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# Highly Sensitive Eight-Channel Light Sensing System for Biomedical Applications

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**Running title:** BBI system

## Abstract

We demonstrate the potential of an eight-channel light sensing platform system, named Black Box I (BBI), for rapid and highly sensitive measurement of low-level light using a nonradioactive optical readout. We developed, normalized, and characterized the photon sensitivities of the eight channels of the BBI using placental alkaline phosphatase (PLAP) as a model imaging reporter. We found that the BBI system had a statistically strong linear correlation with the reference IVIS Lumina II system. When we applied normalization constants, we were able to optimize the photomultiplier tubes (PMT) of all eight channels of the BBI (up to $r^2 = 0.998$). We investigated the biomedical utilities of BBI by: (i) determining alkaline phosphatase activities in mouse plasma samples as a diagnostic secretory biomarker of cancer, and (ii) diagnosing cancer metastases in the organs of mice bearing triple negative breast cancer. We provide an important new addition to low-cost biomedical instruments intended for pre-clinical diagnostic imaging with high sensitivity, high sample throughput, portability, and rapid on-site analysis of low-level light.


**Key words:** 8-channel, luminescence, metastasis, high sensitivity, photomultiplier tubes (PMTs), on-site analysis, Black Box I (BBI)

## Introduction

The photo-electric effect was first discovered by Elster *et al.,* in 1889 [1], but the photomultiplier tube (PMT) was invented only in the mid-1930s. PMT revolutionized the power of optical devices to measure very fast and low-level light (LLL) signals [2]. Because of the unique instrumental advantages of PMTs vis-à-vis light sensitivity and response time, they have been utilized in a wide range of instrumentations such as luminometers, positron emission tomography (PET), gamma cameras, and others [3 4 5].

Many biomedical imaging modalities have degrees of risk when using radioisotopes (e.g., PET, SPECT) or strong magnetic fields (e.g., MRI, MRS) [6]. These instruments are usually large, expensive, and inappropriate for most *in vitro* medical biopsy applications, including cancer biopsies, and pre-clinical small animal studies. The unique advantages of PMTs in optical instrumentation over other modalities include low cost, high sensitivity to LLL, portability, and fast sample throughput.

In this study, we developed a new 8-channel light sensing platform system for determining nonradioactive optical readouts, termed Black Box I (BBI), which consists of: (i) an 8-channel sample stage with a mirror cap, (ii) light control units, (iii) a photon counter assembly, (iv) a power supply, and (v) a controlling system with a personal computer (PC). The 8-channel sample stage is designed to cover a mirror cap with

slantingly bored holes at the side (Figure 1), where the pitches between the 8 channels are designed to exactly fit to the light detection window on the sample stage. The photon counter assembly carries 8 PMTs, discriminators, photon counters, and the controllers. The assembly is finally connected to a personal computer (PC) and controlled with the specific software (IM8 V00-00C) via a USB interface cable.

We first characterized the photon sensitivities among eight PMTs in BBI using placental alkaline phosphatase (PLAP) as a model chemiluminescent light standard, and then normalized by establishing a linear dose-response using a known amount of PLAP. We observed a very strong correlation between the relative luminescence unit (RLU) values of BBI and total flux (p/s) values of an IVIS Lumina II system (simplified to IVIS system hereafter, PerkinElmer) in logarithmic scales, that is, $\log_{10}$ [RLU/s of BBI]= $\boldsymbol{m} \times \log_{10}$ [p/s of IVIS system] + $\boldsymbol{b}$, where the constants $\boldsymbol{m}$ and $\boldsymbol{b}$ represent the unique photon sensitivity of each PMT. Therefore, the PMT channels of the BBI system are highly optimized to provide surrogate measurements for light production (total flux or radiance), which is commonly quantified using the IVIS system (Figure 2, Suppl. Figure 1). The biomedical usefulness of the BBI system was further examined through rapidly investigating the secreted alkaline phosphatase (SEAP) levels in mouse plasma samples as a model biomarker for determining cancer metastases in the tissues of a

mouse model bearing syngeneic triple negative breast cancer (Figure 3).

The present BBI system is an important addition to biomedical instrumentations and diagnostic platforms that are of low cost, high sensitivity to very fast LLL, have high sample throughput, and are compact and suitable for on-site analysis.

## Experimental Methods

### Chemicals and samples

Phosphate buffered saline (PBS) was obtained from Gibco (Thermo Scientific, Waltham, MA). Great EscAPe SEAP Chemiluminescence Kit 2.0 was purchased from Takara Bio (Mountain View, CA), which includes Placental alkaline phosphatase (PLAP) and its specific substrate. Female nude mice (nu/nu) of 4-6 weeks old were purchased from Charles River (Newark, NJ). D-luciferin was purchased from BIOSYNTH International Inc. (IL, USA). The 4T1 triple negative breast cancer cells stably expressing firefly luciferase (FLuc) was used from our previous study [7].

### Assembly of optical and electric parts for constructing Black Box I (BBI)

We designed and made in-house all the metal parts including the sample stage and aluminum mirror cap at the National Institute of Advanced Industrial Science and

Technology (AIST, Tsukuba, Japan). The channels of the mirror cap were carved to have the same pitch as those of an 8-strip PCR tube and light-conduction windows on the sample stage. We also specially designed the light-proof black plastic case for the BBI, which was custom made by Kyoto Jusi (Kyoto, Japan). The following electric parts were obtained from the providers: light conduction path (TOKYO BOX Co., Ltd), shutter (Fujita Electric Works, Ltd), discriminator (Texas Instruments Japan Ltd), amplifier (Analog Devices KK), pulse sharpener (Toshiba Electronic Devices & Storage Co.), counter (Texas Instruments Japan Ltd), and microcomputer (Microchip Technology Japan K.K). The PMTs were purchased from Hamamatsu Photonics (Hamamatsu, Japan), and order-assembled by Nishihara Co. (Kashiwa, Japan). The assembled BBI system was connected to a personal computer (PC) via a USB interface cable, and controlled with specific software (IM8 V00-00C), which was custom developed by Nishihara Co. (Kashiwa, Japan). All the parts were finally assembled, and the fidelity was confirmed with the PC.

**Normalization of the light sensitivities of the integrated eight PMT channels in BBI**

The relative light sensitivity of eight PMT channels in the BBI system was correlated with the IVIS system for each channel through the following procedure (Figure 2B and

Suppl. Figure 1). Before measurements, the BBI system was placed inside the chamber of the IVIS system and connected to the outer controlling PC via a USB cable as shown in Figure 2A. The PLAP standard stock solution (100 μg/mL) from the Great EscAPe SEAP Chemiluminescence Kit 2.0 (Clontech, Mountain View, CA) was diluted in the provided dilution buffer to three final concentrations of 20, 5, and 1 μg/mL. An aliquot (10 μL) of the diluted PLAP solutions was transferred to an 8-lane PCR strip and simultaneously mixed with an aliquot (10 μL) of the provided substrate using an 8-channel micropipette (Gilson), and immediately mounted on the sample stage of the BBI system inside the chamber of the IVIS system. The corresponding optical intensities were simultaneously determined over time using both BBI and IVIS systems with an acquisition time of 2 min, generating a range of signal intensities. Through triplicate measurements, we obtained 168 data points in total (3 PLAP concentrations × 8 channels × 7 replicates/concentration/channel). The statistical parameters correlating the data from the BBI and IVIS systems were analyzed using Prism 8.1 (GraphPad).

**Determination of SEAP activities in mouse plasma using BBI**

A range of SEAP samples in mouse plasma was collected as model biomedical samples for an *in vivo* secreted biomarker, as described before[8]. Their activities were

independently determined using a single-tube luminometer assay to confirm the linear correlation between BBI and IVIS data sets (Figure 2C, Suppl. Figure 2, Suppl. Figure 3). In brief, mice were injected with two different concentrations of SEAP (High: 36 μg/kg, or Low: 200 ng/kg) intravenously (*i.v.*) and the plasma samples were collected by submandibular bleeding at various time points starting 1 min post-injection for up to 10 days, for assessing the plasma half-life of SEAP [8]. The plasma samples were stored at -80˚C until assayed.

Prior to measurement, the plasma samples (n = 8) were thawed on ice, and vortexed. Twenty μl of each plasma sample were mixed with 80 μl of the dilution buffer and heat-inactivated by incubating at 65°C for 30 min. The samples were cooled on ice for 2-3 min and equilibrated to room temperature, and then used for subsequent experiments.

The main body of the BBI system was placed in the dark chamber of the IVIS system as stated above, and connected to the outer controlling PC via a USB interface cable. A 10 μl sample aliquot was transferred to the 8-lane PCR tubes under two different scenarios: 1) each tube contained the same plasma sample (multiple replicates); or 2) each tube contained a different plasma sample. An aliquot of the substrate solution (10 μl) was then simultaneously injected into the 8-lane PCR tubes (Cases 1 and 2) using an

8-channel micropipette (Gilson). Then the PCR tube was immediately mounted on the sample stage of the BBI system, and the corresponding optical intensities were simultaneously determined at 1, 3, 15, 30, 37, and 40 min after substrate addition using both BBI and IVIS systems (Suppl. Figure 3). The dataset from scenario 1 was analyzed to confirm the validity of the established correlation equation (Suppl. Figure 3A), while the dataset from scenario 2 was used for evaluating the correlations between the instruments (Figure 2C, Suppl. Figure 2B and 3B).

In parallel, the SEAP activities of all samples were determined with a conventional single-tube luminometer (Turner Biosystems 20/20n). The SEAP standard curve was firstly obtained by serial dilution of stock solution using a SEAP positive control, as described before [8] in 10 µl of healthy mouse plasma, where no background SEAP activity was expected ([SEAP] = 0). The linear range of the luminometer signal vs. SEAP concentration in plasma was used to determine the linear correlation between luminometer readings and SEAP concentrations, and to convert signal intensities (relative light units, RLU) to concentrations (ng/ml). For the measurement of the SEAP activities in the plasma samples, 10 µl of diluted and heat-inactivated sample was mixed with 10 µl of the substrate solution and vortexed briefly, the light signals were measured using a 10 sec integration time in a single-tube luminometer at two time points (Suppl.

Figure 2AB): 0 and 36 min after substrate solution was added. All the datasets obtained from the same SEAP plasma samples were plotted in Figure 2C and Suppl. Figure 2B.

We also simultaneously determined the SEAP concentration in 2 blinded plasma samples using the BBI system, since the BBI system can accommodate 8 samples simultaneously (Suppl. Figure 4). Two unknown SEAP serum samples (S1 and S2) were prepared with the same procedure as that shown in Figure 2C, while two standard samples (STD1 and STD2) were prepared from the PLAP stock included in the Great EscAPe SEAP Chemiluminescence Kit 2.0 (Takara Bio). A 10μL aliquot of each standard and unknown sample was transferred to the 8-lane PCR tubes and simultaneously mixed with 10 μL of substrate solution (n = 2). The corresponding optical intensities were simultaneously determined with the BBI and IVIS systems.

**Diagnosis of metastases in mice bearing xenografts of triple negative syngeneic breast cancer using the BBI system**

For metastasis diagnosis, we implanted 4 mice with 4T1 triple negative breast cancer cells in the flanks of nude mice and developed syngeneic tumors (Figure 3BC, Suppl. Figure 5). Two million 4T1 cells stably expressing firefly luciferase (FLuc) were subcutaneously (*s.c.*) implanted on either flank of the hind limbs of living nude mice

(*nu/nu*) on day 0. The mice were then kept for two weeks until the tumors metastasized, i.e., when the primary tumor size had reached 500 to 750 $mm^3$. Before sacrificing the mice, we imaged the implanted tumors using *in vivo* (FLuc) imaging by injecting 3 mg of substrate D-Luc in 100 μl of PBS. The detailed experimental procedure is specified in the Suppl. Methods section.

For metastasis diagnosis, the first 3 mice were sacrificed, and their extracted organs (brain, kidney, thymus, heart, spleen, liver, and lung tissues) were imaged ex vivo. The organ tissues were immersed in PBS buffer for 1 h and then their weights were recorded. The tissues were then transferred to an 8-well microstrip. Oversized organ tissue samples were further dissected using a surgical knife to accommodate them into the wells of the microstrip. The microstrip was then simultaneously injected with 100 μL of the specific D-luciferin substrate solution (30 mg/mL) using an 8-channel micropipette, and mounted on the sample stage of the BBI inside IVIS system. The corresponding optical intensities were simultaneously determined using both BBI and IVIS systems, where the optical images were collected with different integration times, such as 30, 10, 5, 2, 1, 0.5, or 0.1 sec.

We further determined the detection limits of the BBI, IVIS, and Lago (Spectral Instruments Imaging) systems for metastases in organ tissues (Suppl. Figure 5B). Organ

tissues extracted from the last (fourth) mouse, and prepared with the same method, were bathed in a D-Luciferin solution (100 μg/mL) on a rectangular Petri dish and the optical intensities were determined using the Lago system (Spectral Instruments Imaging). The tissues were then moved to an 8-well microstrip. The optical intensities of the microstrip were simultaneously determined with the BBI and IVIS systems, immediately after injection of the specific substrate solution (D-Luciferin, 100 μg/mL).

## Results and Discussion

### The BBI system has a strong linear correlation with the IVIS Lumina II system (PerkinElmer) in terms of light sensitivity

To investigate the correlation between the datasets from BBI and IVIS Lumina II systems, 168 data points were obtained and statistically analyzed (Figure 2A, Suppl. Figure 1). The statistical analysis using Prism 8.1 revealed that the correlation coefficient between the two systems was high ($r^2$ = 0.996) (Figure 2B, Left). The analysis also suggested that the correlation between the two datasets can be expressed as $\log_{10}$ [RLU/s of BBI] = $\boldsymbol{m} \times \log_{10}$ [photon/s of IVIS] + $\boldsymbol{b}$, where the constants $\boldsymbol{m}$ and $\boldsymbol{b}$ represent the fitted slope ($m$) and y-intercept ($b$) parameters of each PMT as shown in Suppl. Figure 1. The imaging results showed very high correlation between the BBI

and IVIS systems. Upon analysis of the correlation between the dataset from each channel of the BBI system and that of IVIS system, the average $r^2$ was found to be 0.998.

The sensitivity and measurement correlations were further confirmed with an independent experiment using a series of SEAP plasma samples (Suppl. Figure 3A). The optical intensities from 8-equivalent SEAP plasma samples in an 8-lane PCR tube were simultaneously determined using the 8 channels of the BBI and IVIS systems. We then repeated the same experiment 8 times with varying levels of SEAP in plasma samples. –The results showed that every channel in the BBI system could accurately reproduce the signal achieved with the IVIS system (Suppl. Figure 1A).

The overall results confirmed that: (i) the datasets from the BBI and IVIS systems demonstrate an extremely high level of linear correlation ($r^2$=0.998), (ii) the excellent linear correlation suggests that the RLU counts per second of the BBI system can be converted into more common units of light flux (photons/s) on the basis of the linear correlation function established for each channel.

**The BBI system can determine the SEAP activities in plasma samples, the results of which show a high correlation with those of conventional systems**

The utility of the BBI system for biomedical diagnoses was further validated by measuring biomarker levels in mouse plasma samples. The results were compared with those of the other conventional instrumentations (Figure 2C, Suppl. Figure 2C).

First, the optical intensities of eight different concentrations of plasma SEAP samples were simultaneously monitored with the BBI and IVIS systems. The time-lapse data acquisitions showed that the optical intensities reached a signal plateau at around 37 min (Suppl. Figure 3B). The light signal achieved by the BBI system exhibited a high correlation ($r^2$ = 0.998) with that of the IVIS system at 40 min (Figure 2C). An independent evaluation of SEAP levels using a conventional single-tube luminometer also showed a high correlation ($r^2$ = 0.990) with that of BBI. The 3-dimensional plot from the three datasets also suggested a strong linear trendline (Suppl. Figure 2C).

Second, we determined the SEAP levels in unknown plasma samples using the BBI system (Suppl. Figure 4). We firstly prepared two unknown SEAP plasma samples with the same method as shown in Figure 2C, and simultaneously determined the activity levels using two-point calibration with two different concentrations of SEAP standard solutions from the Great EscAPe SEAP Chemiluminescence Kit 2.0 (Takara Bio) using varying light integration times. The results indicated that the SDs of the dataset from the BBI system was approximately 12-fold smaller than those from the IVIS system on

average: i.e., 0.02 vs. 0.26, respectively. Importantly, measurements achieved using the BBI system were more precise (lower variability) than those obtained using the IVIS system, since the SDs of BBI were less affected by signal integration time.

All the optical imaging systems measure volume independent light output if the sample is in the homogeneous liquid form and the light emission surface is uniformly placed against the photomultiplier (PMT) tube. We are not expecting any volume dependent variation in our experiments since the samples were compared within the same experiment maintained at a constant volume. The volume is crucial only when the cells expressing the reporters are adherent and liquid in various depth is placed in between the PMT and the source of light output due to attenuation factor. In addition, in our 8-channel system we measured light emitted from different samples simultaneously that were uniformly positioned against PMTs in multiple wells at a constant volume, hence we are not expecting any variation in the optical output.

**Metastases in mouse organ tissues can be determined with high sensitivity**

We used 4T1 syngeneic tumors in nude mice (n = 4) for our metastasis study. The animals were sacrificed, and various tissues were used for diagnosis of metastasis using the BBI and IVIS systems (Figure 3B-D).

Mouse 1 had no metastasis in the investigated organs. On the other hand, Mice 2 and 3 had metastasis in multiple organs. Mouse 2 was found to carry metastasis in the brain, thymus, and lungs. Interestingly, Mouse 3 had metastasis in the heart, spleen, and lungs. The correlation coefficient obtained via the BBI and IVIS systems was $r^2$= 0.70 for 23 tissue samples analyzed, which was much lower than those observed with SEAP plasma samples. This is possibly owing to the heterogeneity of the biopsy tissue samples, which show variable depth of metastasis within the organs. This may cause confounding variability owing to distinctive light attenuation levels and variability upon photon acquisition, as illustrated in Figure 3C, Inset '***a***'.

The detection limits of the BBI system to tissue biopsies were further investigated with Mouse 3, which is prepared as stated above with respect to the reduced light integration time (Suppl. Figure 5A). The results indicated that the BBI system can sensitively determine the optical intensities even at 0.1 sec of light integration, whereas the IVIS system reported the optical values with relatively higher SDs at the integration time of approximately less than 10 sec.

The organ metastases of Mouse 4 were further imaged to compare the detection limits of BBI, Lago (Spectral Instrumentations Imaging), and the IVIS systems (Suppl. Figure 5B). The results showed that, although the Lago and IVIS systems did not detect a

considerable load of metastases from the organ tissues, the BBI system clearly reported that the liver and lungs showed malignancy in the organs. Considering metastasis is a challenging clinical problem and the cause of most cancer related deaths[9], these results support the notion that the BBI system provides a technological advance, allowing sensitive and high-throughput analysis of organ tissue biopsies, especially for fast LLL optical signals that have been even below the detection limits of conventional biomedical imaging systems available for small animal models currently.

## Conclusions

We demonstrate the efficiency of the developed 8-channel light sensing platform system, named Black Box I (BBI), for simultaneous determination of eight nonradioactive optical readouts with rapid and portable on-site capability. The BBI system also shows a strong linear correlation with conventional biomedical imaging instrumentations including the IVIS in vivo imaging system and the single-tube luminometer system. It can even be used to determine early-stage metastases emitting LLL in intact organ tissues *ex vivo*. Because the BBI system simultaneously monitors eight different biomedical samples emitting very fast LLL, it can be applicable to various creative experimental setups such as lab-on-chip, flow-batch light monitoring,

and on-site analysis of nonradioactive optical readouts. Our BBI system is an important addition to biomedical instrumentations that determine LLL signals with high sensitivity, high sample throughput, low cost, and rapid on-site analysis. The simultaneous light sensing modality of the BBI system also enables many other applications including lab-on-chip and flow-batch analysis of nonradioactive optical readouts in biomedical samples.

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# Figures

**Figure 1**. Schematic diagram of the electronics of the Black Box I (BBI) for simultaneously sensing of light from multiple samples. The mirror cap is designed to embed an 8-strip PCR tube or a 6-channel microslide on the sample stage. Light signal from each sample is reflected by a mirror cap and collected to the light-conduction window of the sample stage. Inset ***'a'*** shows the images of mirror caps that are designed to accommodate 8-lane PCR tubes or 6-channel microslides (**1**-**3**). Inset ***'b'*** shows the images of a plastic light-conduction path (**4**), PMTs (**5**), and a circuit board for amplifier and counter (**6**). After all assembly, the BBI appears as shown (**7**).

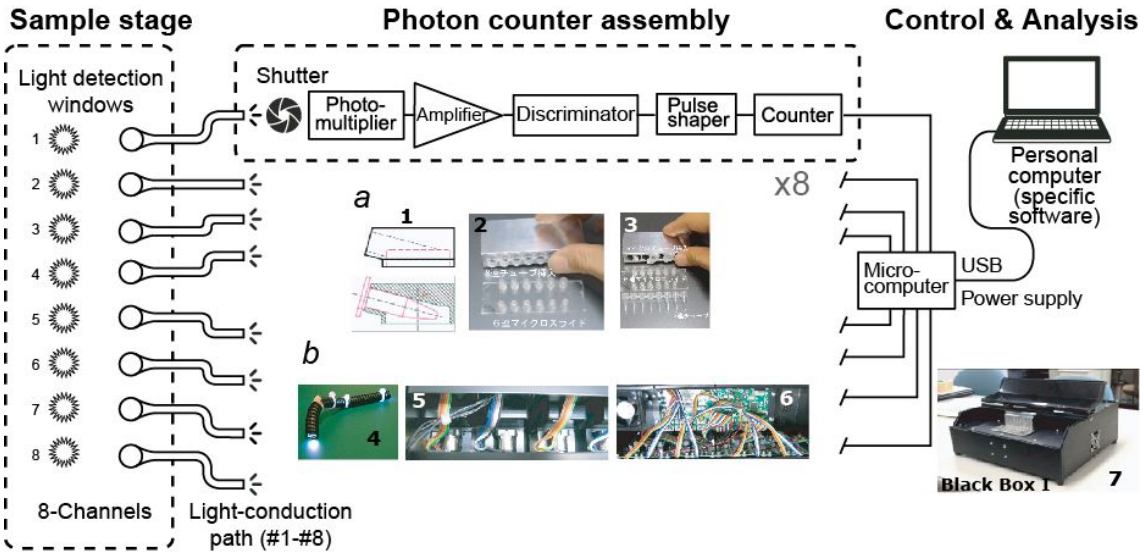


**Figure 2**. Linear correlation of the dataset of the BBI systems with those of conventional systems. (A) Experimental setup of the instrumentations. BBI system was placed inside the chamber of the IVIS Lumina II system for simultaneous determination of light production from samples. The BBI system in the IVIS chamber was controlled by the outer PC through a USB interface cable. Inset ***'a'*** shows the optical images of 8-lane PCR tubes with three different sample concentrations. (B) Normalization of the light sensitivities of 8 PMTs of the BBI. The specific light sensitivity of each PMT of the BBI system was rigorously evaluated with 168 standard placental alkaline phosphatase (PLAP) samples. The correlation between the BBI and IVIS systems was found to be ($\log_{10}$[RLU/s of BBI] = $m$ × $\log_{10}$[photons/s of IVIS] + $b$). The correlation function was applied to normalization of the dataset of the BBI system. (C) Correlation of the optical intensities to SEAP samples by BBI and the other instrumentations. The datasets from BBI system and single-tube luminometer (Turner Biosystems 20/20n) were plotted on the $y$- and $x$-axis, respectively. The number by each data point indicates the photon counts (p/s) of each plasma sample, as determined by the IVIS system (n = 3 replicates per sample).

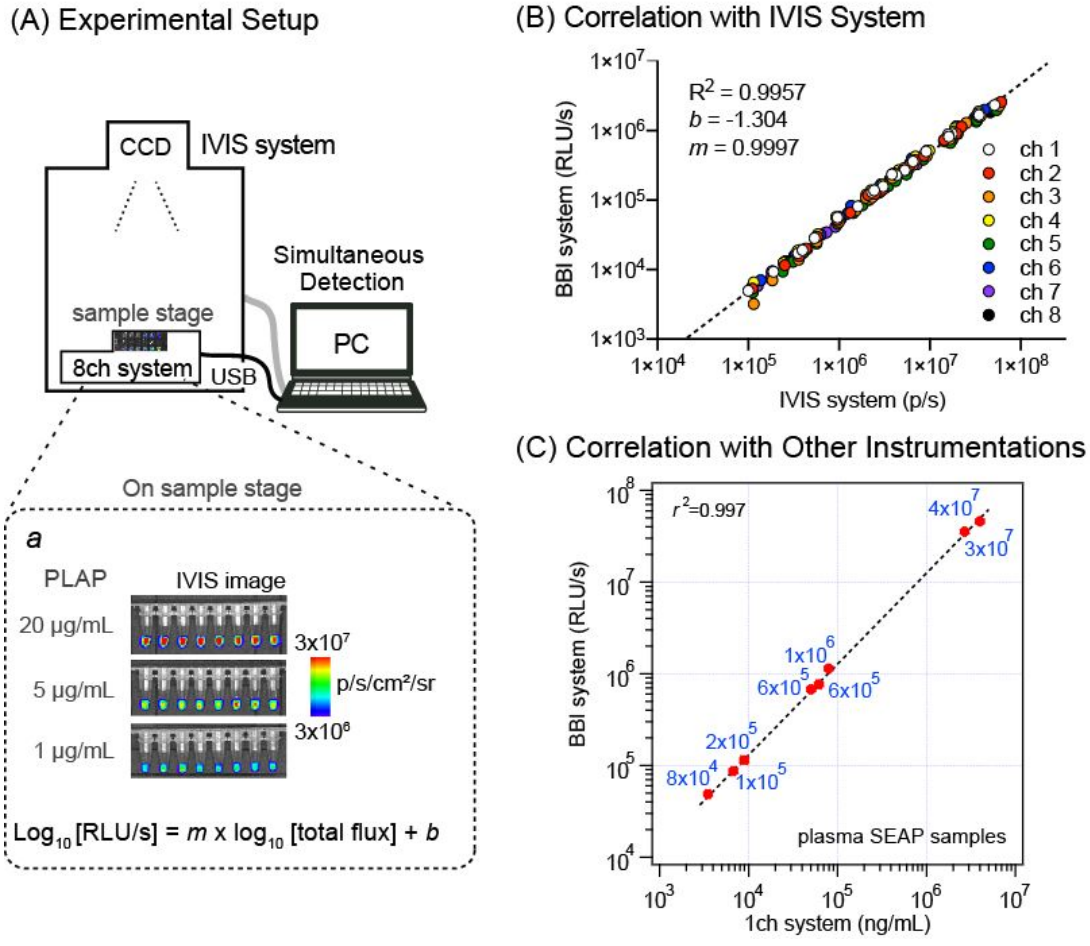
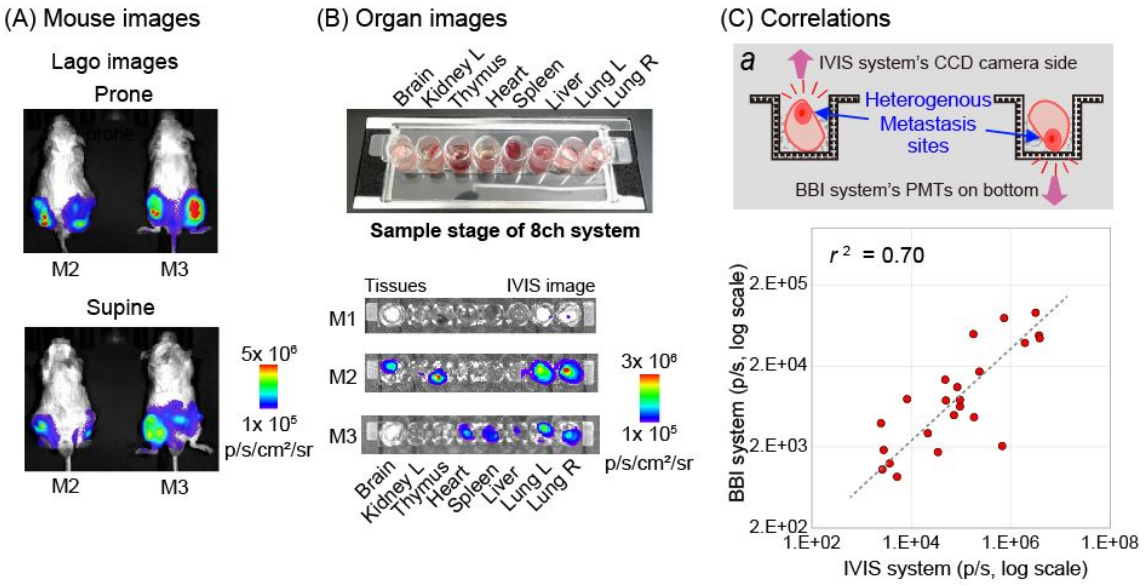


**Figure 3.** Metastasis analysis of mouse tissue xenografts. (A) Mouse images showing tumor xenografts. The mice were imaged with conventional bioluminescence imaging to visualize metastasis. (B) Simultaneous determination of metastases in mouse organ tissues. The organ metastases expressing FLuc were determined with the BBI and IVIS systems (PerkinElmer). (C) Correlation of the optical intensities by the BBI and IVIS systems. The photon count values were normalized by integration time (s). Inset '***a***' illustrates the heterogeneous metastasis features of the xenografts in organs. Abbreviations: M1, M2, and M3 indicate Mice 1, 2, and 3.



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