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Methodology article

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Monitoring immediate-early gene expression through firefly luciferase imaging of HRS/J hairless mice

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Abstract

Background: Gene promoters fused to the firefly luciferase gene (*luc*) are useful for examining gene regulation in live transgenic mice and they provide unique views of functioning organs. The dynamics of gene expression in cells and tissues expressing luciferase can be observed by imaging this enzyme's bioluminescent oxidation of luciferin. Neural pathways involved in specific behaviors have been identified by localizing expression of immediate-early genes such as *c-fos*. A transgenic mouse line with *luc* controlled by the human *c-fos* promoter (*fos::luc*) has enabled gene expression imaging in brain slice cultures. To optimize imaging of immediate-early gene expression throughout intact mice, the present study examined *fos::luc* mice and a second transgenic mouse containing *luc* controlled by the human cytomegalovirus immediate-early gene I promoter and enhancer (*CMV::luc*). Because skin pigments and hair can significantly scatter light from underlying structures, the two transgenic lines were crossed with a hairless albino mouse (HRS/J) to explore which deep structures could be imaged. Furthermore, live anesthetized mice were compared with overdosed mice.

Results: Bioluminescence imaging of anesthetized mice over several weeks corresponded with expression patterns in mice imaged rapidly after a lethal overdose. Both *fos::luc* and *CMV::luc* mice showed quantifiable bright bioluminescence in ear, nose, paws, and tail whether they were anesthetized or overdosed. *CMV::luc* and *fos::luc* neonates had bioluminescence patterns similar to those of adults, although intensity was significantly higher in neonates. *CMV::luc* mice crossed with HRS/J mice had high expression in bone, claws, head, pancreas, and skeletal muscle, but less in extremities than haired *CMV::luc* mice. Imaging of brain bioluminescence through the neonatal skull was also practical. By imaging luciferin autofluorescence it was clear that substrate distribution did not restrict bioluminescence imaging to capillaries after injection. Luciferin treatment and anesthesia during imaging did not adversely affect circadian rhythms in locomotor activity.

Conclusions: Imaging of gene expression patterns with luciferase can be extended from studies of live animals to rapid imaging of mice following a pentobarbital overdose before significant effects from postmortem changes occurs. Bioluminescent transgenic mice crossed with HRS/J mice are valuable for examining gene expression in deep tissues.

Background

Several researchers have described how transgenic mice expressing reporter genes that produce a fluorescent or bioluminescent product can be imaged continuously to assay gene expression, image changes in tumor growth, or follow the progress of infections [1]. Dynamic changes in gene expression have also been monitored in individual cells maintained in culture by imaging fluorescent or bioluminescent reporter genes [2–4]. In general, fluorescence methods are easier to use than those based on bioluminescence and often can be implemented with an existing camera system and fluorescence microscope. Whole-animal fluorescence imaging is, however, complicated by the practical limitations of directing excitation light into organisms, and is limited by the tissue's autofluorescence, which degrades the signal-to-noise ratio (S/N). Rather than administering excitation light, bioluminescence imaging based on firefly luciferase (*luc*) requires introduction of the enzyme substrate luciferin that reaches target areas via the blood supply and diffusion. Furthermore, because bioluminescence signals are nearly absent from most tissues, the S/N can be much larger than with fluorescence methods based on green fluorescent protein (GFP), a popular reporter gene product [5].

For most genes, the time course of expression is usually assayed by harvesting tissue from many animals at multiple time points. In contrast, whole-animal imaging in a single animal enables much simpler ongoing tracking of gene regulation involved in time-dependent processes such as development, growth, circadian rhythms, and disease in individual animals [1,6–13]. By monitoring responses in the same animal before and after onset of gene induction, noise from inter-individual variation is eliminated, and animals are used more efficiently. Furthermore, several treatments can be tested sequentially in the same animal.

Although live mice provide the benefits of serial imaging, they can produce movement artifacts in images because of breathing or twitching. Mice given a lethal overdose of anesthesia would yield more stable imaging, but postmortem metabolic changes in tissue oxygen or ATP levels could affect the luciferase reaction, and cell stress can alter expression of new luciferase. We examined anesthetized and acutely overdosed mice to test whether the bioluminescence pattern shortly after an overdose remains consistent with the live animal signal.

We compared two lines of transgenic mice containing luciferase controlled by immediate-early gene promoters that respond rapidly following a stimulus and produce large changes in brightness. The *luc* gene is controlled by the human *c-fos* promoter in one line (*fos::luc*) and by the human cytomegalovirus immediate-early gene 1 enhancer

and promoter in the other (*CMV::luc*) [14]. Both strains were generated by oocyte injection, so each cell carries the transgene. The distribution of the protein or mRNA of *c-fos* has been used to localize neuronal excitation, cellular correlates of behavior, and cell division in many tissues for over a decade [15–18]. The human CMV promoter/enhancer responds to signals mediating inflammation and cell stress [19–21] and was recently shown to be induced by cell membrane depolarization [22]. Consequently, several tissues of both transgenic mice provide rapid responses to a broad range of stimulation.

To image gene expression throughout transgenic rodents, luciferin is typically injected intraperitoneally or by the tail vein. In either case, it is carried through the circulatory system and by diffusion. If this charged molecule does not readily penetrate all areas, the pattern of the luciferase signal could be influenced by the distribution of luciferin. Although luciferin readily crosses cell membranes, it might remain primarily within blood vessels, in which case these structures could dominate images of tissues and bias accurate interpretation of gene expression patterns. The intrinsic fluorescence of luciferin was used to image its distribution in the brain, evaluating whether it easily passes through some of the least permeable endothelial cells of the body.

It was also important in this study to determine the range of gene expression that could be quantified simultaneously in various areas of individual mice, because previous studies often provided images with unstated dynamic range. Subtle changes in gene expression could be imaged here even when they occurred above elevated background expression rather than the completely dark background of inactive tissue, as shown by logarithmic scaling. Both transgenic lines were crossed with a hairless, albino mouse (HRS/J yielding Hr-CMV and Hr-*fos*) to test whether internal organs and other deep structures could be readily visualized in these strains. Repeatable patterns of expression in specific body areas of hairless mice verified that luciferase bioluminescence imaging of these mice is a promising approach for long-term quantitative measurements of gene expression in live animals.

Results

Overdosed mice

Gene expression patterns were characterized using bioluminescence imaging of intact mice. It was necessary to introduce luciferin into the body so that it could reach all cells and organs at sufficient concentrations to generate measurable light. Images of luciferin autofluorescence suggested that it could readily cross the blood-brain barrier (Fig. 1). The sodium salt of luciferin was administered intraperitoneally (i.p.) at 10 mM in PBS. A volume of 0.1 ml provided bioluminescence images of the skin, brain,

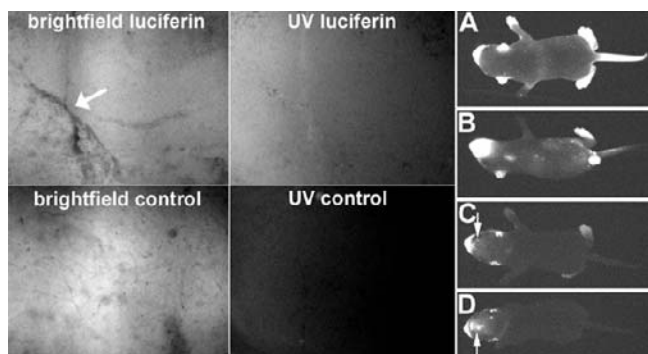


Figure 1
Dispersal of luciferin into brain, skin, and internal organs. **Left:** Brain slices from thalamic area of adult CMV::luc mice injected i.p. with luciferin or not injected (control). Autofluorescence of luciferin in near-UV light showed that it is distributed diffusely and not restricted to the capillaries (arrow in brightfield). **Right:** Bioluminescence in an overdosed fos::luc neonate (postnatal day 1) imaged for 10 minutes. **(A)** Dorsal and **(B)** ventral views of the same mouse showed transgene expression in paws, tail, ears, nose, and internal organs of the abdomen. Light from the brain was visible after first removing skin over the cranium **(C)** and then upper cranium itself in **(D)**. Arrows: brain bioluminescence.

and internal structures in adult CMV::luc and fos::luc mice after an overdose with pentobarbital (Fig. 1, 2). Likewise, overdosed neonatal fos::luc mice showed expression in the brain that was visible through the skull (Fig. 1C) and was clearer after removal of the dorsal cranium (Fig 1D).

Bioluminescence was detected in all neonates and adults of the two original transgenic lines during 10-minute exposures captured immediately after the overdose to minimize any effects from new luciferase synthesis (Fig. 2, Table 1). Quantitative comparisons of signal intensity revealed highest gene expression in the paws, tail, and ears in both lines, and CMV::luc mice exhibited higher signals than age matched fos::luc mice. Using whole animal measurements, CMV::luc neonates were 3-fold brighter than fos::luc neonates ($p < 0.001$, analysis of variance (ANOVA) and Scheffe posthoc test) and 20-fold brighter than CMV::luc adults ($p < 0.001$). Fos::luc neonates were 9-fold brighter than fos::luc adults ($p < 0.05$). CMV::luc adults were brighter on average than fos::luc adults, but these differences were not significant. Light was also detected from around the mouth of neonates and adults of both lines.

Although neonates were surprisingly similar to adults in their signal, there were some distinct differences; both

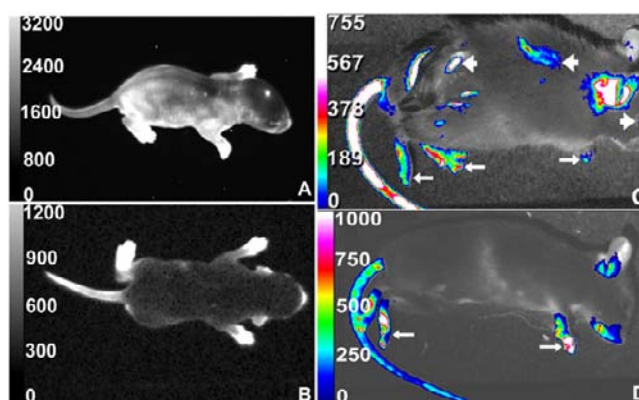


Figure 2
Expression in similar body regions of overdosed CMV::luc and fos::luc mice. **(A)** CMV::luc neonate showing higher expression than **(B)** a fos::luc neonate. Both had highest expression in the paws, tail, ears, and nose, but the CMV::luc mice also showed a bright signal from a point on the dorsal surface of the head. Likewise, adult CMV::luc **(C)** and fos::luc mice **(D)** had similar gene expression patterns, like those of neonates, with a few exceptions. CMV::luc adults also showed expression through the eyes and fur of the abdomen (short arrows). (Paws: long arrows.) Adult signals are shown as pseudocolor overlays on the reference whole-animal images. Bioluminescence intensity is presented in analog-to-digital units (ADUs) of the camera according to scale at left of each image in all figures.

showed expression in the appendages, but many neonatal CMV::luc mice (25%) showed a single bright spot centered dorsally on the head (Fig. 2A and Fig. 3A). This spot was not observed in any fos::luc mouse. The eyes of about one half of the adult CMV::luc mice also had detectable expression (Fig. 2C), but fos::luc mice and CMV::luc neonates did not (Fig. 2A,2B,2D). Furthermore, CMV::luc mice produced abdominal signals visible through their fur (Fig. 2C).

Live mice

To test whether luciferase patterns would be different between overdosed and non-anesthetized freely-moving mice, two neonates were injected with luciferin and imaged continuously. These neonates appeared like overdosed mice. (Compare Fig. 2 and 3.) Therefore, very young live mice imaged by either method reveal similar gene expression patterns.

The pattern of expression in live anesthetized adult mice (5 CMV::luc, 2 fos::luc, Fig. 4A) was not obviously different from signals in mice given an overdose and imaged immediately. Again, highest expression was in the paws, tail, ears, and nose. Both transgenic mice showed normal

Table 1: Comparing bioluminescence intensity in whole body of transgenic mice. Maximum intensity (in ADUs) of mice overdosed with Nembutal. SD: standard deviation. N: number imaged.

Group	Mean	SD	N
CMV::luc neonates	6843.4	4134	23
Fos::luc neonates	2272.0	1747	22
CMV::luc adults	316.8	240	23
Fos::luc adults	227.0	199	23

circadian behavior (Fig. 4B), indicating that their circadian systems were intact. The phase of running-wheel activity was set by the light/dark (LD) cycle where they were maintained before being moved to constant darkness (DD), indicating that they could entrain to the LD cycle. Furthermore, their behavioral activity was organized into circadian rhythms that are typical of mice in DD. Additional experiments to determine the average period of these free-running rhythms are underway.

To examine any effects on signal intensity, five anesthetized CMV::luc adult mice were compared with 13 others given an overdose and imaged during the same phases of the LD cycle as the anesthetized mice, between 2 and 14 hours after light onset. Although the same areas were visible in both groups, the maximum signal was on average 2.3-fold higher in the anesthetized mice but was not significantly different by ANOVA and Scheffe posthoc test. Similarly, two fos::luc mice which were anesthetized and imaged were on average 2.2-fold brighter than overdosed fos::luc mice, but were not significantly different.

Hairless mice

Both original transgenic lines were crossed with hairless albino mice to increase any bioluminescence signals through the skin that originate from deeper structures. The overdosed hairless CMV::luc mice (Hr-CMV) showed luciferase expression in several body regions with clearly detectable signal in the joints of the paws, tail vertebrae, and claws that was not visible in the CMV::luc or fos::luc mice (Fig. 5 and Fig. 2C,2D). Some signal from the dorsal and ventral abdomen of the Hr-CMV mice may have been obscured in the haired mice (Fig. 2C and Fig. 5). Light emanated from the ear canals of the Hr-CMV mice (Fig. 5), possibly from the tympanum, and from the dorsal cranial spot that may be equivalent to the one centered on the head of CMV::luc neonates (Fig. 2A and Fig. 3A).

Logarithmic scaling of intensity proved to be an effective way to reveal fine details including the dark areas that obscured light from deeper areas (Fig. 5). The areas that were visible by eye using any scaling of the images from 21 overdosed Hr-CMV mice, according to prevalence,

were dorsal abdomen (62%), skeletal muscles (43%), tip of nose (33%), ear canals (33%), ventral abdomen (29%), vertebrae of tail (29%), dorsal cranium (20%), eyes (20%), joints of paws (19%), claws (19%), bottom of paws (19%), top of paws (10%), and gonads (10%).

To identify internal organs that express the CMV::luc transgene, both CMV::luc and Hr-CMV mice were dissected 20 minutes after luciferin injection (Fig. 6). The pancreas was the brightest organ and abdominal muscles had weaker expression. To confirm the source of the frequent bright signal in the dorsal abdomen, we removed the pancreas and examined it with a 10× Neofluar lens (Zeiss) attached to the camera (data not shown). The pancreas showed a diffuse signal throughout its extent, and because of light scatter, tissue sections deemed necessary to identify expressing cell types.

An additional five Hr-CMV mice were placed in DD to monitor circadian rhythms in wheel-running activity. Like the CMV::luc and fos::luc mice, they also showed normal circadian rhythms in wheel-running during 14 weeks in DD, and they were anesthetized and imaged up to four times each. The spatial patterns of these mice were indistinguishable from those of overdosed Hr-CMV mice, and efforts are continuing to test for any circadian modulation of bioluminescence.

Three Hr-fos mice were overdosed and imaged. Signals were not as distinct as in the Hr-CMV mice, but all three had expression in the paws and nose. One also expressed the transgene in the eye, and one expressed in the gonads and tail, although without the distinct banding pattern of Hr-CMV tails that corresponds with the vertebrae.

Discussion

Imaging luciferase reporter genes in intact animals enabled observations to be made in a physiologically relevant state without altered promoter control that can occur *in vitro* where cells are in artificial environments and are often dividing faster than in the animal. Because the anesthetized and overdosed mice showed similar high expression in the extremities and ears, this shared pattern

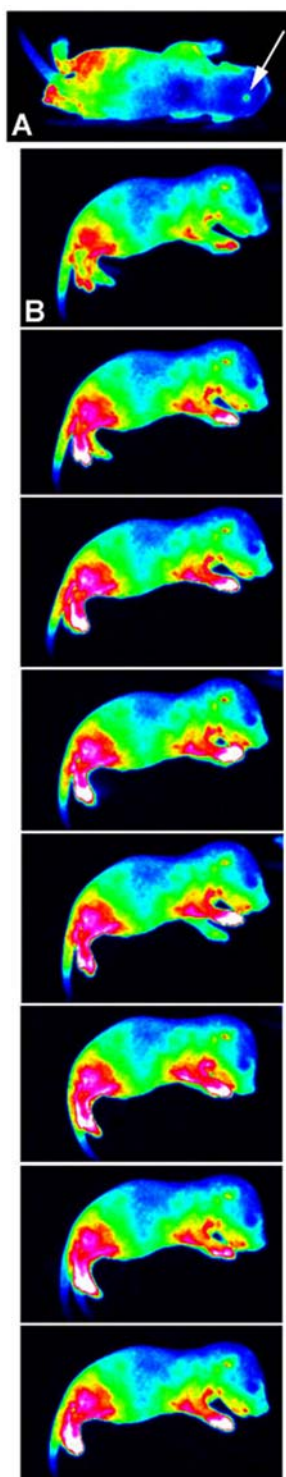


Figure 3
Imaging without anesthesia. Expression in a non-anesthetized CMV::luc neonate (postnatal day 1). **(A)** Frequently observed dorsal cranial spot (arrow). **(B)** Series of consecutive 1-minute exposures of the same mouse showed the persistence of the signal and a similar expression pattern to that of overdosed neonates.

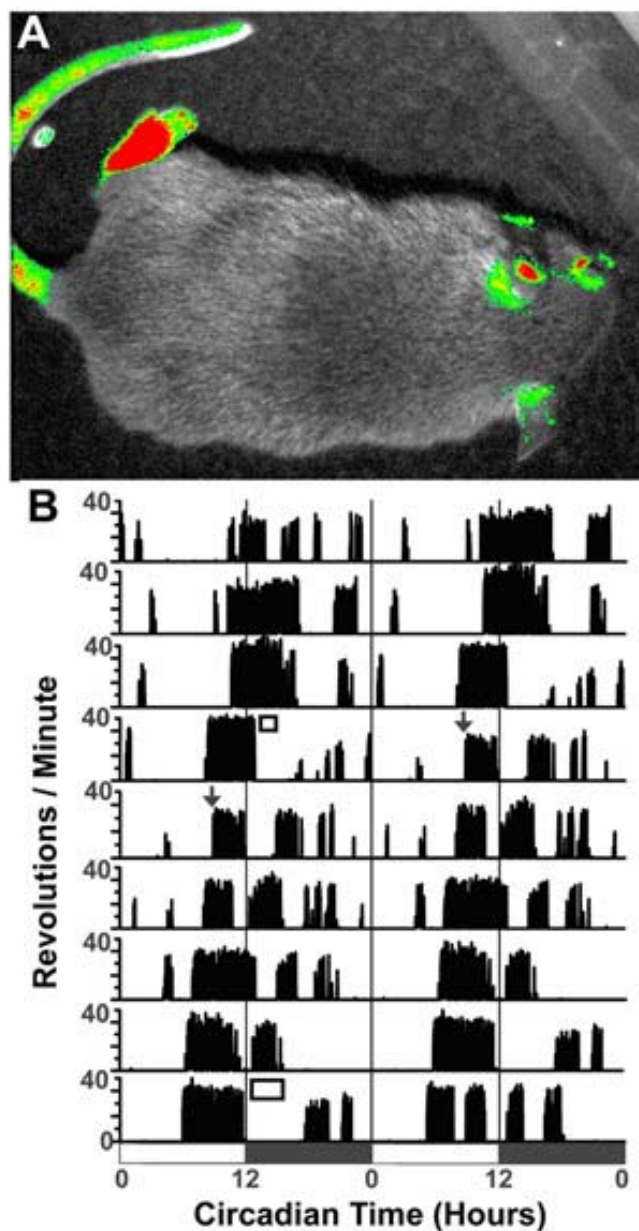


Figure 4
Common gene expression patterns in anesthetized and overdosed mice. **(A)** Adult CMV::luc mouse imaged while under pentobarbital anesthesia lasting one to four hours. Expression was not obviously different from mice given an overdose. Expression shown as an overlay on a reference image. **(B)** Mice recovered within an hour and produced circadian rhythms in wheel running activity in constant darkness. Corresponding phase of previous light/dark cycle shown below. The rhythms in wheel-running activity were typical of mice maintained in darkness with a period less than 24 hours. Open boxes: when mouse was out of cage for imaging. Arrows: one daily activity onset. The data was double-plotted (48 hours across) so that circadian rhythms can be easily followed.

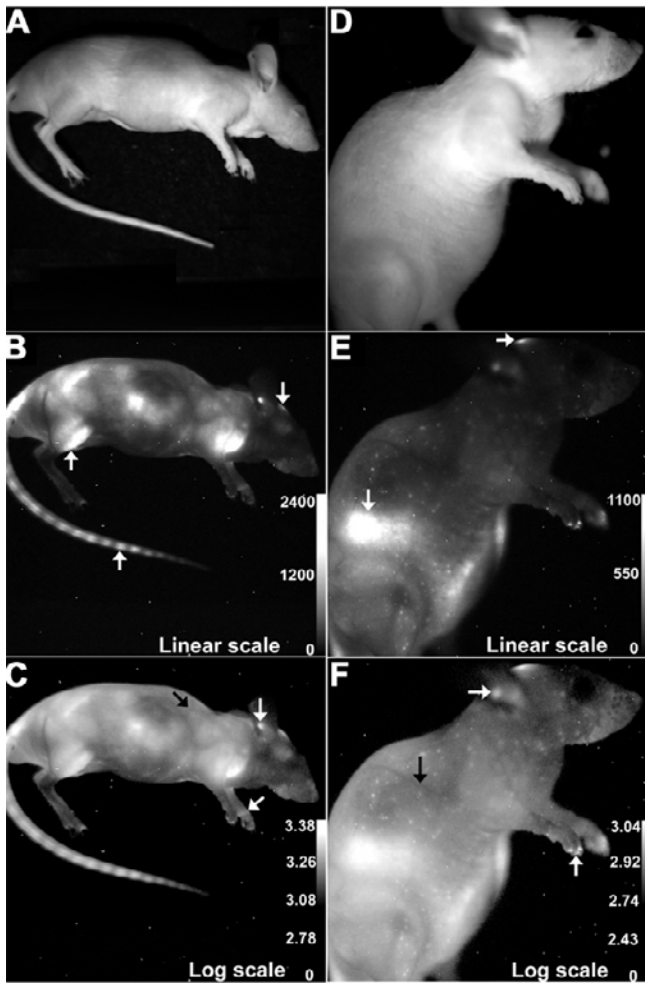


Figure 5
Expression patterns in Hr-CMV mice. When crossed with albino hairless mice, the transgenic mice showed substantially less signal in the skin than the haired transgenic mice and internal structures were visible. (A) Reference image of an overdosed adult Hr-CMV mouse. (B) Linear scaling of intensity showed detail of the brightest areas, and (C) logarithmic scale revealed subtle differences in expression in the ear canal, joints, and muscle in the same image. (D) Reference image of a second adult Hr-CMV mouse taken with f/ 1.2 lens and +4 close-up filter (Promaster), (E) linear scale, and (F) logarithmic scale of same image. Highest signal was from the tail (B), internal organs (E), some skeletal muscles (B), ear canals (C and F), claws (F), skull (B and E), joints (C) and the underside of the paws (C and F). Note that some dark, non-expressing structures that obscure background light are visible (C and F, black arrows).

was not due to effects from loss of heart beat, breathing or body temperature. Furthermore, freely-moving mice had very similar expression patterns to those of overdosed or

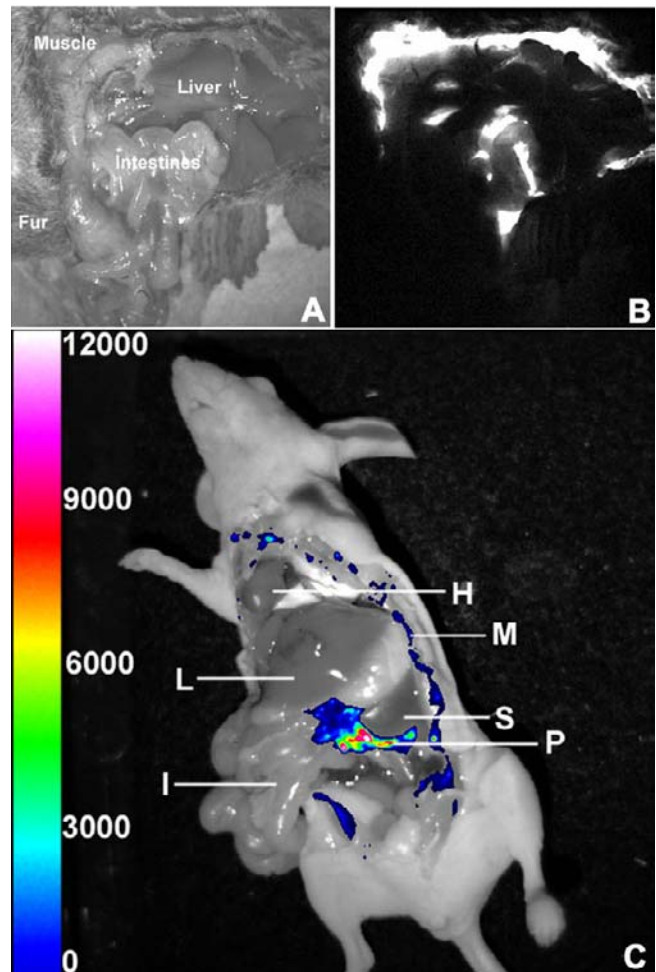


Figure 6
CMV::luc expressed in pancreas and only a few other areas. (A) Reference image of dissected adult CMV::luc mouse with four areas labeled. (B) Expression from the same mouse. (C) Dissection of Hr-CMV mouse. Shown is an overlay of the bioluminescence image on the reference image to identify brightest tissues. Pancreas (P) revealed high expression and muscle tissue (M) showed weak signal, but heart (H), liver (L), spleen (S), and intestine (I) did not.

anesthetized mice and may be more useful in some imaging experiments that require behavioral assays, although these experiments were limited to young neonates that move little. Repeated injections did not influence signals from the anesthetized mice, indicating that they can be sampled repeatedly.

CMV versus c-fos

CMV::luc and fos::luc neonates showed similar overall bioluminescence patterns that were also like those of freely-moving non-anesthetized neonates. The high

peripheral expression in both transgenic lines may be due to elevated skin cell division rates in the tail, ears, and paws [23]. Fos::luc mice were previously reported to have high expression in the skin [14], and both the CMV-IE1 and *c-fos* promoters are active in dividing cells [24,25]. Whether the observed eye signal originated in the retina and why it was not detected in the fos::luc mice needs to be addressed.

Why the CMV::luc neonates and the Hr-CMV mice exhibited the dorsal cranial spot is also not yet known. The ventricular and sub-ventricular brain areas are readily infected by mouse CMV during development [26], and human CMV reactivation from a latent state depends on IE-1 induction [27,28]. Human CMV infects neurons, glial cells, endothelial cells [29,30]. The CMV::luc and Hr-CMV mice may provide insight into gene regulatory factors responsible for viral infection throughout the body.

It was initially expected that the two transgenes (CMV::luc and fos::luc), which were derived from different immediate-early genes, would display distinctly different expression patterns, although the two do have some common gene regulatory elements. The 5' upstream sequence (5' US) of *c-fos* contains a promoter controlled by cAMP or Ca²⁺ signaling through a cAMP response element (CRE), mitogen-activated protein kinases that respond to growth hormones, or a stress-signaling pathway dependent on STATs (signal transducers and activators of transcription) [31]. It is also induced early in G1 during the cell cycle [24].

Similarly, the human CMV 5'US contains a powerful promoter and enhancer that responds through a wide range of signaling pathways and, like *c-fos*, also responds through its multiple CREs [19,20,32]. It is repressed in some tissues, such as liver, and is reactivated by signaling pathways acting through NF-kappaB, a transcription factor that mediates inflammation and stress signals [33,34]. A recent study has shown that the CMV 5'US also responds to membrane depolarization in neurons [22], and so, like *c-fos*, could be a marker for neural activity in the nervous system of CMV::luc mice. Differences in expression pattern and intensity could be attributed to the different combinations and types of gene regulatory elements of the transgenes. Likewise, the common expression in the extremities could be the result of cell turnover in these tissues to which both transgenes should respond. Previously, high expression detected in the skin of the fos::luc mice was tentatively attributed to keratin formation [14]. The lower skin expression in Hr-CMV versus CMV::luc mice was perhaps due to loss of hair follicles, pigmentation, or other effects from the albino and hairless mutations.

The insertion of a transgene can disrupt important genes involved in behavior. Locomotor activity was used to detect possible behavioral effects or disruption of the circadian system, although imaging of hypothalamic explant cultures from the fos::luc and CMV::luc mice has shown circadian rhythms in transgene expression [14,35], so it was unlikely that the mice had major mutations of the circadian system. Both transgenic mice provided circadian rhythms in wheel-running activity and they could entrain to light/dark cycles. Although a more complete analysis of their circadian properties will be presented elsewhere, these first measurements of any behavior in fos::luc or CMV::luc mice indicate that they can be studied at both the cell and organism levels. Clearly, the photoreceptors in the eyes that project to the circadian pacemaker in the hypothalamic suprachiasmatic nucleus are functional in these mice, and the pacemaker's regulation of circadian rhythms in the whole animal persists [36].

Hairless mice

Although, the Hr-fos mice showed less light from internal structures than the Hr-CMV mice, signal in the extremities of Hr-fos mice was visible as reported previously in fos::luc mice [4]. These results are consistent with the known transient expression of *c-fos* [15] and suggest that Hr-fos mice could be useful for detecting induction above these low baseline signals following a sufficiently strong stimulus.

Previously, luciferase bioluminescence has been examined in nude BALB/c mice [9], but these are athymic and lack T-cells, which could alter transgene expression patterns. Nevertheless, the nude mouse study showed the time-course of the luciferase signal after luciferin injection and maximal signals were reached about 20 minutes after injection, the time used in this study. Unlike nude mice, Hr-CMV mice are not immunocompromised and therefore require no special housing. Also, Hr-CMV showed no abnormal behavior and had typical circadian running wheel rhythms.

Because 62% of the Hr-CMV mice examined had a bright dorsal abdominal area and the pancreas was the brightest organ of Hr-CMV and CMV::luc mice dissected immediately after overdosing, we interpret this light as high gene expression in the pancreas. When the pancreas was removed, it bioluminesced throughout its length, and additional cellular studies will be needed to explore exactly where the light originated. The NF-kappaB pathway of pancreatic cells mediates some responses to cholecystokinin [37], suggesting that direct induction of the CMV::luc transgene by NF-kappaB may generate the pancreas signal. Although one early report of transgenic mice did not detect high expression in the pancreas from a transgene regulated by the human CMV IE-1 enhancer

and promoter [38], later studies clearly showed high expression in the pancreas using similar CMV IE-1 constructs [39–43]. Therefore, it is likely that the pancreas signal of Hr-CMV and CMV::luc mice reflects normal pancreas function. The Hr-CMV mice could be useful in a research model for pancreatic cancer studies because of the activation of CMV IE-1 during cell division.

Although heart, kidney, liver, and spleen of Hr-CMV mice were silent in images, tissue harvested from these organs of CMV::luc mice have shown luciferase expression after cell lysis and total luciferase assays [14]. It has been argued that hemoglobin in "dark" organs such as the spleen and heart may absorb luciferase bioluminescence and limit imaging of gene expression [44]. However, this quenching should enable better detection of the nearby pancreas signal in Hr-CMV mice.

Imaging considerations

Deep structures of hairless mice may be better imaged through luciferase than GFP based methods because scattering of light only affects the emission from the luciferase reaction and not the energy needed for excitation, whereas GFP excitation requires penetration of excitation light to the tissue of interest [45]. Light scatter is likely a greater limitation on imaging than signal intensity. As shown in this study, signal intensity from luciferase can extend over several orders of magnitude.

Not surprisingly, signals from bioluminescence can be best exploited by capturing images with cameras that have a comparably large dynamic range, particularly ones with a 16-bit range per pixel. One drawback of the color cameras used by many researchers when imaging GFP is that they are usually limited to only 8 bits for each of the three color channels, providing a dynamic range only slightly better than what the human eye can see when the optical system is ideally arranged to provide maximal distribution of intensities. In contrast, a 16-bit camera, such as the liquid nitrogen-cooled CCD camera used here, enables both the very dim and very bright areas of images to be imaged simultaneously, as shown by logarithmic scaling in Fig. 6. Unfortunately, many researchers do not make full use of camera imaging capabilities and chose to create images that only provide information with the very limited range of about 100 gray levels used in printing or viewing by the human eye [46].

Although the spatial pattern of bioluminescence could conceivably be influenced by the distribution of luciferin, two points suggests that it would not have a major role here. First, images of luciferin autofluorescence showed a diffuse distribution in the brain rather than a signal restricted to the capillaries. Although these images were captured 75 minutes after luciferin injection, much longer

than the time allowed before whole-animal imaging, they do suggest that luciferin diffuses into the brain. This result does not show, however, that the luciferin levels are distributed evenly. Nevertheless, luciferin concentrations could be saturating even at low concentrations in many areas because release of oxyluciferin from luciferase is rate-limiting [47,48]. Second, particular differences in expression between CMV::luc and fos::luc mice (the dorsal cranial spot and eye signal) also suggest that luciferin levels did not dictate expression patterns. Furthermore, other researchers have addressed this issue and concluded that luciferin concentration did not limit their images of bioluminescence in mice [9].

According to the small molecular weight of firefly luciferase, 62 kDa [49] and rough estimates of time required for transcription and translation, images collected during the first 10-minutes after an overdose should adequately reflect luciferase made up to the time of imaging, and shorter exposures would provide even more conservative assays of expression. Previous results with fos::luc hypothalamic cell cultures showed that about 4.6 hours is required for bioluminescence to reach half its maximum following cell depolarization at 36°C [14]. One outcome of these results is that imaging of overdosed mice must be performed as soon after an overdose as possible. Incidentally, the last three images of Fig. 1 may include signal from expression induced by the procedure, because these images were taken in series, but this did not seem to have a large effect on expression patterns.

Finally, the cause of the approximately 2-fold decline in signal immediately after an overdose is not known. Although it could be due to a drop in oxygen availability, many factors may be involved. This reduced signal should be weighed against loss of spatial resolution in live mice due to breathing and other spontaneous movements. We have shown that overdosed mice can provide detailed images particularly after logarithmic scaling, as in Hr-CMV mice (Fig. 5), which is useful for screening expression across the animal in fine detail. Because signal strength of overdosed mice was more than adequate for quantitative measurements, the signal loss does not compromise measurements. The overdose does not alter the signal pattern in mice because the same body areas were visible as in live mice, which suggests that overdosed mice are practical for assaying the distribution of gene expression. Also, mice often need to be dissected immediately after an overdose to identify the source of the signal as shown here with CMV::luc mice. It is important, however, not to make numerical comparisons between live and overdosed mice when correlating luciferase intensity with absolute levels of gene induction.

The bioluminescent mouse models characterized here could serve as important tools for biomedical research. Through this technique, gene expression can be followed in the same animal, and faint signals can be tracked *in vivo* simultaneously with much brighter signals from neighboring tissue [9–11]. CMV IE-1 expression correlates with known sites of human CMV infection [30], and IE-1 expression is required for CMV reactivation [27]. Consequently, CMV::luc mice could be valuable for imaging processes responsible for viral infection [27,28]. This study joins others in describing how to determine the extent of gene expression spatially and temporally in intact animals.

Conclusions

Adult fos::luc and CMV::luc transgenic mice showed luciferase expression in the tail, paws, ears, and nose, suggesting similar control of the two immediate-early gene upstream sequences used in creating these mouse lines. In contrast, distinct differences between the two lines were demonstrated by additional expression in the dorsal cranium and eyes in the CMV::luc mice. Neonates of both lines had higher expression than the adults, which likely reflects increased immediate-early gene expression during development. Both lines continued to show expression in the appendages after mice were crossed with a hairless albino mouse (HRS/J). The hairless CMV::luc mice also provided views of internal structures including bones of the tail and paw, pancreas, and skeletal muscles. Microscopic imaging of luciferin autofluorescence in brain slices, showed that this luciferase substrate diffuses into brain tissue following intraperitoneal injection suggesting, along with the imaging results, that it spreads readily throughout the body. Also, imaging results were not obviously altered by anesthetics used to sedate and immobilize the mice during imaging, although overdosed mice showed an overall decline in signal. Finally, both lines of mice displayed circadian locomotor rhythms that entrained to daily light/dark cycles and persisted following repeated imaging sessions.

Methods

Animals

Transgenic CMV::luc and fos::luc (Tg(Fos::luc)1Rnd, Jackson Laboratories, Bay Harbor, ME) mice *Mus musculus* [14] were bred and maintained on a 12:12 hour light/dark cycle in the Animal Care Facility at Bowling Green State University, Bowling Green, OH. Animals received food and water *ad libitum* in a temperature controlled environment. Light onset for one room was at 9 pm eastern standard time and 7 am for a second room. CMV::luc and fos::luc mice were crossed with male HRS/J *hr/hr* mice (Jackson Laboratories) to create two new lines. Standard tail clip assays were used to verify luciferase expression in the progeny [14]. These mice were then further crossed to

produce mice that were homozygous for the albino and hairless mutations and either homozygous or hemizygous for one of the two transgenes (Hr-CMV and Hr-fos). Animal procedures were approved by the University's animal care committee and met National Institutes of Health guidelines. All efforts were made to minimize animal discomfort.

Imaging luciferin fluorescence

Adult CMV::luc mice were injected i.p. with 0.1 ml sodium salt of luciferin (Molecular Probes, Eugene, OR) at 10 mM in phosphate buffered saline (PBS). The time between luciferin injection and imaging was varied from 10 to 75 minutes. The mice were overdosed with sodium pentobarbital (Nembutal, 0.1 ml, i.p.) and decapitated. Most mice were exposed briefly to isofluorane vapors to temporarily anesthetize the mice before luciferin and Nembutal injections. Brains were removed, sectioned 200 μ m-thick, and immediately viewed with an Axiophot epifluorescence microscope (Zeiss, Thornwood, NY) equipped with a mercury lamp, MicroMax cooled-CCD camera (Roper Scientific, Tucson, AZ), and DAPI and FITC filters to image luciferin autofluorescence. Pixel intensity was measured with MetaMorph software (Universal Imaging, Downingtown, PA).

Transgenic neonate and adult imaging

Neonates (0–10 days postnatal), juveniles (21 days–2 months), and adults (2–8 months) of both sexes from all four transgenic mouse lines (CMV::luc, fos::luc, Hr-CMV, and Hr-fos) were examined at hours distributed evenly across the 24-hour day. The sodium salt of luciferin was administered by intraperitoneal (i.p.) injection (0.1 ml, 10 mM in PBS). After 20 minutes, mice were euthanized with an overdose of Nembutal (0.1 ml, i.p.). Isofluorane was given before injections as when examining autofluorescence. To image internal organs, mice were injected with luciferin, overdosed, and quickly dissected. Particular tissues, skin and pancreas, were removed and imaged briefly in isolation.

Mice, intact or dissected, were imaged with a Nikkor lens (f/1.2 or f/1.8) attached to a liquid nitrogen-cooled CCD camera containing a back-thinned, back-illuminated 512 \times 512 pixel 16-bit sensor (Roper Scientific, Tucson, AZ). Exposures of 10 minutes were used to image bioluminescence. Adults were imaged laterally and neonates were imaged from a dorsal view to have most of each animal within the focal range. Background bias current was subtracted from images, cosmic ray event artifacts were removed with a rank filter and intensity was measured using V++ software (Roper Scientific, Tucson, AZ).

Live transgenic mice were imaged like overdosed animals except that 20 minutes after luciferin injection, an

anesthetizing rather than overdosing Nembutal injection was administered i.p. (50 mg/kg body weight) to produce unconsciousness and immobility for 1–4 hours. Neonatal, juvenile, and adult CMV::luc, fos::luc, Hr-CMV, and Hr-fos mice were imaged for 10-minute exposures at times throughout the day. Anesthetized mice were placed in a box on heating pad while imaging. At the end of experiment, the mouse was observed while it recovered and then returned to the colony. Non-anesthetized neonates were imaged with 1-minute exposures.

Locomotor activity

Mice were given at least 3 days in constant darkness (DD) to adjust to a cage with a running wheel before imaging at particular phases of the circadian cycle. Mice were moved in a light-tight container to the imaging room and were returned to DD after imaging where they recovered completely and began wheel running within a few hours. Before the next images were taken, the circadian system was allowed at least 3 days to produce a stable circadian rhythm after any perturbing effects of the procedure on the phase of the free-running locomotor rhythm had passed. Mice were provided with food and water *ad libitum*. Mouse running wheels were monitored with magnetic switches, a computer data acquisition system, and software to continuously count wheel revolutions occurring during 1-minute intervals. Activity counts were plotted and daily activity onsets were used as phase reference points for circadian rhythms in locomotor activity.

Author's contributions

AC and MG participated in the planning of these experiments and interpretation of the results. AC performed all imaging experiments, tail clips and assisted in breeding. Both collaborated to write the first draft of the manuscript. All authors read and approved the final version of the manuscript.

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