Single-cell bioluminescence imaging of deep tissue in freely moving animals

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Bioluminescence is a natural light source based on luciferase catalysis of its substrate luciferin. We performed directed evolution on firefly luciferase using a red-shifted and highly deliverable luciferin analog to establish AkaBLI, an all-engineered bioluminescence in vivo imaging system. AkaBLI produced emissions in vivo that were brighter by a factor of 100 to 1000 than conventional systems, allowing noninvasive visualization of single cells deep inside freely moving animals. Single tumorigenic cells trapped in the mouse lung vasculature could be visualized. In the mouse brain, genetic labeling with neural activity sensors allowed tracking of small clusters of hippocampal neurons activated by novel environments. In a marmoset, we recorded video-rate bioluminescence from neurons in the striatum, a deep brain area, for more than 1 year. AkaBLI is therefore a bioengineered light source to spur unprecedented scientific, medical, and industrial applications.

Bioluminescence imaging (BLI) is based on the detection of light produced by the enzyme (luciferase)–catalyzed oxidation reaction of a substrate (luciferin) (1, 2). In vivo BLI is a noninvasive method for measuring light output from luciferase-expressing cells after luciferin administration in living animals (3), and this method typically employs firefly luciferase (Fluc) and the natural substrate D-luciferin (Fig. 1A, left) that produces longer-wavelength (green-yellow) light and is more stable for enzymatic reaction after administration than the other commonly used luciferase substrate, coelenterazine (4–7). However, due to its relatively low tissue permeability, D-luciferin has a heterogeneous biodistribution in the body (8). The low affinity (high Michaelis constant, $K_M$) of D-luciferin for Fluc also suggests un- even saturation of the Fluc reporter enzyme with substrate in vivo. In particular, in vivo BLI in the brain has been hampered due to low passage of D-luciferin through the blood-brain barrier (BBB) (8). In recent years, synthetic analogs of D-luciferin were reported (9–11), including AkaLumine (Fig. 1A, right), that when catalyzed by Fluc produces near-infrared emission peaking at 677 nm, which can penetrate most animal tissues and bodies. We previously demonstrated that AkaLumine hydrochloride (AkaLumine-HCl) has favorable biodistribution to access Fluc-expressing cells in deep organs such as the lung and can saturate Fluc more effectively than D-luciferin (12).

We hypothesized that Fluc is not enzymatically optimal for AkaLumine-HCl; therefore, we performed directed evolution on the luciferase gene through successive rounds of mutagenesis, screening, and validation to develop an enzyme that could strongly pair with AkaLumine-HCl. We constructed gene libraries encoding variants of AkaLumine (23) and screened them by selecting for high $K_M$ value for Fluc and the poor tissue permeability of AkaLumine-HCl. AkaLumine-HCl was injected into mice, a dose previously shown to provide a saturating concentration in vivo of AkaLumine-HCl (12).

We localized luciferase expression deep inside the mouse body by two approaches, (1) cell implantation in the vasculature and (2) viral transduction in the brain. In the former approach, HeLa/Akaluc or HeLa/Fluc cells were transplanted. We injected 10$^5$ cells into the tail vein. Using this protocol, the majority of intravenously injected cells are initially trapped in the small capillaries of the lung (14, 15); thus, we imaged the upper part of an anesthetized animal 10 min after cell injection and immediately after intraperitoneal substrate administration. Under these conditions, the AkaLumine-HCl/Akaluc combination yielded a 52 ± 0.5-fold stronger signal than D-luciferin/Fluc (Fig. 1D).

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In the second approach, we imaged bioluminescence from the striatum, a group of contiguous subcortical structures deep in the brain involved in motor control and other functions. Because access of D-luciferin to the brain is limited by the BBB (8) but AkaLumine-HCl is tissue-permeant (22), we expected that brain imaging should benefit from AkaLumine-HCl/Akaluc. We used an adeno-associated virus (AAV)-based tetracycline (TET)-inducible system (10) for expression of Akaluc or Fluc (fig. S9) in the striatum. Two weeks after viral infection, mouse heads were comparatively imaged after substrate administration (intraperitoneal). First, the activity of stratially expressed Fluc was examined with different substrates, indicating that AkaLumine-HCl was more accessible to the brain than another reported synthetic analog CycLuc1 (30), as well as D-luciferin (fig. S10). In a pair of mice, strong and faint bioluminescence signals were observed for the AkaLumine-HCl/Akaluc and D-luciferin/Fluc combinations, respectively (Fig. 1E, top). We verified that a 100-µl solution of 30 mM AkaLumine-HCl was sufficient for saturating stratially expressed Akaluc (fig. S11) and that both the AkaLumine-HCl/Akaluc and D-luciferin/Fluc signals developed in a similar sustained pattern, peaking around 10 to 20 min after substrate administration (intraperitoneal) (fig. S12). Statistical analysis revealed that AkaLumine-HCl/Akaluc yielded a 1408 ± 375-fold stronger signal than D-luciferin/Fluc (Fig. 1E, middle, fig. S10), which is a more prominent improvement than that observed for pulmonary localization (Fig. 1D).

The all-engineered BLI system composed of AkaLumine-HCl and Akaluc is hereafter referred to as AkaBLI. AkaBLI with intravenous administration allowed the monitoring of brain striatal bioluminescence at video rate in a freely moving mouse for >1 h (Fig. 1E, bottom right; fig. S13; and movie S1), demonstrating the practical applicability of AkaBLI for studying naturally behaving animals. The same measurement was not possible with D-luciferin/Fluc (Fig. 1E, bottom left). We also monitored the signal development of striatal Akaluc via three major systemic administration routes (fig. S8). We found that the maximal intensity was higher in the order of intravenous, intraperitoneal, and oral administration and that intravenous gave the same temporal profile as intraperitoneal, whereas oral gave the most persistent bioluminescence (fig. S14). In a trial experiment, after 1 day of water deprivation we gave the mouse ad libitum oral access to an AkaLumine-HCl solution and recorded striatal AkaBLI. About 5 min after several fluid intakes, a substantial amount of bioluminescence became apparent on the head and was monitored at video rate for more than 1 h (fig. S15 and movie S2). Such painless and voluntary self-administration of substrate to awake animals will be useful for BLI experiments under natural conditions and to assess sensitive behavioral changes.

Based on the bright bioluminescence signals from HeLa/Akaluc cells in the mouse lung (Fig. 1D), we examined the cell sensitivity of AkaBLI as a quantitative method to examine pulmonary cell trapping by titrating down the number of injected HeLa/Akaluc cells. By observation of Venus fluorescence, we prepared solutions that contained 1, 2, 3, or 10 HeLa/Akaluc cells (fig. S16). Twelve mice were injected with a 1-cell-containing solution (nos. 1 to 12). A focal bioluminescent signal was observed over the upper back of mice 6 and 9 (Fig. 2, 1 cell), presumably derived from the one HeLa/Akaluc cell trapped
in the lung. No signal was detected from the other 10 mice, suggesting efficient pulmonary passage of single cells. Likewise, mice injected with 2, 3, and 10 cells were imaged. Bioluminescence signals were observed in 5 of 9 mice given the 2-cell injection (Fig. 2, 2 cells) and in 9 of 10 mice given the 3-cell injection (Fig. 2, 3 cells), and all of them appeared as single foci. The 10-cell injection produced a focal fission in mouse 32 and single foci in mice 33, 35, 36, 37 were injected with 1, 2, 3, and 10 cells, respectively. (A) Representative images with substantial bioluminescent signals. Mouse sample numbers are indicated inside. The color bars indicate the total bioluminescence radiance (photons/sec/cm²/sr). (B) Bioluminescence was quantified for each mouse and plotted. Numbers of samples with a significant amount of signal are underlined. The bioluminescent signals (mean ± SEM) of the underlined samples are plotted as a function of cell number (inset).

Fig. 2. Analysis of single-cell and sparse-cell AkaBLI of implanted tumorigenic cells trapped in the mouse lung. Detection (all-or-nothing) of bioluminescence from a small number of Akaluc-expressing HeLa cultured cells trapped in the mouse lung. An anesthetized mouse was intravenously injected with the indicated number of cells and 30 min later with 100 μl of AkaLumine-HCl (30 mM). Bioluminescence images were acquired using a cooled CCD camera (1-min exposure time). Mouse samples nos. 1 to 12, 13 to 21, 22 to 31, and 32 to 37 were injected with 1, 2, 3, and 10 cells, respectively. (A) Representative images with substantial bioluminescent signals. Mouse sample numbers are indicated inside. The color bars indicate the total bioluminescence radiance (photons/sec/cm²/sr). (B) Bioluminescence was quantified for each mouse and plotted. Numbers of samples with a significant amount of signal are underlined. The bioluminescent signals (mean ± SEM) of the underlined samples are plotted as a function of cell number (inset).
Fig. 3. Noninvasive monitoring of neuronal ensemble responses to novel environments in mouse hippocampal CA1 using AkaBLI. (A and B) c-fos-tTA mice were injected with AAV-TRE-Venus-Akaluc (A) targeting the right hippocampal CA1 region. (B) While off-Dox, exposure to a novel environment induces c-fos-dependent expression of tTA, which binds to the TRE and drives the expression of Venus-Akaluc, labeling a subpopulation of activated neurons. (C) Experimental setup to study brain responses to two novel environments: a white, square styrofoam box with an alcohol odor (#1), and a blue, round plastic bucket with an acetic acid odor (#3). The familiar context was a home cage (#2). Shown below is the experimental scheme containing three consecutive observation epochs: 1 to 3. BLI: Mice were anesthetized, administered (intraperitoneally) with AkaLumine-HCl (75 nmol/g body weight), and then imaged using a cooled CCD camera (5-min exposure time). Depletion was performed during the on-Dox period (green shade). (D and E) BLI data of a representative mouse, which showed a novel-context dependent increase in the bioluminescence signal. R.S.I., relative signal intensity. BLI time points correspond to BLI symbols shown in (C). Photographs of the first, second, and third BLIs during observation 1 [i, ii, and iii, respectively (D)] with bright-field image (BF) are shown (E). The color bar indicates the total bioluminescence counts/5 min. (F and G) Three-dimensional reconstructions of infected neurons with Venus expression (green) in a cleared brain sample stained for nucleic acid with SYTO 61 (red). Maximum projection images of the region containing the cortex (CX) and the hippocampal CA1. A magnified micrograph (G) (80-μm volume, 0.5-μm step size) corresponding to the box shown in (F) (910-μm volume, 6.1-μm step size). Scale bars, 100 μm. (H and I) Statistical analysis of BLI data. Bioluminescence signals after novel exposure (red) compared with those taken 1 day earlier (gray) and >4 day later with Dox (black) (H). Bioluminescence signals after novel exposure (red) compared with those after home cage return (blue) (I). Data are presented as mean ± SEM (n = 6 mice). *P < 0.05, **P < 0.01. Significance was calculated by means of two-sided paired t tests.
production for research, industry, and medical applications.

REFERENCES AND NOTES


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Author contributions: S.I. and A.M. conceived and designed the whole study. S.I. performed all the experiments and analyzed the data. M.S. analyzed Fluc mutants in cultured cells. S.S. performed gene construction. H. Hama designed and performed histological experiments. K.N. acquired light-sheet microscopy images. T.F. studied the temporospatial organization of Fluc expression. T.K. and S.K.-K. performed AkaBLI experiments of pulmonary tumor cells. M.T., Y.I., and H. Hioki performed AkaBLI experiments with the marmoset. T.K. and S.K.-K. performed AkaBLI experiments of pulmonary tumor cells. M.T., Y.I., and H. Hioki performed AkaBLI experiments with the marmoset.

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Improved spy tactics for single cells

Bioluminescence imaging is a tremendous asset to medical research, providing a way to monitor living cells noninvasively within their natural environments. Advances in imaging methods allow researchers to measure tumor growth, visualize developmental processes, and track cell-cell interactions. Yet technical limitations exist, and it is difficult to image deep tissues or detect low cell numbers in vivo. Iwano et al. designed a bioluminescence imaging system that produces brighter emission by up to a factor of 1000 compared with conventional technology (see the Perspective by Nasu and Campbell). Individual tumor cells were successfully visualized in the lungs of mice. Small numbers of striatal neurons were detected in the brains of naturally behaving marmosets. The ability of the substrate to cross the blood-brain barrier should provide important opportunities for neuroscience research.

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