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Jack K. Tung  
Ken Berglund  
Claire-Anne Gutekunst  
Ute Hochgeschwender  
Robert E. Gross

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Jack K. Tung,<sup>a,b</sup> Ken Berglund,<sup>b</sup> Claire-Anne Gutekunst,<sup>b</sup> Ute Hochgeschwender,<sup>c</sup> and Robert E. Gross<sup>a,b,\*</sup>

<sup>a</sup>Georgia Institute of Technology, Coulter Department of Biomedical Engineering, 313 Ferst Drive, Room 2127, Atlanta, Georgia 30332, United States

<sup>b</sup>Emory University, Department of Neurosurgery, 101 Woodruff Circle, WMRB Rm 6337, Atlanta, Georgia 30322, United States

<sup>c</sup>Central Michigan University, College of Medicine and Neuroscience Program, Department of Neuroscience, 1280 S. East Campus Street, Mt. Pleasant, Michigan 48859, United States

**Abstract.** The use of bioluminescent reporters in neuroscience research continues to grow at a rapid pace as their applications and unique advantages over conventional fluorescent reporters become more appreciated. Here, we describe practical methods and principles for detecting and imaging bioluminescence from live cells and animals. We systematically tested various components of our conventional fluorescence microscope to optimize it for long-term bioluminescence imaging. High-resolution bioluminescence images from live neurons were obtained with our microscope setup, which could be continuously captured for several hours with no signs of phototoxicity. Bioluminescence from the mouse brain was also imaged noninvasively through the intact skull with a conventional luminescence imager. These methods demonstrate how bioluminescence can be routinely detected and measured from live cells and animals in a cost-effective way with common reagents and equipment. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: [10.1117/1.NPh.3.2.025001](https://doi.org/10.1117/1.NPh.3.2.025001)]

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## 1 Introduction

Conventional optical approaches for live cell imaging have generally relied on the use of various fluorescent proteins or synthetic molecules. These imaging techniques typically require an exogenous light source to excite the fluorescent molecules, where they enter a higher-energy state and subsequently emit light of a specific wavelength as they return to their ground state.<sup>1</sup> Fluorescent molecules have proven to be exquisitely versatile reporters for live cell imaging because they span a broad spectrum of colors and can be detected with very high spatial and temporal resolution. Many important neuroscience questions regarding cellular anatomic structures, neuronal circuitry, molecular interactions, brain dynamics, and brain pathology have been addressed with the use of fluorescent molecules.

In contrast to fluorescence, bioluminescence is light generated from a chemical substrate and is routinely demonstrated in nature by various bioluminescent marine species, arthropods, fungi, and bacteria.<sup>2</sup> These organisms generate light via an enzymatic reaction, in which a chemical substrate (e.g., luciferin) is oxidized by an enzyme (e.g., luciferase).<sup>2,3</sup> Bioluminescence is therefore produced without any excitation light source and persists as long as the substrate is present. A variety of bioluminescent proteins spanning a broad spectrum of colors and emission properties have been identified and their genes cloned; the major ones used in neuroscience research are summarized in Table 1.

Bioluminescence imaging differs from fluorescent readouts in several aspects that, depending on the specific application, can be advantageous or disadvantageous. One major advantage of fluorescent molecules is that they can be far brighter than bioluminescent proteins;<sup>27</sup> they can be made brighter by simply increasing the amount of excitation light, whereas bioluminescence intensity is strictly limited by the number of

substrate molecules being catalyzed by the luciferase. Due to the relative dimness of bioluminescent proteins, longer exposure times are generally needed to collect a number of photons comparable to that of a fluorescence molecule. Bioluminescence imaging therefore generally has a limited temporal resolution compared to that of fluorescence imaging.<sup>27</sup>

On the other hand, several unique properties of bioluminescence make it an attractive imaging modality. First, bioluminescent signals generally have a higher signal-to-noise ratio (SNR). This is due to the fact that background luminescence is negligible compared to the signal produced from the luciferase reaction.<sup>28</sup> Bioluminescent signals can therefore be much more sensitive than fluorescent signals, which generally have to compete with background auto-fluorescence.<sup>29</sup> Second, bioluminescence does not require excitation light, eliminating the risk of photobleaching and phototoxicity that is associated with fluorescence imaging.<sup>30</sup> Bioluminescent signals are therefore well suited for live cell imaging and can be recorded for much longer timescales compared to fluorescent signals without damaging reporter molecules or cells. Lastly, since bioluminescence requires no exogenous excitation light sources, it is a suitable optical readout for imaging light-sensitive cells such as retinal neurons.

Luciferase proteins have undergone significant evolution in their versatility as genetically encoded reporters for neuroscience research. Similar to their fluorescent counterparts, luciferase proteins can be targeted to specific regions in the cell with the use of trafficking or localization signal sequences to allow for imaging of subcellular structures over time.<sup>15</sup> The concept of fluorescence resonance energy transfer has also been translated to bioluminescent proteins to measure molecular interactions. In this instantiation, both the intensity and spectral properties of bioluminescent proteins are altered when they are associated with fluorescent proteins in a process termed bioluminescence resonance energy transfer.<sup>31–33</sup> Luciferase proteins have also

\*Address all correspondence to: Robert E. Gross, E-mail: [rgross@emory.edu](mailto:rgross@emory.edu)

**Table 1** Summary of major bioluminescent proteins used in neuroscience research.

	Protein	Species	Emission peak	Mg, ATP?	Substrate	References
Luciferases	FLuc	Firefly	560	Y	D-Luciferin	4–14
	VLuc	<i>Cypridina noctiluca</i>	460	Y	Vargulin luciferin	5
	RLuc	<i>Renilla reniformis</i>	480	N	Coelenterazine	8,15–17
	GLuc	<i>Gaussia princeps</i>	480	N	Coelenterazine	5,18,–21
Photoproteins	Aequorin	<i>Aequorea victoria</i>	470	N	Coelenterazine	22–26

been engineered to respond to small molecules such as calcium and ATP,<sup>15</sup> allowing them to be used for measuring changes in cellular dynamics such as neuronal activity. Protein engineering techniques have led to the development of brighter and longer wavelength luciferases that are well suited for *in vivo* imaging.<sup>34–38</sup>

Given the expanding toolbox of bioluminescent proteins and their wide variety of applications (see reviews by Badr and Tannous<sup>39</sup> and Saito and Nagai<sup>40</sup>), it is timely to look at methods of detection of bioluminescence in laboratories not necessarily set up for bioluminescence imaging *per se* or unwilling to purchase commercially available bioluminescence imagers (e.g., Olympus LV200 microscope, IVIS Spectrum animal imager). In this paper, we discuss advantages and disadvantages of the various methods we have utilized for detection and quantification of bioluminescent signals from live cells and animals.

## 2 Materials and Methods

### 2.1 Preparation of Coelenterazine Substrate

The substrate for *Renilla*- and *Gaussia*-based luciferases, coelenterazine (CTZ), is typically dissolved in nonpolar solvents such as ethanol or methanol. These solvents are not ideal for live cell imaging due to their inherent toxicity. We therefore recommend solubilizing CTZ in aqueous solution with the help of inert chemical agents such as  $\beta$ -cyclodextrin as described by Teranishi and Shimomura<sup>41</sup> or by utilizing commercially available solvents (e.g., Fuel-Inject from Nanolight Technology). Solutions of CTZ can be made at any desired concentration, although we routinely use a stock concentration of 600  $\mu$ M for *in vitro* use. After solubilization, CTZ can be aliquoted and stored at  $-20^{\circ}\text{C}$  for several months. Since the amount of CTZ needed for *in vivo* applications is relatively higher, we freshly prepare CTZ before each use following the manufacturer's recommendations. It is important to note that CTZ should be protected from light to prevent auto-oxidation.

### 2.2 Cell Culture and Transfection

HEK293 cells were passaged regularly in Dulbecco's Modified Eagle Medium (with 10% FBS, 1% penicillin/streptomycin) and seeded to 90% confluency on glass cover slips the day before transfection. HEK cells were transfected with expression vectors encoding membrane-localized *Renilla* luciferase<sup>16</sup> and membrane-localized *Gaussia* luciferase protein<sup>18</sup> using Lipofectamine 2000 (Invitrogen). Transgene expression was confirmed by fluorescence microscopy the following day.

Dissociated cortical neuron cultures were derived from E18 rat embryos. Cortical tissue was digested with 2 mg/mL papain

and dissociated by mechanical trituration before seeding onto 18-mm diameter German glass coverslips coated with 50  $\mu$ g/mL poly-D-lysine (Sigma). Neuronal cultures were grown in serum-free Neurobasal media (w/1 $\times$  B27 supplement, 0.5 mM glutamine) and media was changed (half-volume) every 3 to 4 days. Cortical neuron cultures were transduced with viral vectors encoding luciferase 2 to 3 days after plating.

### 2.3 Live Cell Bioluminescence Imaging

Bioluminescence images were taken on an Olympus inverted fluorescence microscope equipped with a variety of objectives, c-mount adaptors, and cameras (Table 2).

Cells cultured on glass coverslips were transferred to a perfusion chamber (Warner Instruments) containing phenol-free media at the time of imaging to minimize light absorption and maximize transmission of bioluminescence through the media (this is especially important for upright microscopes). The cells were then checked under fluorescence to confirm transgene expression and determine the right depth of focus for bioluminescence imaging. The microscope was then switched to an empty filter position to collect whole-spectrum bioluminescence.

Images were collected using the open-source Micromanager image acquisition software. Camera settings were standardized and optimized by cooling the chip to the lowest temperature (to minimize dark current) and maximizing the gain. Binning was used only when it was necessary to produce visible images. All of the background light sources in the room (windows, doors,

**Table 2** Objectives, adaptors, and cameras tested.

Objectives	Adaptors	Cameras
PlanApo 60 $\times$ oil, 1.4NA	0.5 $\times$	Coolsnap-ES CCD (Photometrics)
UPlanFI 40 $\times$ oil, 1.3NA	0.3 $\times$	Coolsnap-FX CCD (Photometrics)
LUMPlanFLN W 60 $\times$ , 1.0NA		ImagEM C9100-13 EMCCD (Hamamatsu)
UPlanSApo 20 $\times$ , 0.75NA		iXon Ultra 897 EMCCD (Andor)
LucPlanFL 60 $\times$ , 0.7NA		QuantEM EMCCD (Photometrics)
		OptiMOS sCMOS (Photometrics)

and electronics) were covered with blackout material and images were collected at various exposure times (1 to 40 s). Long-term bioluminescence images were acquired using the multiacquisition feature of Micromanager and a perfusion system to deliver CTZ during the imaging period (up to 3 h). Imaging was done under controlled conditions that included using the same batch of transfected cells, CTZ concentration, media, exposure times, and imaging conditions to reduce variability between experiments. We did not attempt to normalize bioluminescence signals between different sets of experiments, although we employed the same camera settings (i.e., exposure time and binning) for a given combination of the microscope, camera, objective lens, and luciferase used. Thus, the bioluminescence signal was comparable within a given set of the experiments.

For the comparison of camera models in Fig. 3, bioluminescence imaging was repeated using an upright microscope (Eclipse E600-FN, Nikon) and a water-immersion objective lens (40 $\times$ , NA 0.8). CTZ was applied to the same batch of HEK cells transiently transfected with a membrane-bound *Gaussia* luciferase cultured on glass coverslips. Images were taken every 5 s with exposure time of 4.5 s and CTZ (100  $\mu$ M) was bath-applied 50 s after the start of an imaging session. The camera models compared were Photometrics CoolSNAP ES [noncooled scientific charge-coupled device (CCD); digitization: 12 bit; gain 0.3 $\times$ ], Photometrics CoolSNAP fx (cooled scientific CCD; digitization: 12 bit; gain 2 $\times$ ), Hamamatsu ImagEM C9100-13 (EMCCD; digitization: 16 bit; EM gain: 1200 $\times$ ), and Andor iXon Ultra 897 (EMCCD; digitization: 16 bit; EM gain 1000 $\times$ ). Each camera was binned to roughly 256  $\times$  256 pixels, resulting in a similar pixel size of 25.8  $\mu$ m (CoolSNAP ES), 26.8  $\mu$ m (CoolSNAP fx), 32  $\mu$ m (ImagEM), and 32  $\mu$ m (iXon Ultra 897).

Image quality was estimated by calculating the SNR of captured images at the peak luminescence time point. SNR was calculated by dividing the mean pixel intensity of the bioluminescence image to the standard deviation of the pixel intensity from a background image (or region with no cells) in ImageJ.

## 2.4 In Vivo Bioluminescence Imaging

For imaging the mouse brain, an adeno-associated virus (AAV) encoding *Renilla luciferase* (RLuc) was injected into the cortex (−1.58 AP, −0.75 ML, −0.8 SI, and −1.58 AP, −1.75 ML, −0.5 SI) of 5- to 6-week old CD1 white mice (Harlan). After 2 weeks, the fur was shaved off the head and the animals were anesthetized with ketamine/xylazine before bioluminescence imaging. 500  $\mu$ g of CTZ was administered either intraperitoneally or intravenously (via tail vein injection). Bioluminescence images were captured with the animals still under ketamine/xylazine anesthesia using a conventional luminescence imager (Fuji LAS-3000). Sequential images of 20 s exposure time were captured and the total signal intensity of each image was quantified in ImageJ to achieve a bioluminescence signal time course. Images at the time of maximum signal intensity are displayed showing the relative bioluminescence signal pseudocolored by a RGB lookup table in ImageJ. All procedures were performed in accordance with the US National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at Emory University.

## 3 Results and Discussion

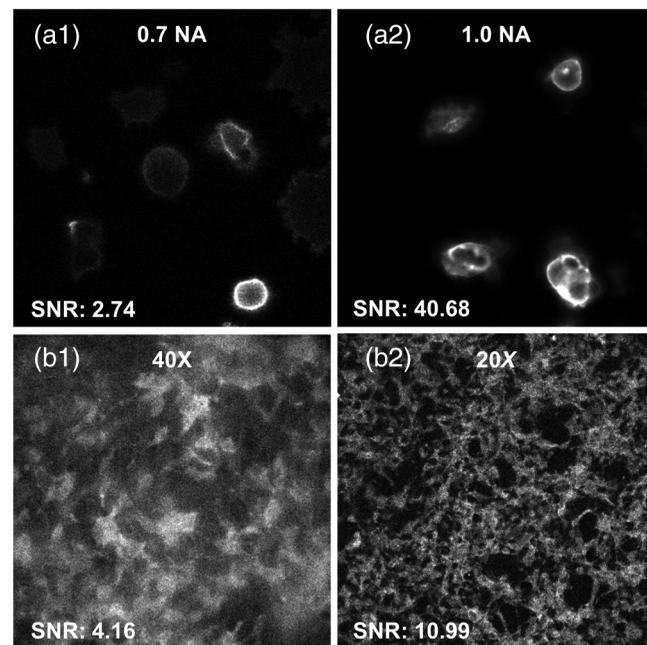
### 3.1 Live Cell Bioluminescence Imaging

Image brightness is directly related to the light-gathering power of the objective (numerical aperture, NA) and inversely related to the image magnification (M): brightness  $\propto$  (NA/M).<sup>2</sup> We have therefore maximized the image brightness of our samples by optimizing three components of our microscope: the objective, camera, and intermediate optics.

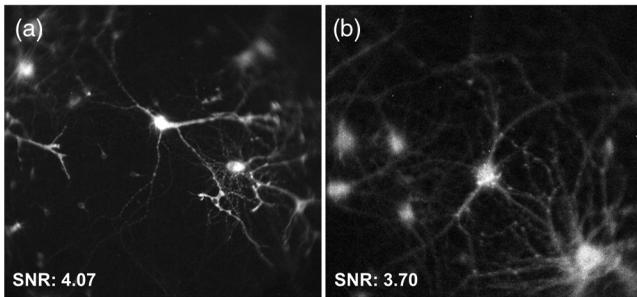
In selecting an appropriate objective lens for bioluminescence imaging, we aimed at finding one with the highest NA and lowest workable magnification. In comparing various objective lenses, we found that objectives with higher NA produced brighter, higher resolution images [Figs. 1(a2) versus 1(a1)]. Lower magnification objectives were also able to produce higher resolution images [Figs. 1(b2) versus 1(b1)].

We utilized an intermediate demagnifying lens on a camera mount in order to allow more light to be focused onto a smaller area of the camera chip. Each pixel in the illuminated area of the chip therefore receives more light, producing a brighter image. We found that greater demagnification produced brighter images with sufficient spatial resolution to visualize detailed cellular morphology [Figs. 2(a) versus 2(b)]. Note that silhouetting at the periphery of the image may occur with greater demagnification, so the field of view requirements must be carefully considered.

There are numerous camera options currently available for low-light optical imaging. CCD cameras are the most popular choice and rely on the photoelectric effect to convert a light signal into an electrical signal. The readout noise of CCD devices was significantly reduced with the advent of electron multiplying charge-coupled devices (EMCCD), making these cameras



**Fig. 1** Objective lens comparison. Bioluminescence images from cultured HEK cells expressing *Gaussia* luciferase (a1 and a2) and *Renilla* luciferase (b1 and b2) with the following objectives: (a1) LUCPlanFL 60 $\times$  0.7 NA, (a2) LUMPlanFLN/W 60 $\times$  1.0 NA for comparison of NA, (b1) UPlanFI 40 $\times$  oil 1.3NA, and (b2) UPlanSApo 20 $\times$  0.75 NA for comparison of different magnification together with NA.



**Fig. 2** Intermediate lens comparison. Bioluminescence images of dissociated cortical neuron cultures expressing *Renilla* luciferase using a 0.3× intermediate lens (a) and a 0.5× intermediate lens (b). Both images were taken with a UAPO 40× 1.35NA oil objective and a sCMOS (OptiMOS, Photometrics) camera.

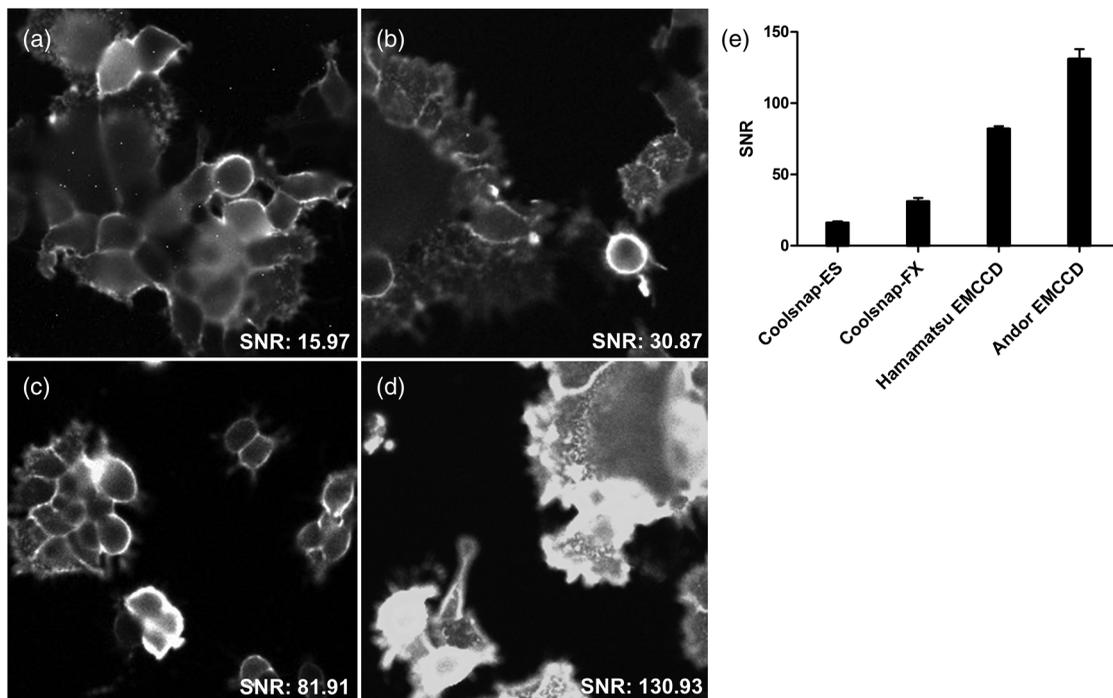
especially well suited for low-light imaging applications. Scientific CMOS (sCMOS) devices are a relatively new type of sensor that also offers extremely low readout noise and wide dynamic range, making them a cost-effective alternative for imaging in low-light conditions. We have therefore compared several CCD, EMCCD, and sCMOS cameras for bioluminescence imaging (Fig. 3). All of the cameras tested were able to produce bioluminescence images with relatively low background and short exposure times (1 to 10 s). Even though EMCCD cameras generally outperform sCMOS devices at very low light levels, our images were qualitatively similar (most likely due to the fact that *RLuc* and *GLuc* are relatively bright luciferases). One should therefore select a camera based on the level of sensitivity required, as the price differences between EMCCD, sCMOS, and CCD cameras can be quite significant.

Due to the relatively long-exposure times required for bioluminescence imaging, we found that it was important to reduce the amount of ambient light in the room as much as possible to reduce background noise. We routinely turned off or covered light sources (such as an arc lamp for fluorescence observation) near the microscope before bioluminescence imaging. We found that blackout curtains were especially effective at isolating the microscope and camera from any potential light contamination. Cooling the camera to the lowest temperature setting also helped reduce background by limiting dark current noise.

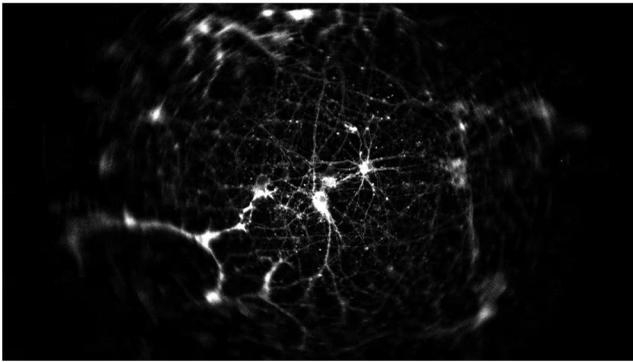
The danger of phototoxicity and photobleaching limits the effectiveness of long-term live-cell imaging with fluorescent molecules. In contrast, we have demonstrated that bioluminescent reporters can be used to image live cells for extended periods of time without any apparent adverse effects (Fig. 4). The substrate was supplied by a perfusion system, resulting in a long-lasting bioluminescent signal which could be detected over several hours. We did not observe any apparent adverse effects during this period, making this approach suitable for imaging cellular processes that are directed over long timescales, such as cellular trafficking, synaptogenesis, and migration.

### 3.2 *In Vivo* Bioluminescence Imaging

*In vivo* fluorescence imaging is often compromised by high non-specific background from tissue and cells (auto-fluorescence). In contrast, background bioluminescence from tissues not expressing luciferase is negligible. This property of bioluminescence makes it an ideal signal for imaging whole animals where the number of luciferase-expressing target cells is generally few compared to the surrounding non-expressing tissue. We assessed the usability of *Renilla* luciferase for *in vivo* imaging

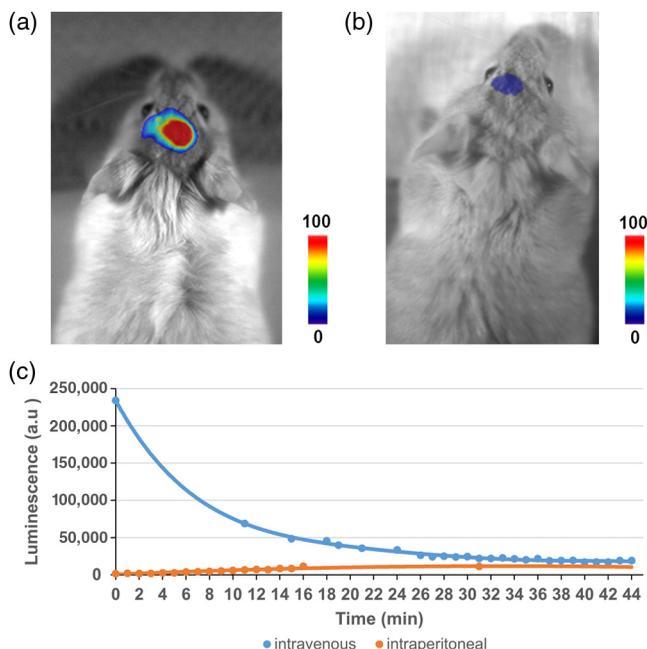


**Fig. 3** Camera comparison. Bioluminescence images of HEK293 cells transfected with *Gussia* luciferase taken with various cameras: (a) Noncooled scientific CCD (Coolsnap-ES, Photometrics); (b) cooled scientific CCD (Coolsnap-FX, Photometrics); (c): EMCCD (ImagEM C9100-13, Hamamatsu); (d): EMCCD (iXon Ultra 897, Andor). Contrast was adjusted for each camera using the highest and lowest intensity obtained in each camera. (e) Average SNR calculated at the time of peak luminescence for each camera.



**Fig. 4** Long-term bioluminescence imaging *in vitro*. Time-lapse bioluminescence images illustrate the ability to conduct live cell imaging over extended periods of time without the threat of phototoxicity or photobleaching. Cortical neurons expressing *Renilla* luciferase were imaged over a period of 3 h. Note that the bioluminescent signal decreases over time (as substrate availability decreases) but is able to be refreshed again by the addition of more substrate. (Video 1, WMV, 1014 KB) [URL: <http://dx.doi.org/10.1117/1.NPh.3.2.025001.1>].

and found that the bioluminescence signal was strong enough to allow detection of signals through the intact skull [Figs. 5(a) and 5(b)]. Special consideration of the route of substrate administration is needed when selecting a luciferase to use for *in vivo* bioluminescence imaging. Firefly luciferase (Fluc) has been frequently used for bioluminescence imaging in animals due to the relatively ease of the intraperitoneal route of substrate (D-luciferin) administration. Although we have demonstrated that coelenterazine can also be delivered intraperitoneally, *Renilla* luciferase is maximally effective when the substrate is administered via intravenous routes [Fig. 5(c)]. This may be



**Fig. 5** *In vivo* bioluminescence imaging. Bioluminescence imaging of an adult mouse injected with AAV encoding *Renilla* luciferase in the cortex 2 weeks prior. 500  $\mu$ g CTZ was delivered to the same mouse either intravenously (a) or intraperitoneally (b). Color bar depicts relative luminescent signal. (c) Bioluminescence images were taken over time and quantified in ImageJ to create a plot of bioluminescence time course.

due to the fact that the bioavailability of substrate in the brain is reduced when it is administered intraperitoneally, where it needs to drain through the lymphatic system before entering the bloodstream. Since beetle (e.g., firefly) and marine (*Renilla*, *Gaussia*) luciferases utilize different luciferin substrates, it is therefore feasible to multiplex them together due to absence of crosstalk between the two systems.

The low background with bioluminescence makes *in vivo* bioluminescence imaging particularly sensitive for detecting signals from small areas over extended periods of time. In fact, bioluminescence imaging has been successfully used for applications from tracking transplanted cells<sup>42,43</sup> to monitoring cellular processes such as neurodegeneration, inflammation, and neurogenesis.<sup>44</sup> Although *in vivo* bioluminescence signals provide limited spatial information directly, the cell-type specificity of luciferase expression provides indirect spatial information because any detected bioluminescence should ostensibly be coming only from cells expressing luciferase.

## 4 Conclusion

We have demonstrated how bioluminescent proteins can be effectively used as an optical reporter for both *in vitro* and *in vivo* settings. Given the proper detection equipment and scientific question, bioluminescence offers several unique advantages over conventional fluorescence readouts. The major advantage of using bioluminescent signals is that they are detected over little to no background noise, enabling long term imaging applications with no risk of phototoxicity or artifact. With the development of brighter and more responsive luciferase proteins, the use of bioluminescent reporters in neuroscience research will continue to grow more robust and versatile.

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**Jack K. Tung** is an MD/PhD student currently pursuing his PhD in biomedical engineering at Georgia Institute of Technology and Emory University. His current research interests include developing and applying novel technologies for the treatment of neurological disorders. His dissertation comprised developing novel optogenetic probes driven by bioluminescence (inhibitory luminopsins) and applying them in various animal models of epilepsy to control seizure activity.

**Ken Berglund** is a research associate at Emory University School of Medicine. His research focus is development of novel optogenetic tools and application of his tools in studies of neurophysiology and neuropathology in the rodent brain.

**Claire-Anne Gutekunst** received her PhD in psychobiology and is currently assistant professor in Neurosurgery, Emory School of Medicine. She is a behavioral scientist and anatomist who specializes in developing and validating gene therapies for various CNS disorders. She has developed a gene therapy for neurodegenerative diseases using C3 transferase, a bacterial exoenzyme with RhoA inhibition activity. She uses C3 expressing vectors to promote axon regrowth in the otherwise nonpermissive adult CNS with applications to various disorders including optic nerve injury, glaucoma, spinal cord injury, stroke, and other neurodegenerative disorders.

**Ute Hochgeschwender**, MD, is currently an associate professor of neuroscience at Central Michigan University's College of Medicine. Her long-term interest has been the molecular basis of brain function resulting in behavior. Over the past several years she developed a method to integrate the optogenetic and chemogenetic analyses of neural circuits by combining optogenetics with bioluminescence. Her current research interests are in further advancing the concept of bioluminescence-driven optogenetics and in utilizing these tools for research into the mechanisms and potential, noninvasive treatment of neurodegenerative and psychiatric diseases.

**Robert E. Gross**, MD, PhD, is the MBNA/Bowman Endowed Chair in Neurosurgery at Emory University School of Medicine, with appointments in Neurology and the Coulter Department of Biomedical Engineering at Emory/Georgia Institute of Technology. He is a neurosurgeon specialized in functional neurosurgery, and director of the Emory Neuromodulation Technology Innovation Center (ENTICE). He runs the Translational Neuroengineering Research Lab, developing and refining new treatments for neurological and psychiatric disorders, using molecular and electrical techniques for neuromodulation, including novel optogenetic techniques.