

control. Luciferase reporters were stable for at least several weeks in mice and remained responsive to the adenovirus-mediated delivery of exogenous p53. The proposed noninvasive imaging of p53 transcriptional activity may greatly enhance and accelerate the testing of different therapeutic combinations in preclinical studies.

Another example of the application of bioluminescence reporter genes is the assessment of laser-induced tissue damage through expression of stress-related proteins. A transgenic reporter mouse was created where expression of firefly luciferase was controlled by the regulatory region of the inducible 70-kDa heat shock protein (HSP).⁴ The expression of HSP-70 as an indicator of cell injury was monitored noninvasively following pulsed laser irradiation in an attempt to refine laser parameters appropriate for laser surgery and diagnostics. After thermal injury was induced with a pulsed diode laser, expression of shock proteins was activated and was accompanied, in this case, by expression of luciferase and emission of light upon luciferin addition. Bioluminescence levels correlated well with the actual HSP-70 concentration, as determined by enzyme-linked immunosorbent assay (ELISA).

4.1.2 Bioluminescent gene expression reporters for physiology research

Taking advantage of the fact that variants of firefly luciferases emit light of a different color but use the same substrate, attempts have been made to create a multiplex assay for simultaneous monitoring of multiple gene expressions. Murine fibroblasts were successfully transformed with three different firefly luciferases emitting green, orange, and red light. The luciferase activities related with expression of three different genes were measured simultaneously using optical filters to separate emissions (Fig. 4.1).⁵ This system enables one to simply and rapidly monitor multiple gene expressions in a one-step reaction and directly compare two or more transcriptional activities and/or interactions with transcription factors in the same cell population and investigate acute expression profiles under the applied therapy.

The activity of a lactase promoter was investigated noninvasively in the small intestine of developing mice.⁶ To identify regions of the lactase gene involved in mediation of expression, transgenic mice harboring 0.8-, 1.3-, and 2.0-kb fragments of the 5' flanking region cloned upstream of a *luc* gene were generated. Expression was assessed non-invasively in living mice. It was shown that both 1.3- and 2.0-kb promoter reporters were expressed at relatively high levels based on observed bioluminescence intensity, with the maximum expression occurring in the middle section of the intestine. Post-weaned 30-day transgenic offspring showed a 4-fold decline in luciferase expression, which corresponded to the known maturation decline in lactase expression. In contrast, a 0.8-kb promoter-reporter construct demonstrated low luciferase activity that was not restricted to the intestine. These results demonstrate that a distinct 5' region on the lactase promoter directs intestine-specific expression, and that regulatory sequences are located on a 1.2-kb region upstream of the lactase transcription start site.

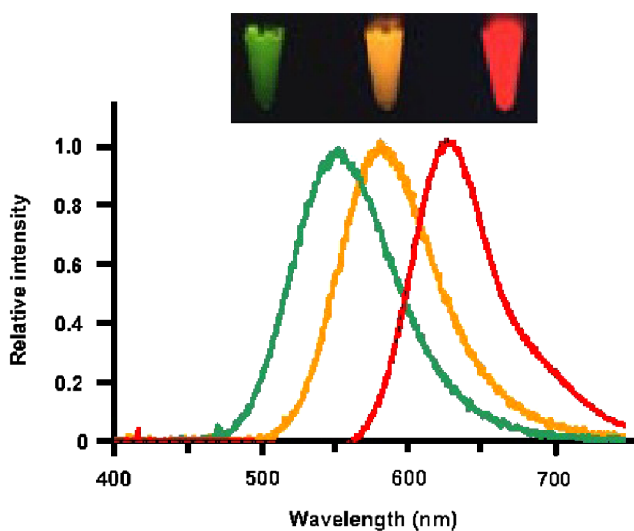


Figure 4.1 Bioluminescence of NIH/3T3 cells expressing green, orange, and red firefly luciferases: Top: photo image—exposure of 1 min using Superia Venus 1600 film (Fujifilm, Japan); Bottom: bioluminescence spectra (green, orange, and red lines). (Adapted from Ref. 5 with permission from BioTechniques, © 2009.)

Many publications exist that describe the investigation of circadian rhythms using luciferase as a reporter gene. The phenomenon of naturally bioluminescent circadian rhythms of the dinoflagellate *Gonyaulax* was used as the model for this research. The main advantage of using this approach is that by detecting light, it is possible to monitor oscillations of metabolic processes without accumulation of the product of bioluminescent reactions, i.e., photons of light. Artificial bioluminescent circadian reporters were constructed for both eukaryotic and prokaryotic organisms, and the mechanisms of the circadian clock were revealed.^{7,8} Recent work on the mechanisms of the circadian clock of the unicellular green alga *Chlamydomonas reinhardtii* describes approaches used to engineer a luciferase-based real-time reporter of circadian rhythms.⁹ When the performance of the firefly luciferase reporter gene was compared with a fluorescent reporter, GFP, and with other luciferases, such as the bacterial luciferase and *Renilla* luciferase, it was shown that firefly luciferase is better suited for this application, as it has a half-life of only a few hours and does not require external illumination of live cells, thus avoiding the problem of autofluorescence and photodamage to the cells. In addition, firefly luciferin is more soluble and affordable than other luciferins, thus facilitating development of an automated high-throughput platform for monitoring circadian rhythms. Matsuo and colleagues engineered a *C. reinhardtii* strain expressing firefly luciferase under the control of the circadian-regulated *psbD* gene promoter.¹⁰ It was shown that (1) the emitted light signal maintains a circadian period for several days under constant light and constant darkness; (2) the signal is temperature compensated (an innate property of all circadian clocks); and (3) the circadian phase is sensitive to light pulses. The created bioluminescent reporter

strains can now be used in conjunction with other molecular approaches, such as reverse genetics, to test *C. reinhardtii* genes homologous to those involved in other circadian systems.

4.1.3 Bioluminescent gene expression reporters for viral research and bacteriology

Another area where expression of bioluminescent reporter genes has found wide application is in the investigation of viral pathogenesis. Studies of viral invasiveness and pathogenesis generally rely on murine models that require the sacrifice of infected animals to determine viral distribution and titers. Viral particles do not possess the machinery for expression of genes encoded in their genome; they rely on the metabolic pathways of their hosts for replication. During the time course of infection, the viral genome is inserted into the host cell, and expression of viral proteins begins. This feature of viral infection was used to develop a bioluminescence method for tracking infection. Luciferase genes were inserted into the genome of the herpes simplex virus, and with bioluminescence, invasiveness and spread of infection were monitored in live infected mice by measuring spatiotemporal distribution of the emitted light (Fig. 4.2).¹¹ The results of bioluminescence monitoring correlated well with the results obtained by real-time PCR detection of viral DNA. The main advantages of this bioluminescence approach are the ability to detect infection at any site of a living mouse, the

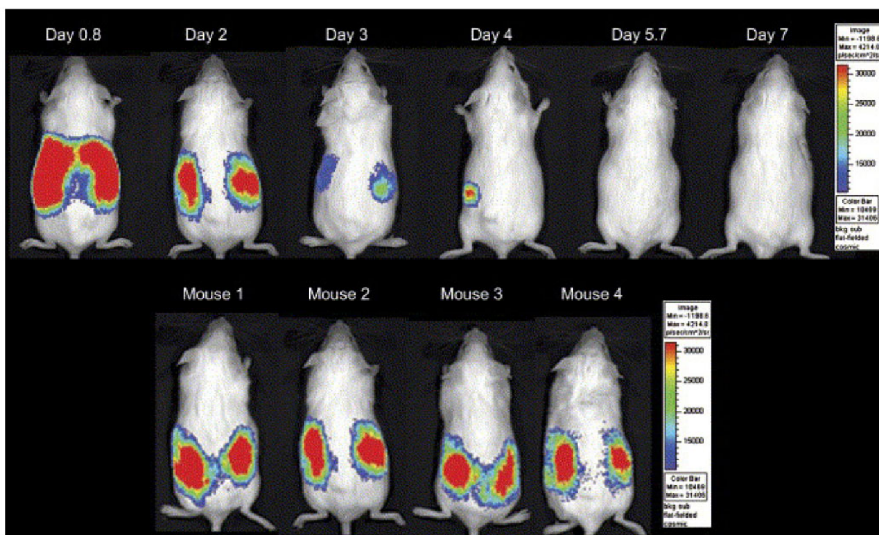


Figure 4.2 (Top) Time course of hematogenous infection of mice by recombinant herpes simplex virus type 1 monitored by bioluminescence. Four 9-week-old CD-1 female mice were infected by IP injection of 2×10^7 PFU of HSV, and bioluminescence was monitored for 7 days. Bioluminescence images of a mouse are overlaid on grayscale photo images. (Bottom) Variability between animals is represented by images of 4 animals on the 2nd day post infection. (Adapted from Ref. 11, © 2006 Elsevier.)

possibility of repeatedly imaging the same mouse, and easy quantification of the signal. Limitations of the method include animal tissues partially attenuating the light signal through scattering or absorption and the possibility of overlapping infection sites in 2D images. These circumstances would make it impossible to distinguish infection sites with bioluminescence monitoring alone and could result in underestimating or overestimating the infection.

Recombinant bacterial viruses—bacteriophages, carrying luciferase genes—were created for the purpose of detecting the presence of target bacterial pathogens. The principle that was used to monitor viral infection in mammals also underlies this application. The phages are not able to express the genes, so they remain dark. When the phage infects the host cell, however, the luciferase is synthesized, causing the bacteria cells to light up and thus be detected. This approach was first suggested by Ulitzur and Kuhn in 1987.¹² They put those genes that were encoding a bacterial luciferase (*lux* genes) into phages specific for *E. coli*. The proposed approach is based on the property of a lysogenic bacteriophage to insert its genome into a bacterial chromosome and use the cellular machinery for expression of the encoded genes intracellularly. This is represented schematically in Fig. 4.3. After the recombinant bacteriophage is brought into contact with the host bacteria, infection occurs, followed by expression of bacterial luciferase in the bacterial cells. The addition of long-chain aldehyde to the sample results in bright bioluminescence. Later bioluminescent reporter bacteriophages were constructed for detection of *Enterobacteriaceae*, *Listeria monocytogenes*, *Salmonella*, and *Mycobacterium tuberculosis*.¹³ Bioluminescent phage-based methods for enumeration of pathogens were successfully used for testing food and environmental samples. The system allowed *Salmonella* cells to be detected directly in artificially contaminated whole eggs without disruptive sampling.¹⁴ After incubation for 24 hours, eggs inoculated with 10^2 – 10^3 colony forming units (CFU) per egg became luminescent after spraying with the recombinant bacteriophage (Fig. 4.4).

While the application of this technology for clinical on-spot diagnostic purposes has not been reported, the technique was used for rapid testing of antibiotic susceptibility. The method proved to be extremely useful, especially for slow-growing bacteria such as *Mycobacterium tuberculosis*.¹⁵ Temperate (lysogenic) *M. tuberculosis*-specific bacteriophage with a firefly luciferase gene inserted into its genome was used to infect an isolated bacterial culture at a multiplicity of infection of approximately 10^3 for 4–6 hours at 37 °C. A luciferin-containing buffer was then added to the sample, and light output was registered. The higher light output was from the samples containing strains that were more resistant to the antibiotic tested. In a comparison study performed on 25 clinical isolates, an excellent correlation was observed between the luciferase reporter-phage (LRP) technique and conventional cultural methods. The essential advantage of the LRP-based method is that the results are obtained within hours compared to the weeks required in traditional testing.

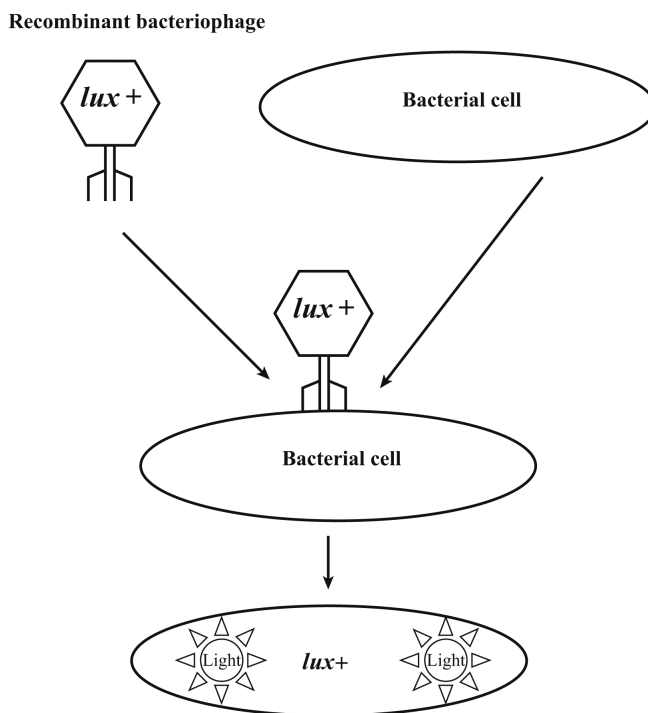


Figure 4.3 Schematic representation of bacteriophage-mediated bioluminescence detection of bacteria.

4.1.4 Bioluminescent gene expression reporters for toxicity testing

Toxicity assays based on whole-cell viability assessment represent the general type of nonspecific acute toxicity measurements (see Chapter 3). Moving a step further in specificity, a variety of semi-specific, stress-responsive whole-cell biosensors were developed (for review, see Ref. 16).

During the course of evolution, bacteria developed the ability to sense and respond to various adverse environmental conditions. The ultimate stress-response result is a repair, restoration, or degradation of the damaged elements. The schematic representation of response events is presented in Fig. 4.5. In general, the cellular damage leads to the generation of a signal for activated transcription of specific gene-encoding proteins that combat the toxic agent or repair the caused damage.

Thus, monitoring the up-regulated gene expression can reveal the nature of biological damage caused by an agent. Identification of responsive elements in the bacterial genome through large microarray experiments allowed for identifying specific regulatory sites that are involved in response to particular stress. This knowledge served as a foundation for the creation of a variety of cellular biosensors that 'reported' the presence of pollutants and toxic compounds in the environment by initiation of reporter gene expression. Evidently, the reporter gene product

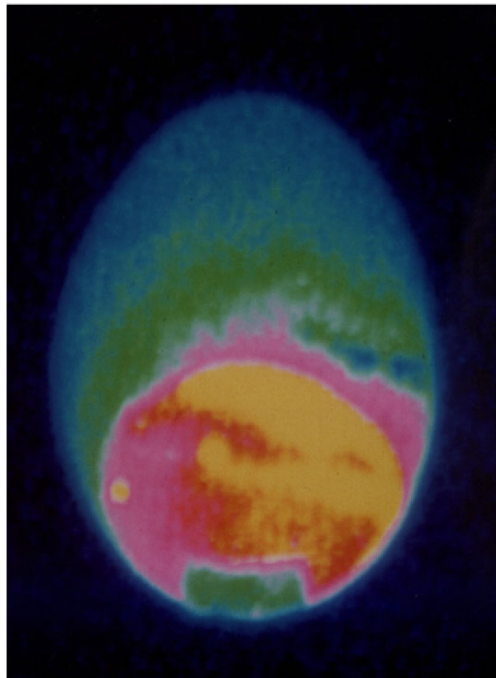


Figure 4.4 Bioluminescent image of the egg inoculated with 10^3 CFU of *Salmonella* sp. and incubated at 37 °C for 24 hours. Recombinant (*lux AB+*) phage was added to the egg, and the bioluminescent image was taken 30 min after infection.¹⁴

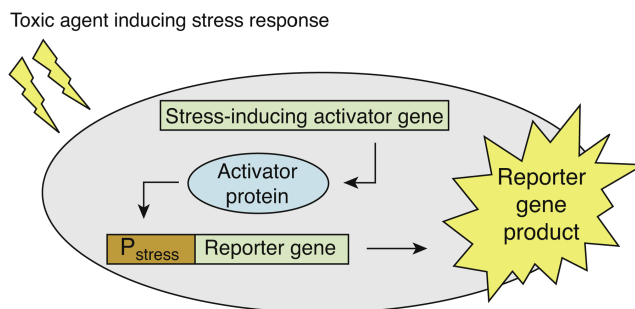


Figure 4.5 Schematic presentation of cell-based semispecific toxicity biosensor.

should be easily assayed, which is why the reporter of choice mainly involves genes encoding bioluminescence or fluorescence (Table 4.2).

The use of stress responses for toxicity monitoring provides more information on the nature of toxicity and greater sensitivity than does monitoring approaches based on cellular viability. The specificity level of these biosensors depends on the specificity of the responsive element. In a case when the reporter gene is fused to general stress-response elements initiated by, for example, DNA damage (SOS response) or by protein damage (heat shock response), the resultant biosensors are referred to as semi-specific. Several types of semi-specific biosensors with different

Table 4.2 Selected reporter gene systems for toxicity testing (adapted from Ref. 16).

| Gene | Product protein | Detection |
|------------------|---------------------------|---|
| <i>luc</i> | Firefly luciferase | Light after addition of substrate (Luminometer, CCD camera, photographic film, scintillation counter) |
| <i>lux AB</i> | Bacterial luciferase | Light after addition of substrate (Luminometer, CCD camera, photographic film, scintillation counter) |
| <i>lux CDABE</i> | Bacterial bioluminescence | Light (Luminometer, CCD camera, photographic film, scintillation counter) |
| <i>ruc</i> | <i>Renilla</i> luciferase | Light after addition of substrate (Luminometer, CCD camera, photographic film, scintillation counter) |
| <i>gfp</i> | Green fluorescent protein | Fluorescence by fluorescence-activated cell sorting, fluorescence microscopy, visually |
| <i>dsRed</i> | Red fluorescent protein | Fluorescence by fluorescence-activated cell sorting, fluorescence microscopy, visually |
| <i>lacZ</i> | β -galactosidase | Product of enzymatic reaction after addition of appropriate substrate (luminometry, fluorometry, spectrophotometry, visually) |

stress-inducible pathways can be applied in one screening to obtain more specific information on the type of toxicity, which is a clear advantage for environmental toxicity testing. The stress promoter–reporter gene fusions can be present in separate strains, and the assays are run in parallel in this case. Alternatively, a biosensor strain can carry two inducible reporters with different gene products that can be assayed separately.

The significant advantages of cell-based semi-specific biosensors is that they react only to a bioavailable fraction of pollutants, they provide information on the nature of toxicity, and they can be run in a high-throughput format. On the other hand, the concept of using positive response as a measure of toxicity could make it more difficult to avoid false-negative results. The exposure of a biosensor too close to the lethal concentration of a toxic compound could result in the decrease or even lack of a signal due to the loss of viability. This problem could be mitigated in two ways: (1) measuring the viability of the cell and the stress response simultaneously, or (2) combining features of a general and semispecific biosensor help to circumvent the problem of false-negative results. If the biosensor contains two distinct reporter genes, one of which is fused to a constitutively expressed promoter and the other to a stress-related promoter, a constant basal level of expression of both genes indicates the true negative result; whereas, a decline in the former gene implies that the biosensor is inhibited or inactivated and is not able to produce the stress response.

Using this approach, the recombinant SWITCH test was developed for the determination of geno- and cyto-toxicity of ground water and sediment.¹⁷ The test is based on the recombinant *Salmonella typhimurium*, a strain that carries the SWITCH plasmid, which is a combined construct of the SOS-*lux* and *lac*-GFPuv. In this construct, the entire *lux* operon (*lux CDABE*) is fused to the promoter *pcolD*, which is dependent on the inducible SOS-DNA repair system in bacteria. Upon exposure to genotoxin, the SOS-DNA repair system and its associated