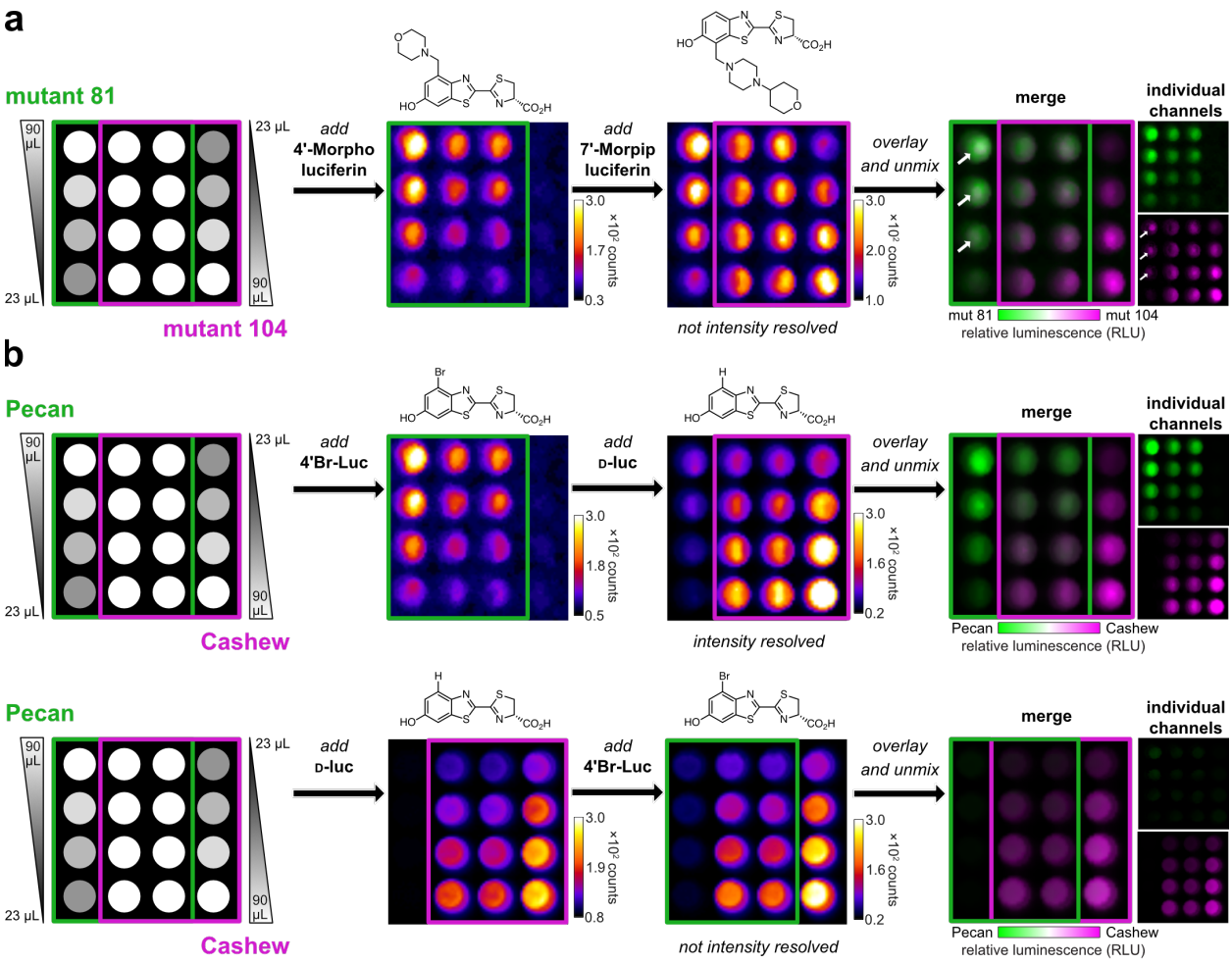

Figure S2. Substrate unmixing requires probes that are intensity resolved. (a) An orthogonal pair comprising mutant 81/4'-Morpho luciferin and mutant 104/7'-Morpip luciferin is not intensity resolved,[1-2] and thus not amenable to rapid BLI. Gradients of the mutants (expressed in bacterial lysate) were plated in a 4x4 matrix. 4'-Morpho luciferin (the preferred substrate for mutant 81) was then administered, followed by 7'-Morpip luciferin (the preferred substrate for mutant 104). Final luciferin concentrations were 100 µM. Substrate unmixing was not successful. Strong residual signal (from 4'-Mopho luciferin) in the 7'-Morpip luciferin image can be observed. The presence of white pixels in the merged image (arrows) is not consistent with the composition of the well (since only one luciferase was present). (b) Gradients of Cashew and Pecan were plated in a 4x4 square. When the dimmer analog (4'-BrLuc) was added prior to the brighter one (D-luc), the signals can be readily unmixed (top). When D-luc was added first, though, the signals cannot be distinguished (bottom). Final luciferin concentrations were 100 µM in each case.

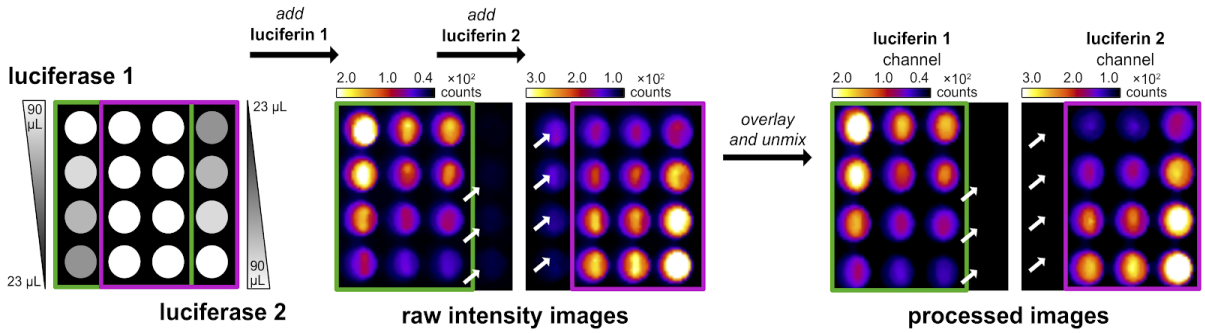

Figure S3. Residual signals are removed by substrate unmixing. Gradients of mutant luciferases in bacterial lysate were plated in a 4x4 matrix. The corresponding luciferins (100 μM) were added sequentially. Pixels containing residual signal are highlighted by the white arrows. These signals are removed upon imaging processing.

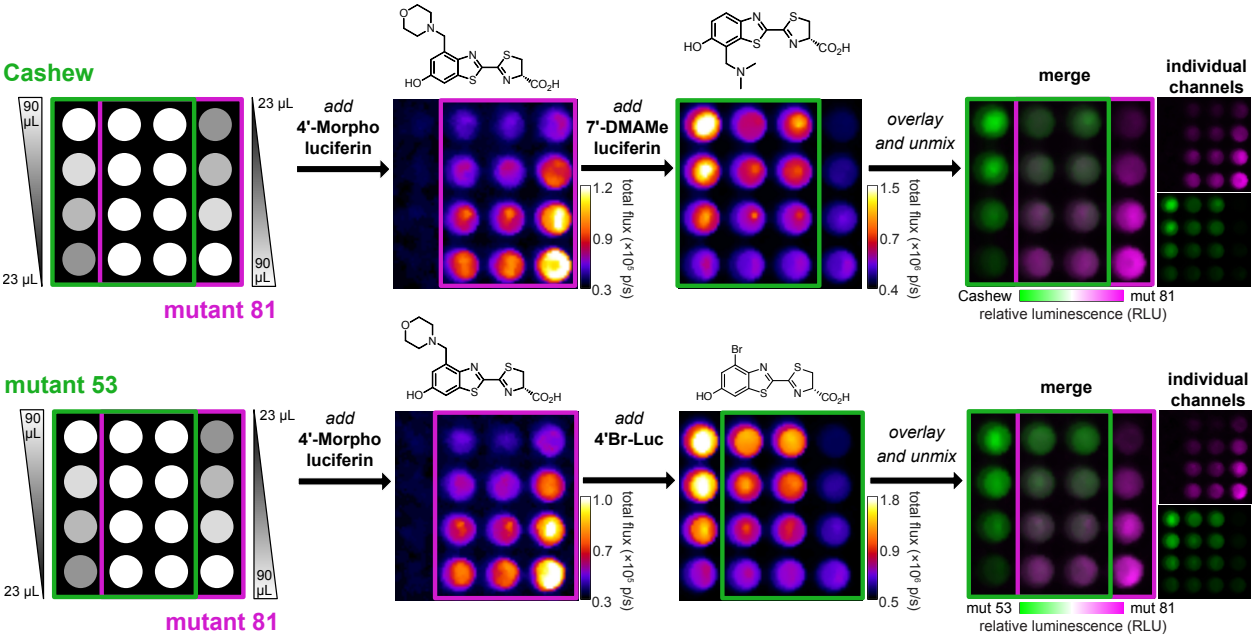

Figure S4. Multiple orthogonal pairs can be rapidly unmixed. Luciferases examined include mutant 81 (and its corresponding substrate 4'-MorphoLuc), Cashew (and its corresponding substrate 7'-DMAMeLuc), and mutant 53 (and its corresponding substrate 4'-BrLuc).[1-2] Gradients of engineered luciferases were plated as shown. The corresponding substrates (100 μM) were administered, beginning with the dimmest luciferin. Images were acquired after each addition. The raw data were stacked and unmixed.

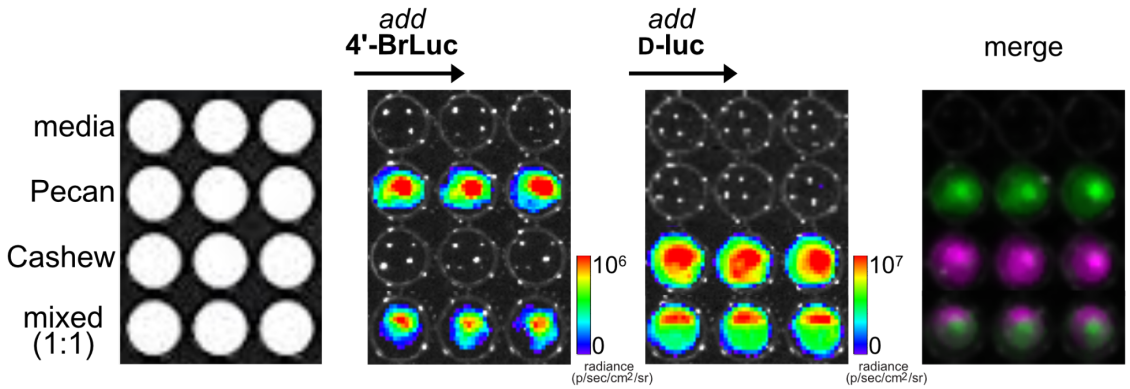

Figure S5. Sequential substrate administration enables multicomponent bioluminescence imaging *in cellulo.* DB7 cells expressing Cashew, Pecan, or no luciferase (media) were plated (1 x 10^5 cells/well). Some wells contained a 1:1 mixture Cashew- or Pecan-expressing cells (5 x 10^4 of each cell type per well). All samples were first treated with 4'-BrLuc, followed by D-luc. Images were acquired after each substrate addition. Raw photon values are shown, along with the merged image following substrate unmixing.

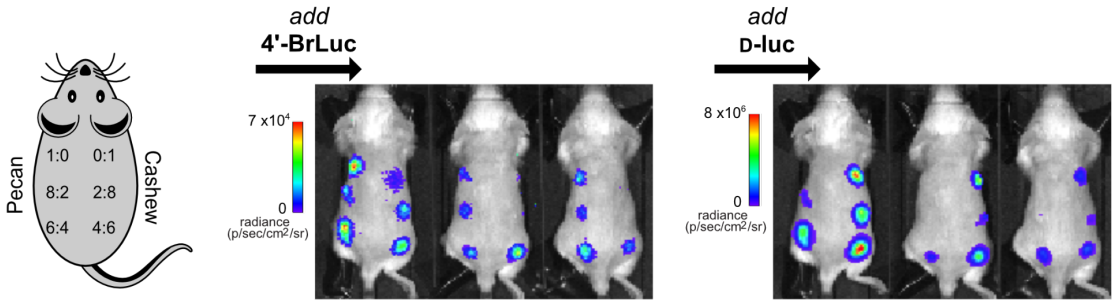

Figure S6. Multicomponent BLI in mouse models. Images used to generate the false colored pictures in Figure 5 are shown. Sequential application of 4'-BrLuc and D-luc enabled different ratios of Pecan- and Cashew-expressing cells to be visualized.

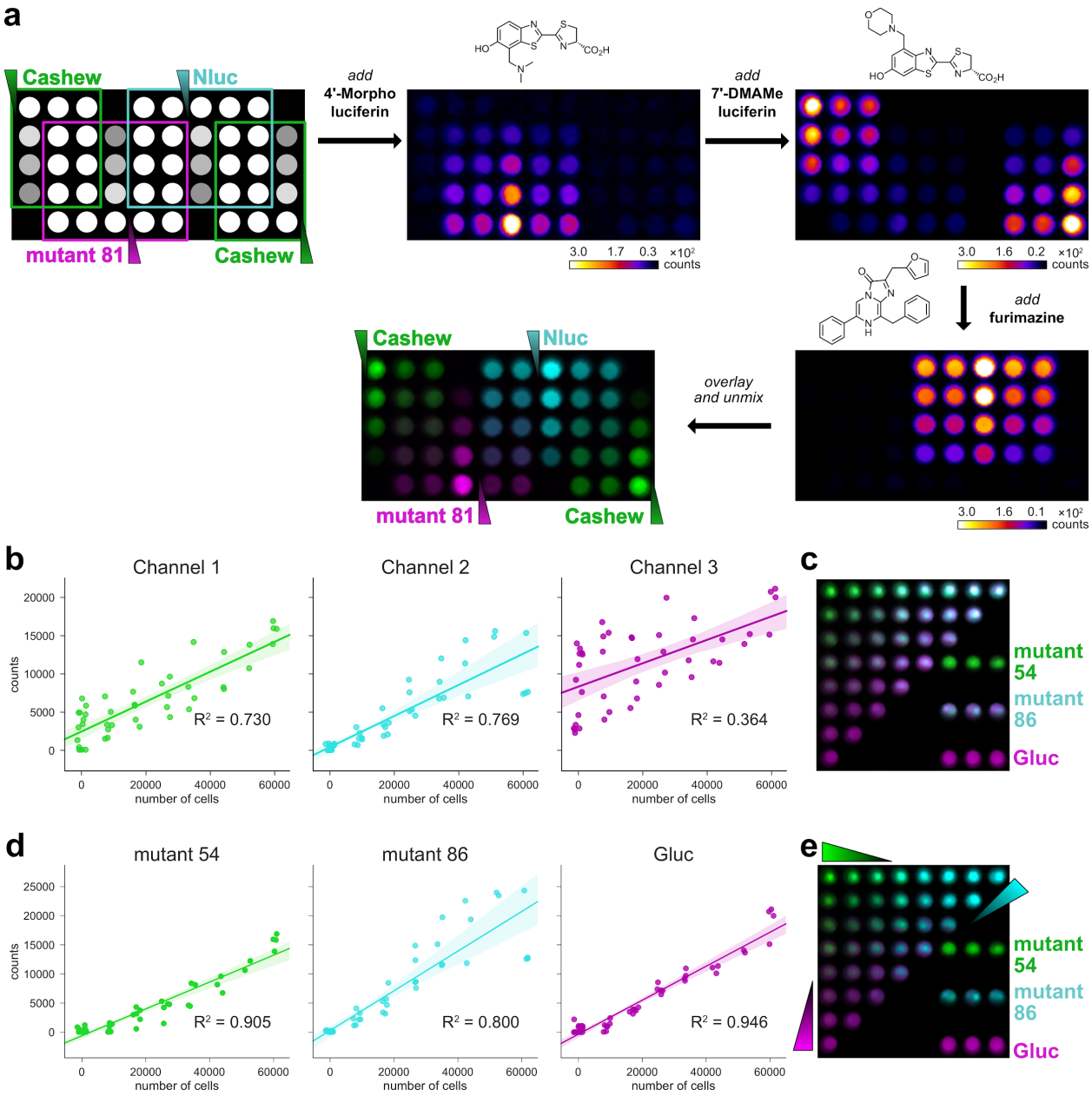

Figure S7. Three orthogonal probes can be distinguished in bacterial lysate and mammalian cells. (a) Gradients of luciferases in bacterial lysate were plated in a 96-well plate. 7'-DMAMeLuc luciferin[1-2] (250 μM), 4'-Morpho luciferin[1-2] (250 μM), furimazine (1:100 dilution of commercial stock) were added in sequence. Images were acquired after each addition, and the raw data were stacked and unmixed. (b-e) Gradients of cells expressing luciferase mutants 51 and 86, or *Gaussia* luciferase were plated in a triangle, with 60,000 cells per well. 4'-BrLuc (500 μM), D-luc (500 μM), and coelenterazine (40 μM) were added in sequence. (b) Quantification of each channel from (c) fit via linear regression. The shaded area represents the 95% confidence interval of the fit. (c) Overlay of raw signal from mixed images. (d) Quantification of each channel from the unmixed image in (e) fit via linear regression. The shaded area represents the 95% confidence interval of the fit. (e) Overlay of the unmixed channels.

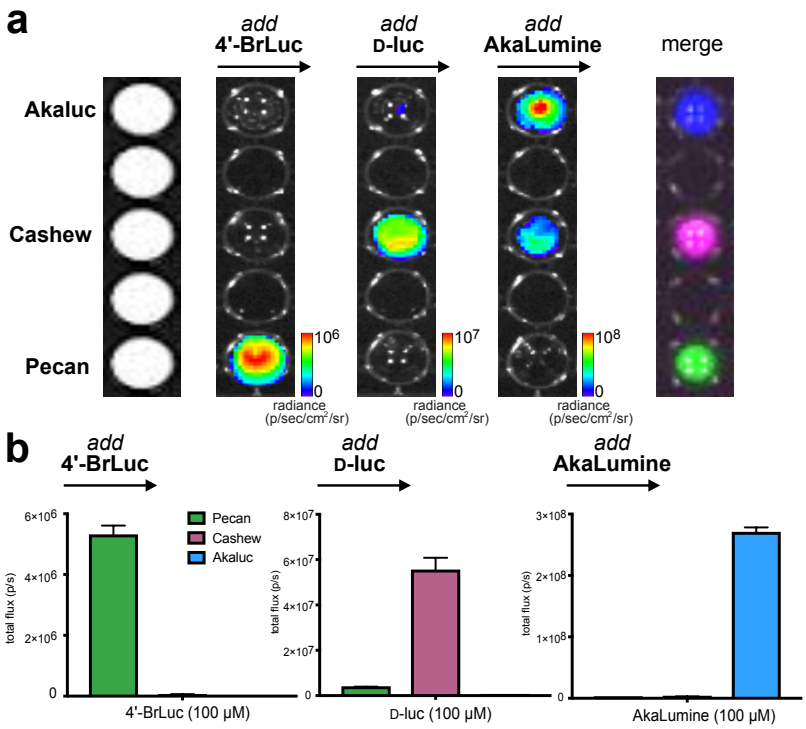

Figure S8. Rapid BLI with three insect-derived luciferases and luciferins. (a) Cells expressing Pecan, Cashew, or Akaluc were seeded in a 96-well plate. Sequential substrate administration (4'-BrLuc, followed by D-luc, then AkaLumine all at 100 μM), and unmixing enabled three-component imaging. Raw images were acquired after each substrate addition. The substrate-specific signals were unmixed, assigned false colors and overlaid. Data are representative of n = 3 replicates. (b) Quantified photon outputs for the images in (a). Error bars represent the standard error of the mean for n = 3 experiments.

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Supplementary Discussion - Spectral Unmixing Algorithm

Linear Unmixing

Spectral unmixing was developed to automate the analysis of images that contain multiple components of overlapping spectra. It is commonly used in fluorescence imaging to deconvolute fluorophores that are not spectrally resolved.[1] Due to resolution constraints, each pixel in a fluorescence image has the chance to contain different fluorophores. A fundamental assumption of linear unmixing is that the signal of each pixel in an image is a linear combination of the absolute contents of that pixel.

$$l_1 = (0.5)\mathrm{GFP} + (0.5)\mathrm{CFP} + (0)\mathrm{mCherry} \tag{1}$$

$$l_2 = (0)\mathrm{GFP} + (0.25)\mathrm{CFP} + (0.75)\mathrm{mCherry} \tag{2}$$

Where l_1 and l_2 represent the fractional composition of individual pixels in an image. These pixels can be represented as vectors where each component of the vector is a different fluorophore.

$$\vec{l_1} = \begin{matrix} \\ \mathrm{GFP} \\ \mathrm{CFP} \\ \mathrm{mCherry} \end{matrix} \begin{bmatrix} 0.5 \\ 0.5 \\ 0 \end{bmatrix} \qquad \vec{l_2} = \begin{matrix} \\ \mathrm{GFP} \\ \mathrm{CFP} \\ \mathrm{mCherry} \end{matrix} \begin{bmatrix} 0 \\ 0.25 \\ 0.75 \end{bmatrix} \tag{3}$$

Each pixel in these fluorescence images is collected with spectral information (with filters, for example). We can represent the raw pixel data as a different vector $\vec{p}$ of signal intensities, i, at each wavelength (or wavelength range) that was measured:

$$\vec{p} = \begin{matrix} \\ \lambda_1 \\ \lambda_2 \\ \lambda_3 \\ \lambda_4 \end{matrix} \begin{bmatrix} i_1 \\ i_2 \\ i_3 \\ i_4 \end{bmatrix} \tag{4}$$

Where λ_1 to λ_4 are measurements at each wavelength, and i_1 to i_4 are intensities measured at each of those wavelengths. Each component of this vector is dependent on the fraction of fluorescent labels that comprise the pixel, $\vec{l}$, and the spectrum of each of those fluorophores, K. Thus, for each pixel in an image:

$$\vec{p} = K * \vec{l} + \mathrm{error} \tag{5}$$

Written out, this would look like:

$$\begin{matrix} \\ \lambda_1 \\ \lambda_2 \\ \lambda_3 \\ \lambda_4 \end{matrix} \begin{bmatrix} \vec{p} \\ i_1 \\ i_2 \\ i_3 \\ i_4 \end{bmatrix} = \begin{matrix} \\ \lambda_1 \\ \lambda_2 \\ \lambda_3 \\ \lambda_4 \end{matrix} \begin{bmatrix} m_1 & m_2 & m_3 \\ r_{11} & r_{12} & r_{13} \\ r_{21} & r_{22} & r_{23} \\ r_{31} & r_{32} & r_{33} \\ r_{41} & r_{42} & r_{43} \end{bmatrix} * \begin{matrix} \\ m_1 \\ m_2 \\ m_3 \end{matrix} \begin{bmatrix} \vec{l} \\ frac_1 \\ frac_2 \\ frac_3 \end{bmatrix} + \mathrm{error} \tag{6}$$

Where m_1 to m_3 are the spectra of the various fluorescent labels that might comprise the pixel, for example, CFP, GFP, and mCherry, and $frac_1$, $frac_2$, and $frac_3$ are the fractional amounts of each of these fluorophores (as in 3). Thus, solving for $\vec{l}$:

$$K^{inv} * \vec{p} = K^{inv} * K * \vec{l} + K^{inv} * \mathrm{error} \tag{7}$$

and rearranging:

$$\vec{l} = K^{inv} * \vec{p} - K^{inv} * \mathrm{error} \tag{8}$$

For an entire image (list of pixels), $\vec{l}$ and $\vec{p}$ become matrices L and P:

$$\begin{matrix} \\ m_1 \\ m_2 \\ m_3 \end{matrix} \begin{bmatrix} \vec{l_1} & \vec{l_2} \\ frac_1 & \dots \\ frac_2 & \dots \\ frac_3 & \dots \end{bmatrix} = K^{inv} * \begin{matrix} \\ \lambda_1 \\ \lambda_2 \\ \lambda_3 \\ \lambda_4 \end{matrix} \begin{bmatrix} \vec{p_1} & \vec{p_2} \\ i_1 & \dots \\ i_2 & \dots \\ i_3 & \dots \\ i_4 & \dots \end{bmatrix} - K^{inv} * \mathrm{error} \tag{9}$$

or

$$L = K^{inv} * P - K^{inv} * \text{error} \tag{10}$$

By arranging the equation in this way, we can measure the spectrum at each pixel, $\vec{p}$ to calculate the fractional makeup of each pixel, $\vec{l}$.

Bioluminescence Imaging (previous work)

Similarly, these concepts can be translated to bioluminescence imaging (BLI). In the case of BLI, the signal from each pixel represents a mixture of the various labeled cell types. Previously, linear unmixing with bioluminescence has been used in a similar fashion to fluorescence imaging; the various components of $\vec{p}$ were wavelengths (or ranges of wavelengths) measured with filters.[2–4]

$$\vec{l} = \begin{matrix} \text{cells}_{\text{CBR}} \\ \text{cells}_{\text{CBG}} \end{matrix} \begin{bmatrix} 0.5 \\ 0.5 \end{bmatrix} \qquad \vec{p} = \begin{matrix} \lambda_1 \\ \lambda_2 \\ \lambda_3 \\ \lambda_4 \end{matrix} \begin{bmatrix} l_1 \\ l_2 \\ l_3 \\ l_4 \end{bmatrix} \tag{11}$$

Where $\text{cells}_{\text{CBR}}$ and $\text{cells}_{\text{CBG}}$ are cells expressing click beetle red luciferase and click beetle green luciferase respectively, and λ_1 to λ_4 are various filters used by the *in vivo* imaging system.

Substrate-resolved Bioluminescence (this work)

This work utilizes the same basic algorithm (equation 8), and (in a similar fashion as equation 11) defines the components of $\vec{l}$ as cells labeled with various luciferase mutants. The major difference from all other applications of linear unmixing is that the wavelengths of $\vec{p}$ have been replaced with various luciferin substrates. Thus, instead of taking images at a variety of wavelengths, we are imaging after the addition of each luciferin substrate.

$$\vec{l} = \begin{matrix} \text{cells}_{\text{p}} \\ \text{cells}_{\text{c}} \\ \text{cells}_{\text{n}} \end{matrix} \begin{bmatrix} 0.4 \\ 0.4 \\ 0.2 \end{bmatrix} \qquad \vec{p} = \begin{matrix} \text{luciferin}_{\text{Br}} \\ \text{luciferin}_{\text{Dl}} \\ \text{luciferin}_{\text{frz}} \end{matrix} \begin{bmatrix} l_1 \\ l_2 \\ l_3 \end{bmatrix} \tag{12}$$

Where cells_{p}, cells_{c}, and cells_{n} are cells expressing pecan, cashew, and nanoluc respectively, and $\text{luciferin}_{\text{Br}}$, $\text{luciferin}_{\text{Dl}}$, and $\text{luciferin}_{\text{frz}}$ are the substrates 4'-BrLuc, D-Luc, and furimazine respectively. Thus, we can rewrite equation 9 as:

$$\begin{matrix} \\ \text{cells}_{\text{mut1}} \\ \text{cells}_{\text{mut2}} \\ \text{cells}_{\text{mut3}} \end{matrix} \begin{matrix} \vec{l_1} & \vec{l_2} \\ \end{matrix} \begin{bmatrix} frac_1 & ... \\ frac_2 & ... \\ frac_3 & ... \end{bmatrix} = K^{inv} * \begin{matrix} \text{luciferin}_1 \\ \text{luciferin}_2 \\ \text{luciferin}_3 \\ \text{luciferin}_4 \end{matrix} \begin{matrix} \vec{p_1} & \vec{p_2} \\ \end{matrix} \begin{bmatrix} i_1 & ... \\ i_2 & ... \\ i_3 & ... \\ i_4 & ... \end{bmatrix} - K^{inv} * \text{error} \tag{13}$$

In this implementation, K can be determined by measuring the response of each mutant individually across the sequential addition of luciferin substrates:

$$K = \begin{matrix} \\ \text{cells}_{\text{p}} \\ \text{cells}_{\text{c}} \\ \text{cells}_{\text{n}} \end{matrix} \begin{matrix} \text{luciferin}_{\text{Br}} & \text{luciferin}_{\text{Dl}} & \text{luciferin}_{\text{frz}} \end{matrix} \begin{bmatrix} i_{\text{p,Br}} & i_{\text{p,Dl}} & i_{\text{p,frz}} \\ i_{\text{c,Br}} & i_{\text{c,Dl}} & i_{\text{c,frz}} \\ i_{\text{n,Br}} & i_{\text{n,Dl}} & i_{\text{n,frz}} \end{bmatrix} \tag{14}$$

In practice, finding K is a matter of including calibration sites in the image where each mutant is segregated, and receives each compound in the same manner as the rest of the experiment (just as if filters were being applied to calibration wells to determine spectral response).

Intensity Resolution

The technique described herein for multicomponent bioluminescence imaging via sequential substrate administration also relies on *intensity* resolution amongst the substrates. In fact, we have found that for the success of the technique, intensity resolution is just as important as substrate resolution. We define intensity resolution as the difference in brightness between two probes. Probes that are intensity resolved enable sequential administration in a single imaging session because the highest possible signal in the probe that is administered first minimally overlaps with the lowest possible signal in the second probe that is administered. If the three probes, p, are administered in sequence (1–3), the total signal intensity, I, from the contributions of each individual probe, i, following each addition can be illustrated as follows:

$$\xrightarrow{p_1} I = i_1 + e_1 \xrightarrow{p_2} I = i_1 + i_2 + e_2 \xrightarrow{p_3} I = i_1 + i_2 + i_3 + e_3 \tag{15}$$

Where e is the random error associated with each image acquired. In order to be able to resolve all the individual signals, it is necessary that:

$$i_1 << i_2 << i_3 \tag{16}$$

When the intensity resolutions (differences) between each probe are large enough that the signal of the previous probe is similar to the error, the signals can be resolved. Written out:

$$\begin{aligned} i_1 &\approx e_2 \\ i_2 &\approx e_3 \end{aligned} \tag{17}$$

This enables us to eliminate the residual intensity terms from equation 15 to give:

$$\begin{aligned} p_1 &: I = i_1 + e_1 \\ p_2 &: I = i_1 + e_2 \\ p_3 &: I = i_1 + e_3 \end{aligned} \tag{18}$$

Any systematic error that is contributed by the previous probe can be eliminated through linear unmixing, described above. Large amounts of intensity overlaps cannot be solved by unmixing, however. If the relationship in the above equation (16) is not true, we risk not having enough information to resolve the individual signals.

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