ABSTRACT: Luciferases are enzymes that emit light in the presence of oxygen and a substrate (luciferin) and which have been used for real-time, low-light imaging of gene expression in cell cultures, individual cells, whole organisms, and transgenic organisms. Such luciferin–luciferase systems include, among others, the bacterial lux genes of terrestrial Photorhabdus luminescens and marine Vibrio harveyi bacteria, as well as eukaryotic luciferase luc and ruc genes from firefly species (Photinus) and the sea panzy (Renilla reniformis), respectively. In various vectors and in fusion constructs with other gene products such as green fluorescence protein (GFP; from the jellyfish Aequorea), luciferases have served as reporters in a number of promoter search and targeted gene expression experiments over the last two decades. Luciferase imaging has also been used to trace bacterial and viral infection in vivo and to visualize the proliferation of tumour cells in animal models. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: luciferases; gene expression; low-light imaging; luciferase expression constructs

INTRODUCTION

From time immemorial, seamen and fishermen have observed ‘lights’ on the water. In the nineteenth century it was realized that the most frequent cause of such luminous oceanic phenomena are minute marine organisms emitting light—bioluminescence. About 35 years ago, various lucifers began to be characterized (1, 2) which, in their many forms, in the presence of a substrate, a luciferin, emit light in the visible range under physiological conditions. Some eukaryotic organisms, such as the firefly (Photinus), have their own luciferin–luciferase light-emitting systems. Many marine organisms, however, such as mid-depth fishes and invertebrates such as molluscs, emit light because of symbioses with luciferase-producing bacteria occurring in highly specialized light organs. These luminescent bacteria include taxa such as Photobacterium phosphoreum, P. leiognathi, Vibrio logei, V. harveyi and V. fischeri.

It is to be expected that a costly characteristic like biological production of light would be retained only if luminescent visualizing were advantageous. Bioluminescence is used as a disguise for fleeing prey, for ventral light emission to efface an organism’s shadow and render it invisible from below (3, 4), for luring prey (ceratioid fish), for signalling for courtship and mating, and in stress-induced light emission (bioluminescent plankton). One could argue that ever since such metazoan bioluminescent bacteria symbioses and other bioluminescent organisms appeared in the oceans with their unique light emission systems, there has been in vivo luminescent ‘imaging’ or visualization.

NATURAL LUMINESCENT ‘VISUALIZATION’

Marine bioluminescence may be considered one of the most widespread forms of communication on the planet. Organisms emit light that other organisms detect or ‘visualize’ and to which they give some behavioral response (5). Behavior based on natural bioluminescence imaging may be classified under three general headings (5): offence (luring, baiting); defence (startle, camouflage); and communication (courtship and mating). Some striking uses of natural bioluminescent ‘visualization’ include the following: some squids with bacterial symbionts use shadow-effacing, or modulation of their ventrally-emitted light, to match ambient sunlight or moonlight; crustaceans, similar to fireflies, may use a repetitive mating ‘Morse code’ of blinks; some jellyfishes deposit an adhesive glow upon contact with predators, leaving the predator visible and vulnerable; some squids flee, leaving a luminescent cloud of ‘ink’ in a predator’s face; some dragonfishes (Malcosteidae) emit blue-green light, but also emit a ‘night-vision’ long-wavelength red light by which they can...
detect prey (using reverse fluorescence energy transfer) without their prey seeing them (6–12).

The purpose here is to review the representative scientific imaging applications to which these naturally occurring visible light bioluminescent systems, the genes encoding the proteins and their modifications have been put. However, we first present an overview of the luciferin–luciferase light emission systems.

PHYLOGENY AND EVOLUTION

Luciferase is a generic name because none of the major luciferases share sequence homology with each other (5). Luciferases occur in bacteria, fungi, dinoflagellates, radiolarians and about 17 metazoan phyla and 700 genera, mostly marine (5, 12, 13). These include Annelida (segmented worms), Chordata (some elasmobranchiomorphs or sharks, many teleosts or bony fishes), Cnidaria (jellyfishes, anhozoans such as the sea pansy, Renilla), Chaetognaths (one species of arrow-worm), Crustacea (many, including ostracods and euphausiid shrimps or krill), Ctenophora (comb jellies), Echinodermata (sea stars, brittle stars), hemichordate worms, Insecta (fireflies, click beetles), Mollusca (squids, octopods, nudibranchs), Nemertean worms (one species), Pycnogonids (sea spiders), Urochordata (larvaceans, radiolarians, and some fish taxa). Coelenterate luciferase activity is controlled by the concentration of Ca\(^{2+}\) and shares homology with the calcium-binding protein calmodulin (5).

Firefly luciferin (a benzothiazole) is found exclusively in fireflies (Photinus or Luciola). It has the unique property of requiring ATP as a co-factor to convert it to an active luciferin (5). It was realized early that firefly luciferin–luciferase could be used to determine the presence of ATP (23). This has become a standard ATP assay. For one example, since nickel alloys have been shown to have an adverse effect on respiratory metabolism in eukaryotic cell lines, the firefly luciferin–luciferase system has been used to document depressed levels of ATP in cells exposed to the alloys (24).

The mechanisms of bioluminescence utilized by amphipods, bivalves, earthworms, fresh-water limpets, fungus gnats, larvaeceans, nemertean worms, polychaete worms and tunicates are currently unknown. Luciferin–luciferase bioluminescence systems are multiform phenomena and polyphyletic in origin.

GENES AND cDNAs ENCODING DIFFERENT LUCIFERASES

Science has entered into the field of bioluminescent visualization in far more recent times. In the last few decades, many luciferase genes have been isolated, sequenced at least in part, and used to build DNA vectors. In Table 1 we summarize the DNA fragments and cDNAs that encode the different luciferases significant in scientific imaging.

The luciferases most commonly used in experimental bioluminescent imaging applications include the bacterial
Table 1. A summary of known luciferase genes, cDNAs, and proteins. Among these are the prokaryotic luciferases (Lux), eukaryotic luciferases (Luc, Ruc and their regulatory proteins) both of which are commonly used in imaging of luciferase expression in living cells, tissues, and organisms

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Gene – cDNA (size in bp)</th>
<th>Protein product (size in number of amino acids)</th>
<th>Gen Bank accession no. (DNA and amino acid)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio harveyi</em></td>
<td>luxA, 1067 bp</td>
<td>z subunit, 355 aa</td>
<td>M10961</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AAA88685</td>
<td></td>
</tr>
<tr>
<td></td>
<td>luxB, 947 bp</td>
<td>β subunit, 324 aa</td>
<td>M10961.1</td>
<td>223</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AAA88686</td>
<td></td>
</tr>
<tr>
<td></td>
<td>luxE, 1136 bp</td>
<td>acyl-protein synthetase, 378 aa</td>
<td>M28815.1</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AAA27531</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio fischeri</em></td>
<td>luxA, 1064 bp</td>
<td>alkanal mono-oxygenase z-chain, 354 aa</td>
<td>X06758</td>
<td>224</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>CAA29931</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio fischeri</em></td>
<td>luxB, 980 bp</td>
<td>alkanal mono-oxygenase β-chain, 326 aa</td>
<td>X06797</td>
<td>224</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>CAA29932</td>
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</tr>
<tr>
<td><em>Vibrio fischeri</em></td>
<td><em>LuxRICDABEG</em> operon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>luxR, 752 bp</td>
<td>regulatory protein LuxR, 250 aa</td>
<td>AF170104</td>
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</tr>
<tr>
<td></td>
<td>luxI, 581 bp</td>
<td>autoinducer synthesis protein LuxI, 193 aa</td>
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<td>luxC, 1439 bp</td>
<td>acyl-CoA reductase LuxC, 479 aa</td>
<td>AAA48474</td>
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</tr>
<tr>
<td></td>
<td>luxD, 923 bp</td>
<td>acyl transferase LuxD, 307 aa</td>
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<td></td>
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<td>luxA, 1077 bp</td>
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</tr>
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<td>luxB, 993 bp</td>
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</tr>
<tr>
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<td>luxE, 1136 bp</td>
<td>long chain fatty acid luciferin component ligase LuxE, 378 aa</td>
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<tr>
<td></td>
<td>luxG, 722 bp</td>
<td>probable flavin reductase LuxG, 236 aa</td>
<td>AAA48480</td>
<td></td>
</tr>
<tr>
<td><em>Photobacterium luminescens</em> = <em>Xenorhabdus,</em> since 1999 reduced to synonymy (225)</td>
<td><em>LuxCDBAEG</em> operon</td>
<td>fatty acid reductase LuxC, 480 aa</td>
<td>M62917</td>
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<td>luxC, 1442 bp</td>
<td>acyl transferase LuxD, 307 aa</td>
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<td></td>
<td>luxD, 923 bp</td>
<td>alkanal mono-oxygenase z-chain LuxA, 362 aa</td>
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<td>luxA, 1088 bp</td>
<td>alkanal mono-oxygenase β-chain LuxB, 324 aa</td>
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<td>luxB, 974 bp</td>
<td>acyl-protein synthetase LuxE, 116 aa</td>
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<tr>
<td></td>
<td>luxE, 347 bp</td>
<td></td>
<td>AAA63567</td>
<td></td>
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<td>Taxa</td>
<td>Gene – cDNA (size in bp)</td>
<td>Protein product (size in number of amino acids)</td>
<td>Gen Bank accession no. (DNA and amino acid)</td>
<td>Reference</td>
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</tr>
<tr>
<td><em>Photinus pyralis</em></td>
<td><em>luc</em>, 2387 bp</td>
<td>Luciferase, 550 aa</td>
<td>M15077</td>
<td>226</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AAA29795</td>
<td></td>
</tr>
<tr>
<td><em>Luciola cruciata</em></td>
<td><em>luc</em>, 1985 bp</td>
<td>Luciferase, 548 aa</td>
<td>M26194</td>
<td>227</td>
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<tr>
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<td>AAA29135</td>
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<tr>
<td><em>Vargula hilgendorfii</em> (sea</td>
<td><em>vuc</em>, 1834 bp</td>
<td>Vargulin, 611 aa</td>
<td>E02749</td>
<td>228</td>
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<tr>
<td>firefly)</td>
<td><em>vuc</em> mRNA, 1818 bp</td>
<td>Vargulin, 555 aa</td>
<td>M25666</td>
<td>39</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AAA30332</td>
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</tr>
<tr>
<td><em>Aequorea victoria</em></td>
<td><em>aeq1</em>, 672 (590) bp</td>
<td>Aequorin 1; calcium-binding protein, 196 aa</td>
<td>M16103</td>
<td>229</td>
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<tr>
<td></td>
<td><em>aeqprec</em>, 568 bp</td>
<td>Aequorin precursor, ~189 aa</td>
<td>AAA27719</td>
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<td><em>aeq2</em>, 531 bp</td>
<td>Aequorin 2, 177 aa</td>
<td>M11394</td>
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<tr>
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<td><em>aeq3</em>, 551 bp</td>
<td>Aequorin 3, 177 aa</td>
<td>AAA27716</td>
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<td><em>aq440</em>, 925 bp</td>
<td>Aequorin, calcium binding - 196 aa</td>
<td>M16104</td>
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<td><em>aqua</em>, 587 bp</td>
<td>Luminescent protein Aqualine, ~196</td>
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<td></td>
<td></td>
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<td>M16105</td>
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<td></td>
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<td>AAA27718</td>
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<td></td>
<td></td>
<td>L29571</td>
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<td>E02319</td>
<td></td>
</tr>
<tr>
<td><em>Oplophorus gracilorostris</em></td>
<td><em>luc</em>, 590 bp</td>
<td>Oplophorin, oxygenase, imidazopyrazinone luciferase, 196 aa</td>
<td>AB030246</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td><em>luc</em>, 1079 bp</td>
<td>Oplophorin, oxygenase, imidazopyrazinone luciferase, ~359 aa</td>
<td>BAB13776</td>
<td></td>
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<tr>
<td><em>Renilla muelleri</em></td>
<td><em>ruc</em>, 1208 bp</td>
<td><em>Ruc</em>, 311 aa</td>
<td>AY015988</td>
<td>Szent-Gyorgyi CS, Bryan BJ. cDNA encoding <em>Renilla muelleri</em> luciferase (unpublished manuscript).</td>
</tr>
<tr>
<td><em>Renilla reniformis</em></td>
<td><em>ruc</em>, 1196 bp</td>
<td><em>Ruc</em>, oxygenase, 311 aa</td>
<td>M63501</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AAA29804</td>
<td></td>
</tr>
</tbody>
</table>
luciferases (lux) from the marine genera *Photobacterium* and *Vibrio*, firefly luciferase (*Photinus*), aequorin (luciferase from the jellyfish *Aequorea*), vargulin (luciferase from the marine ostracod *Vargula*), oplophoran luciferase (deep-sea shrimp *Oplophorus*) and *Renilla* luciferase (anthozoan sea pansy, *Renilla reniformis*).

- **Bacterial luciferase.** Bacterial luciferase proteins were purified and isolated from the light organs of mid-depth fishes in the ocean (25, 26). It was known early that the catalytic site was on the α subunit (27). Belas et al. (1982) isolated and expressed luciferase genes from *Vibrio harveyi* in *E. coli* (28). Olsson et al. (1988) characterized the activity of the luxA subunit of *Vibrio harveyi* luciferase by visualizing various luxA and luxB truncations, as well as a luxAB fusion expressed in *E. coli* (29). Olsson et al. (1989) furthermore made monomeric luxAB fusions and expressed them also in *E. coli* (30). The *Vibrio harveyi* luxA and luxB cDNAs were cloned and sequenced in the mid-1980s (31–33). The luxCDABE operon from the terrestrial bacterium *Photorhabdus luminescens* was cloned and sequenced and its product, luciferase, was characterized and published in 1991 (34).

- **Firefly luciferase.** The active sites and properties of firefly luciferase (*Photinus*) began to be characterized about 35 years ago (35–37). Firefly luciferase was purified and characterized in 1978 (19). The cDNA encoding the luciferase (*Luct*) from the firefly *Photinus pyralis* was cloned and expressed in *E. coli* by De Wet et al. (1985) (38).

- **Vargulin.** A cDNA for the luciferase gene from the marine ostracod *Vargula hilgendorfii* was cloned, sequenced and expressed in mammalian cells by Thompson et al. (1989) (39). They also discovered that *Vargula* luciferase expression requires only its substrate and molecular oxygen (but no co-factors), thus making it potentially more useful for mammalian expression systems (40). The activity of *Vargula* luciferase is not dependent on a pyrazine structure, as has been demonstrated by cross-reaction experiments with the *Oplophorus* luciferin (41).

- **Aequorin.** The aequorin protein was first extracted from the hydromedusa *Aequorea*, purified and characterized in part by Shimomura et al. (1962) (42). In 1975, Shimomura and Johnson described what was known about the mechanisms of various coelenterate luciferins, including aequorin (22). Ward and Cormier (1975) reported the isolation of various *Renilla*-type luciferins, including aequorin (43). A few years later, it was discovered that *Renilla* luciferin analogues were catalysed by luciferase to excited energy states to transfer energy to a green fluorescence protein or GFP (44). Ward and Cormier (1979) characterized the *Renilla* green fluorescence protein (RGFP) and showed that a natural energy transfer was occurring from the isolated *Renilla* luciferase (Ruc) bioluminescence to RGFP (45). In 1985, the cDNA for aequorin was cloned, sequenced and expressed in heterologous systems (46, 47). The aequorin gene from the jellyfish *Aequorea victoria* was cloned in 1990 (48). It is now known that many cnidarians have GFPs that serve as energy-transfer acceptors fluorescing in response to excited oxy-luciferin–luciferase complexes or to a Ca$^{2+}$-activated phosphoprotein. The cDNA encoding the GFP of *Aequorea victoria* has also been cloned and sequenced (49).

- **Oplophorus luciferase.** The general reaction mechanisms and properties of the luciferin–luciferase system of the deep-sea shrimp *Oplophorus graci- lorosstris* were reported by Shimomura et al. (1978) (50). An empirical formula and structure has been suggested for *Oplophorus* luciferin using spectroscopy and cross-reaction with the luciferase of the ostracod *Vargula hilgendorfii* (40). By 1997, *Oplophorus* luciferase was known to have a more intense light emission than either *Renilla* luciferase or the recombinant aequorin. However the *Oplophorus* luciferase cDNA, not yet cloned, could not be used as a reporter gene (51). Recently, Inouye et al. (2000) succeeded in cloning the *Oplophorus* luciferase cDNA (52).

- **Renilla luciferase.** In 1966, Hori and Cormier described some of the properties and a hypothetical partial structure for the *Renilla reniformis* luciferase protein (Ruc) (53). Kreis and Cormier (1967) showed that light could inhibit the activity of Ruc (54). The isolation of Ruc was first done and further properties elucidated by Karkhanis and Cormier (1971) (55). DeLuca et al. (1971) demonstrated that the *Renilla* bioluminescent system involves the oxidative production of CO$_2$ (56). It was further shown that Ca$^{2+}$-triggered a luciferin binding protein, thus inducing the Ruc system (57). Ruc was first purified and characterized by Matthews et al. (1977) (58). The cDNA of *ruc* was isolated and later expressed in *E. coli* by Lorenz et al. (1991) (59). The *ruc* cDNA was also expressed in a number of transgenic plant tissues (60). In 1996, Lorenz et al. expressed Ruc in simian COS-7 cells and in murine C5 cells (61).

In retrospect, it might be noted that since their discovery, Luc (*Photinus*), aequorin (*Aequorea*) and GFP have been used in a multitude of successful experiments. In combination the three have even been useful in assaying or imaging the spatial–temporal concentrations of Ca$^{2+}$ (62). Combinations of multiple
Table 2. A summary of selected luciferase constructs and vectors useful for imaging. (Construct/vector nomenclature not standardized in the literature).

<table>
<thead>
<tr>
<th>Construct/Vector</th>
<th>Luciferase genes or cDNAs</th>
<th>Promoters/enhancers</th>
<th>Organisms/Cells</th>
<th>Substrate requirement</th>
<th>Imaging application and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB101, pB102, pB105, pB110, pB23, pB128, pGMC12</td>
<td>luxA, luxB (V. harveyi)</td>
<td>Phage λ promoters P&lt;sub&gt;j&lt;/sub&gt; and P&lt;sub&gt;k&lt;/sub&gt;</td>
<td>E. coli</td>
<td>None</td>
<td>Expression of lux, luxB in E. coli (28) — first transgenic expression of lux (26)</td>
</tr>
<tr>
<td>Transposon mini-MuLux</td>
<td>luxE, B, A, D, C (V. parahaemolyticus)</td>
<td>lux promoter</td>
<td>E. coli</td>
<td>Bradyrhizobium japonicum in Glycine max</td>
<td>Decanal</td>
</tr>
<tr>
<td>pFT001, pPPE001</td>
<td>luxA, luxB (V. harveyi)</td>
<td>Amp-Tet (P1), nifD, nifB promoters</td>
<td>E. coli; Bradyrhizobium japonicum</td>
<td>Visualization of N-fixation in soybean nodules via Bradyrhizobium japonicum (210)</td>
<td></td>
</tr>
<tr>
<td>Agrobacterium binary vector</td>
<td>luxA &amp; B (V. harveyi)</td>
<td>T&lt;sub&gt;C&lt;/sub&gt; promoter</td>
<td>Daucus carota; Nicotiana tabacum</td>
<td>Visualization of tissue-specific chimaeric expression (169)</td>
<td></td>
</tr>
<tr>
<td>pDO432, pDO435; pDO446</td>
<td>lux (Photinus)</td>
<td>CaMV 35S RNA promoter</td>
<td>Nicotiana tabacum</td>
<td>Visualization of luc expression in tobacco plants (168)</td>
<td></td>
</tr>
<tr>
<td>pPCV701luxA &amp; B</td>
<td>luxA; luxB (V. harveyi)</td>
<td>T&lt;sub&gt;D&lt;/sub&gt;-DNA P&lt;sup&gt;1&lt;/sup&gt; and T&lt;sub&gt;D&lt;/sub&gt;-DNA P&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Nicotiana tabacum; Daucus carota</td>
<td>Assembly and expression of functional luxA and B genes in plants (170)</td>
<td></td>
</tr>
<tr>
<td>pRSVL</td>
<td>luc but imaging by immunofluorescence</td>
<td>Promoter in Rous Sarcoma Virus long terminal repeat (RSV LTR)</td>
<td>Mouse kidney cells (CV-1)</td>
<td>NA</td>
<td>Luciferase peroxisomal localization visualized by immunofluorescence (109)</td>
</tr>
<tr>
<td>pMRP1; pMRD2</td>
<td>luxAB fusion</td>
<td>P&lt;sub&gt;I&lt;/sub&gt; promoter</td>
<td>Soybean (Glycine max)</td>
<td>Decanal</td>
<td>Successful imaging of LuxAB fusion expression in soybean root nodules using photographic film and low-light intensified video microscopy (211)</td>
</tr>
<tr>
<td>pCLLUC → pSCLUC → pVV·luc</td>
<td>luc</td>
<td>7.5 kDa viral promoter. Vaccinia α gene fragments</td>
<td>BSC-40 cells (African green monkey kidney cells)</td>
<td>Firefly luciferin</td>
<td>Imaging of various luc luciferase and LuxF fusion in cells (29)</td>
</tr>
<tr>
<td>plX vector series</td>
<td>luxA, luxB, luxAB (luxF)</td>
<td>T&lt;sub&gt;7&lt;/sub&gt; promoter</td>
<td>B12(ΔE3) cells</td>
<td>Streptomyces coelicolor</td>
<td>Visualization of lucA &amp; B expression in Streptomyces (91)</td>
</tr>
<tr>
<td>pRS1105</td>
<td>luxA, luxB (V. harveyi)</td>
<td>EndoH, N&lt;sub&gt;1&lt;/sub&gt;αluc (luc tRNA), WhiG (uncharacterized), SapA (all Streptomyces promoters)</td>
<td>Decanal</td>
<td>Luminescence from luciferin</td>
<td>Visualization of Lux expression in individual mammalian cells (112)</td>
</tr>
<tr>
<td>plX, pCLLX, pCV702 and p35Slux vector series</td>
<td>luxAB, luxB fusions</td>
<td>T&lt;sub&gt;7&lt;/sub&gt; gene 10 and CaMV 35S promoter</td>
<td>E. coli; tobacco calli</td>
<td>Decanal</td>
<td>Bacterial luciferase signalling functional as a monomer (65)</td>
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<tr>
<td>plX vector series</td>
<td>luxAB</td>
<td>T&lt;sub&gt;7&lt;/sub&gt; gene 10 promoter</td>
<td>E. coli</td>
<td>Decanal</td>
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NA, not applicable.
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<tr>
<th>Construct/vector</th>
<th>Luciferase genes or cDNAs</th>
<th>Promoters/enhancers</th>
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<td>pPCVGluxA and B</td>
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<td>Promoter search expression of LuxA and LuxB in N. tabacum</td>
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</tr>
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<tr>
<td>pCASL40aa-luc</td>
<td>Mas promoter–luxF (V. harveyi)</td>
<td><em>Solanum tuberosum</em></td>
<td>Luciferin (firefly)</td>
</tr>
<tr>
<td>pWH1520SF-luc</td>
<td>AcNPV–luxAB (V. harveyi)–xylA</td>
<td><em>Bacillus thuringiensis</em></td>
<td>Xylose</td>
</tr>
<tr>
<td>pMV16-luc</td>
<td>HIV-1 LTR enhancer–promoters</td>
<td>Single mammalian cells</td>
<td>Luciferase fusions</td>
</tr>
<tr>
<td>pOCMV-luc</td>
<td>CMV promoters</td>
<td>CHO 3T3 cells</td>
<td>Luciferase fusions</td>
</tr>
</tbody>
</table>

**Luminescence (firefly)**

Table 2. Continued

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<tr>
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<td>CMV promoters</td>
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<td>Luciferase fusions</td>
</tr>
</tbody>
</table>

**Firefly luciferase**

Table 2. Continued

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<tr>
<td>pCASL40aa-luc</td>
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<td>pWH1520SF-luc</td>
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<td><em>Bacillus thuringiensis</em></td>
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<td>pMV16-luc</td>
<td>HIV-1 LTR enhancer–promoters</td>
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<td>pOCMV-luc</td>
<td>CMV promoters</td>
<td>CHO 3T3 cells</td>
<td>Luciferase fusions</td>
</tr>
</tbody>
</table>

**Autographa californica nuclear polyhedrosis virus–vectored**

Table 2. Continued

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<td>pCASL40aa-luc</td>
<td>Mas promoter–luxF (V. harveyi)</td>
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<td>pWH1520SF-luc</td>
<td>AcNPV–luxAB (V. harveyi)–xylA</td>
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<td>pMV16-luc</td>
<td>HIV-1 LTR enhancer–promoters</td>
<td>Single mammalian cells</td>
<td>Luciferase fusions</td>
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<tr>
<td>pOCMV-luc</td>
<td>CMV promoters</td>
<td>CHO 3T3 cells</td>
<td>Luciferase fusions</td>
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<td>pCEP4</td>
<td>ruc–gfp fusion, and individually</td>
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<tr>
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<td>CMV, β-actin, (for pCEP4 vector), and 77 (pGEM-5zf+ vector) promoters –</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Construct/vector</td>
<td>Luciferase genes or cDNAs</td>
<td>Promoter/enhancers</td>
<td>Organism/cells</td>
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<tr>
<td>RD29A-luc</td>
<td>luc</td>
<td>RD29A promoter</td>
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</tr>
<tr>
<td>pLPL–LuxΩ; pL4–LUX–pL2; pNS–luxΩ</td>
<td>luc, humanized luc; ruc</td>
<td>Rat L–PK (liver-type pyruvate kinase)</td>
<td>Pancreatic (MIN6) β-cell islets</td>
</tr>
<tr>
<td>pLPL–LuxΩ; p–150–LPL–LuxΩ; pNS–Luc; pRL-CMV; pPGK4-Luc</td>
<td>luc, humanized luc; ruc</td>
<td>Rat L–PK, human insulin, Herpes simplex minimal TK and CMV promoters</td>
<td>Pancreatic (MIN6) β-cell islets</td>
</tr>
<tr>
<td>pND3 Ruc/GFP; pND2 Ruc/GFP</td>
<td>ruc/gfp; secreted ruc/gfp (Streptomyces)</td>
<td>CMV promoter</td>
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</tr>
<tr>
<td>pGL3</td>
<td>luc</td>
<td>–</td>
<td>9L-luc rat gliosoma cells in vivo in Fischer 344 rats</td>
</tr>
<tr>
<td>Tn5001 luxABCDE Km'</td>
<td>luxABCDE Km' operon (Photobacterium luminescens; Gram+)</td>
<td>Promoteless - a promoter search vector: Random transposon insertion into the S. pneumoniae genome behind stronger or weaker promoters</td>
<td>Lux-transformed Gram + Streptococcus pneumoniae infection in BALB/c mice</td>
</tr>
<tr>
<td>AdCMV Luc; Ad5LucRGD</td>
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<td>A549 cells</td>
<td>Cell membrane-permeable acetoxy methyl ester derivative of n-tuliferin</td>
</tr>
<tr>
<td>AAV-EFIα-luciferase (rAAV); pSV-EIα-luciferase; pXQ2; pXQ6</td>
<td>luc</td>
<td>EF1α promoter</td>
<td>Mouse (CD-1)</td>
</tr>
<tr>
<td>AdCMVLuc; pLuc; AdRepKluc; pneoAC; pCMVLuc; pAdTrackCMV; pAdCMVmAq</td>
<td>Aequorin (aeq1; humanized)</td>
<td>CMV and human preproinsulin (PPI) promoters</td>
<td>MIN6 β-cell islets</td>
</tr>
<tr>
<td>pAM401 ASGX pSB2035</td>
<td>lucCDABE (Photobacterium luminescens) fused to gfp (Aequorea) luxCDABE (Photobacterium luminescens); Aequorea gfp</td>
<td>SOD (superoxide dismutase) promoter</td>
<td>E. coli and other Gram-negative bacteria; visualized in murine mouse</td>
</tr>
<tr>
<td>pAM401 ASGX pSB2035</td>
<td>lucCDABE (Photobacterium luminescens) fused to gfp (Aequorea) luxCDABE (Photobacterium luminescens); Aequorea gfp</td>
<td>77 and CMV promoters</td>
<td>Simian COS-7 cells</td>
</tr>
<tr>
<td>Ad–CMV–Luc</td>
<td>CMV promoter</td>
<td>Muscles of immunoconunpant Swiss Webster mice</td>
<td>Luciferin (freely)</td>
</tr>
<tr>
<td>AdSV40Luc; AdHIVLuc; rLNC/AdLuc; pLNC–AdLuc</td>
<td>CMV, CMV/CMV (P82); SV40, CMV, BGLAP (osteocalcin); BHL–LHR, SV40 and CMV, SV40 and H19 promoters</td>
<td>Transduced cell lines: Hep2/D2Luc human hepatitis 2 cell line, DM2Luc breast carcinoma cell line, P384Luc/3 (clone of human human prostate adenocarcinoma), T50FLuc</td>
<td>Beetle luciferin</td>
</tr>
</tbody>
</table>
photoproteins as simultaneous reporters will probably become more common in the future.

**Fusions**

The papers cited above show that luciferases have been very useful as reporter genes in living cells and in bioluminescent immunoassays (63). In fact there were about 30 photoprotein fusions and conjugates reported between 1988 and 2000 (63–65). The first such fusion was that of the *Vibrio harveyi* luxA and luxB into the luxAB (luxF) fusion, which was expressed as a monomer in *E. coli*, *Bacillus*, yeast and plant systems (30, 66). A luxAB fusion has also been made from the luciferase of *Photorhabdus luminescens* (67). A gfp–luxAB fusion construct expressed in *E. coli* DH5α and *Pseudomonas fluorescens* SBW25 was used to assay bacterial numbers and nutrient-based bacterial metabolic activity in soil samples (68). Assaying the luxAB expression required luminometry (For more information, see section on *Bacterial luciferase imaging in environmental health assessments*, below).

An ruc–gfp fusion construct was first engineered and expressed in murine LM–TK fibroblast cells, in embryonic stem (ES) cells and in early stage embryos by Wang et al. (1996) (69). A ruc-modified gfp fusion was found to be functionally expressed in murine LM–TK cells, whereas a reverse modified gfp–ruc fusion showed no GFP expression, probably because of misfolding (70). In 1999, Wang et al. suggested that chemiluminescent energy transfer Ruc to GFP could be used to image protein–protein interactions (71).

Using an ruc–gfp fusion construct, Wang et al. (2001) imaged the luminescence resonance energy transfer (RET) phenomenon from *Renilla* luciferase (Ruc) emission to a humanized *Aequorea* GFP to document protein–protein interaction in eukaryotic cells (72). cDNAs of ruc and insulin-like growth factor binding protein 6 (Ruc–IGFBP-6) were expressed, along with fused cDNAs for gfp and insulin-like growth factor II (gfp–igf-II). The interaction of the recombinant IGF-II and IGFBP-6 resulted in LRET (luminescence resonance energy transfer) from Ruc to GFP. In 2000, Liu et al. visualized and quantified protein secretion using an Ruc–GFP fusion in the COS-7 and Chinese hamster ovary (CHO) cell lines (73). In simian COS-7 cells, intensified CCD camera imaging has been used to detect LRET from Ruc to aequorin GFP in an Ruc–GFP fusion protein (72).

An important recent development is the use of *P. luminescens* luxCDABE gene fusions in a collection of 8066 individual *E. coli* transformants to assay genome-wide expression profiles in response to environmental stress (74).

**LOW-LIGHT IMAGING TECHNOLOGY**

In 1989, Wick (75) reviewed the growing usage of single photon-counting visualization being used to assay luminescence in microtitre plates, to image metabolite distribution in tumour tissues, to visualize single cell gene expression, and even to visualize the faint chemiluminescence resulting from oxidative metabolism in phagocytes.

In a series of papers from 1992 to 2000, Stanley reviewed the commercially available luminometers, radiometers, low-light imaging CCD cameras, immunoassays, ATP rapid microbiology, hygiene monitoring, molecular probes, labels, nucleic acid hybridizations and reporter genes available for bio-imaging applications, as well as units and standards of bioluminescence (76–86).

Hill and Stewart (1994) reviewed the applied developments of bacterial luciferases as real-time, non-invasive reporters using low-light and photon-counting video cameras (87). They noted their sensitivity and real-time, non-invasive nature and their amenability to imaging by photon-counting and low-light video cameras. Contag et al. published two reviews on the various molecular imaging technologies for the detection and tracking of molecules and cells *in vivo* (88, 89). They also described briefly the work done in imaging tumors using luciferase expression *in vivo*.

It is to the imaging experiments that we now turn (see Table 2).

**APPLICATIONS OF LUCIFERASE IMAGING**

We attempt to summarize the imaging of luciferase expression in individual cells and cell cultures, in individual bacteria, yeasts, algae, insect cells, plant cells, or mammalian cells. We also focus on some of the applications and types of questions that can be answered by such imaging. These imaging experiments include the imaging of luciferases in transformed cells and cell systems in real time.

Next, we consider imaging of luciferase expression in multicellular organisms *in vivo*. This includes the expression and visualization of luciferase in permanently transformed or transgenic organisms.

Finally, we present representative studies using luciferase expression imaging to investigate host–pathogen interactions in whole plant and animal models.

**IMAGING OF LUCIFERASE EXPRESSION IN TRANSFORMED SINGLE CELLS AND CELL CULTURES**

**Imaging of prokaryotic (bacterial) luciferases in cells and cell cultures**

Imaging of bacterial luciferase in bacteria and cell culture has proved to be a fruitful venture. Some representative
papers are overviewed below. The monitoring of bioluminescent bacterial pathogens and symbionts in hosts is discussed in the section on symbiosis and host–pathogen interactions.

In a landmark paper, Engebrecht and his co-workers showed, in 1985, that the bacterial luciferase operon luxCDABE (i.e. luxICDABE under the control of luxR and luxI) from Vibrio fischeri could be vectored by a transposon and expressed in E. coli without the need to add the lux substrate, decanal, because of the presence of the full operon (26). Ulitzur and Khun (1987) also discussed the use of introduced luciferase genes in bacteria as an assay for the presence of particular bacteria and their susceptibility to a given antibiotic (90). In 1988, Schauer et al. visualized Vibrio harveyi luxA and luxB expression in Streptomyces coelicolor (91). Olsson et al. (1988) have shown that fusion gene products can be added to the luxA of Vibrio harveyi as long as the N-terminal hydrophobic sequences of the /C97-subunit are preserved intact, in order to retain enzymatic activity (29).

Olsson et al. (1989) constructed luxAB and luxBA fusions of the V. harveyi luciferase genes and expressed them in E. coli (Fig. 1) and in calli of Nicotiana tabacum, indicating their possible application as reporter genes in eukaryotic cells (30). The authors showed that luxAB has higher expression levels than luxBA. This was an important first. Escher et al. (1989) described a fusion of the luxAB genes of V. harveyi and showed it to be capable of functioning as a monomer in E. coli (66). Using video-imaging and spectroscopy, they found that luxAB has an emission spectrum comparable to the wild-type luxA and luxB, but is more sensitive to elevated temperature. In 1991, Langridge et al. provided an overview of the bacterial luciferase gene expression system and its applications, using low-light imaging in other bacteria and in eukaryotic cells, namely plant cells (92).

In a series of studies (93–97) it was shown that Vibrio harveyi luxA, luxB and a luxAB fusion could be successfully expressed under various bacterial, mammalian and viral promoters and visualized by photon-counting imaging in Gram-positive Bacillus thuringiensis and B. megaterium (cf. Fig. 2), Arabidopsis thaliana, Nicotiana tabacum, Trichoplusia ni (cabbage looper) (386) cells and Manduca sextans (tobacco hornworm; Fig. 3a, b; see the section a Imaging of host–pathogen interactions, below) (95). These lux constructs were vectored into transgenic plants by Agrobacterium tumefaciens T-DNA. Through T-DNA integration, promoterless constructs bearing luxA and luxB were randomly inserted into transgenic Nicotiana tabacum. Lux activity was visualized at different developmental stages in different organs (Fig. 4) (96). Bacterial luciferase expression was also successfully imaged in tomato leaves and fruit (Fig. 5a, b) (97). Firefly luciferase (Pluc) was also expressed in some of these experiments.

When promoterless V. harveyi luciferase (luxAB) was introduced downstream of the promoter for the cyanobacterium Synechococcus psbAI gene (a photosystem II protein), its varying expression under constant bioluminescent imaging revealed that prokaryotes also have circadian rhythms (98). Furthermore, when luxAB was inserted randomly by conjugation and subsequent hom-ologous recombination into the Synechococcus genome and transformed clones were monitored by the then newly developed cooled-CCD camera system, it was found that luciferase expression in these cyanobacteria exhibited not only circadian rhythmicity but a
wide variety of amplitude and waveform cyclicity (99). Liu et al. also discovered that random insertion of promoterless luciferases by homologous recombination is an extremely sensitive assay for differential gene expression levels. Thus, bacterial luciferase imaging was useful in documenting a complex gene expression phenomenon such as cyanobacterial circadian rhythms (100).

In 2000, Kunert et al. transfected Synechocystis with gfp and luxAB (64). Under different media conditions, luminescence imaging revealed that GFP expression initiated and dissipated at a slower rate, while LuxAB expression had a much more rapid response reaction time. The advantage of GFP in Synechocystis is that it has no substrate requirement. The advantage of LuxAB is its rapid expression response time. So, in combination with another reporter gene, such as GFP, LuxAB can be used as a sensitive measure of gene expression in bacteria.

Waddell and Poppe (2000) developed a mini-transposon bearing Vibrio harveyi luciferase (luxAB) in order to produce a luciferase-transfecting bacteriophage to detect E. coli strain 0157:H7 (101). The E. coli colonies of interest could be visualized by image quantifier about 1 h post-transfection. The ability to use a vectored luxAB to detect the presence of bacteria has led to important environmental and food safety applications, of which we review a few.

Bacterial luciferase imaging in environmental health assays

One of the earliest uses of luciferase imaging to detect microbial contamination involved eukaryotic firefly (Photinus) luciferase (Luc), not bacterial luciferase.
Since that time, both luc and bacterial luxAB assays have been used in environmental sampling. Prosser et al. (1996) reviewed how luminometry and CCD image-enhanced microscopy may be useful in detecting naturally luminescent bacteria in environmental samples, to monitor their growth and metabolism on soil particles, microbial survival and recovery, microbial predation, plant pathogenicity, rhizosphere colonization and the reporting of gene expression in environmental samples (103). They suggested that this technology may also be used to distinguish genetically modified bacteria from indigenous bacteria in environmental samples.

Using a mycobacteriophage T5–luc construct, Sarkis et al. (1995) infected Mycobacterium smegmatis to produce luciferase expression both in the bacteria and in the post-infection lysogen (104). Such luciferase reporter phages may be used to test for the presence of drug-resistant or drug-sensitive M. smegmatis strains, as well as for the rapid identification of other effective antmycobacterial agents.

Loessner et al. showed (1996, 1997) that the Listeria monocytogenes-specific bacteriophage A511, when transformed with the Vibrio harveyi luxAB gene, can be a sensitive detector of viable Listeria cells in environmental and food samples within 24 h, rather than the usual 3 days required for traditional culturing (105, 106). Also, competitive PCR and imaging analysis can be used to quantify the number of luciferase gene copies in sediment samples to which Synechocystis 6803-luc cells were added (107). The authors suggest that this technique should have applications for quantifying genetically modified cyanobacteria in nature.

Although not directly imaging luciferase expression, Unge et al. used a mini-transposon construct bearing a gfp–luxAB fusion to simultaneously assay bacterial cell numbers and populational metabolic activities in specific populations of E. coli DH5z and Pseudomonas fluorescens SBW25 in soil samples (68). Bacterial numbers were determined by flow cytometric monitoring of GFP-expressing cells. LuxAB expression, as determined by luminometry, was shown to be dependent on nutrient levels and hence metabolic activity.

**Bacterial luciferase imaging in food contamination assays**

A bioluminescent reporter strain of Escherichia coli (O157:H7) containing the full bacterial luciferase (luxABCDE) operon was inoculated in buffer and in fecal slurry, which were both placed on surfaces of beef carcases to determine the interaction between potential bacterial pathogens and human food animal tissues (108). A sensitive photon-counting camera was used to visualize the presence of bacteria in real time. The full lux operon in O157:H7 renders substrate addition unnecessary.

**Imaging eukaryotic luciferase expression in cells and cell cultures**

Imaging of luciferase expression in plant and animal cell lines has found more diverse applications than has imaging of luciferase in bacteria. These applications include the imaging of protein site-specific secretion, protein trafficking and protein targeting to the imaging of
transgenic promoter expression, real-time gene activation, cell injury-induced expression and regulation, the determination of ATP and free Ca\(^{2+}\) concentrations, and the visualization of immune response.

Keller et al. showed in 1987 that a firefly luciferin–luciferase cDNA construct could be expressed in monkey kidney cells and that the gene product was targeted to the peroxisomes via a putative peroxisomal targeting protein translocation sequence (109). Using cDNAs of luc with the peroxisomal targeting sequences, Gould et al. helped to demonstrate that such sequences for protein transport are conserved from yeasts to plants, insects and mammals (110, 111). The expression of firefly luciferase (Luc) has been imaged in single COS-7 cells by White et al. (1990) (112).

A method for photographic film detection of firefly luciferase expression regulated by the simian virus 40 promoter in mammalian cells was developed for use with a polyester mesh replica plating technique to determine luciferase expression in mammalian cells (113).

Site-specific protein secretion from transformed Chinese hamster ovary (CHO) cells, containing luciferase from the marine crustacean ostracod Vargula hilgendorfii (Luc), was visualized in real time using an image-intensifying system (114).

Transient expression of luciferase fusion proteins has been an important tool in cell imaging. Luciferase cDNA fused to the 5′-flanking region of the rabbit collagenase gene containing a wild-type promoter showed an increased expression after mechanical injury to the smooth muscle Rb-1 cell line (115). In hormone studies, a modified luciferase expression system was used for real-time measuring of gene expression in endocrine cells (116). Jausons-Loffreda et al. (1994) used single photon-counting technology to document steroid hormone activity in transformed cell lines expressing firefly luciferase, activated by chimeric constructs of the binding domain of the Gal4 yeast protein fused to the hormone-binding domains of various steroid receptors (117). Using promoter–luciferase reporter constructs, dynamic gene expression was visualized in real time and quantified in nursing rat lactotrope cells by Castano et al. (118), who proposed that similar constructs could be used to visualize gene expression in any normal cell type. The Renilla reniformis luciferase cDNA was expressed transiently in simian COS-7 cells and stably in murine C5 fibroblasts and in extracts (61). A firefly luciferase–aequorin fusion protein was used in HeLa cells to detect rapid changes in ATP and free Ca\(^{2+}\) levels, based on light emission in response to C9 complement attack (119). Quantification of ATP concentrations has also been carried out in rat cardiac myocytes by Dorr et al. (1989) (120).

Rat pituitary tumour cells (GH3) were transformed with a construct containing the firefly luciferase gene and 5000 bp of the 5′ flanking region of the human PRL (prolactin) gene, subjected to luciferin, and then imaged by CCD photon-counting for time periods up to 72 h (121). Basal PRL promoter–luciferase activity was compared to stimulated activity after the addition of such stimuli of the PRL promoter as thyrotropin-releasing hormone (TRH), forskolin, calcium channel agonist Bay K8644, and basic fibroblast growth factor (bFGF). Individual cells could be imaged.

Visualization of longer-term transient luciferase expression in mammalian cells has also been possible. Various modified polylsine constructs with coupling reagent sulpho-LC SPDP were transfected with luciferase into HuH7 human hepatoma cells and imaged 2–16 days after transfection (122).

Real time-intensified CCD camera imaging of firefly luciferase (Luc) expression has been utilized to confirm that glucose may induce insulin gene transcription through increases in intracellular Ca\(^{2+}\) concentration in MIN6 β-cells when glucose, insulin or the Ca\(^{2+}\) channel inhibitor verapamil were added (Kennedy et al. 1999a) (123). Furthermore, Kennedy et al. (1999b) used recombinant firefly luciferases and photon-counting imaging to visualize concentration changes in free ATP in subdomains of single living MIN6 and primary β-cells (124). When control was made for pH, free ATP levels could be visualized in real time in the cytosol, at the plasma membrane and in the mitochondrial matrix by using luciferases specifically targeted to these three subdomains (cLuc, pmLuc, and mLuc), respectively. This was an excellent example of subcellular imaging of luciferase expression.

Chinese hamster ovary (CHO) cells with stable expression of both a CRE–luciferase reporter construct and the human pituitary adenyl cyclase-activating peptide (PA-CAP) receptor were exposed to receptor agonists for each. Visualization of luciferase light emission, as well as fluorescence, was used to visualize Ca\(^{2+}\) mobilization and the induction of adenyl cyclase activity (125).

Pancreatic β-cell activation of phosphatidylinositol 3′-kinase has been visualized by photon-counting imaging with an intensified CCD camera, using intranuclear injection of recombinant promoter fused to firefly and Renilla luciferase cDNAs (126). Elevated glucose levels induced pre-proinsulin (PPI) and liver-type pyruvate kinase (L-PK) promoters fused to firefly and Renilla luciferase cDNAs, thus producing light emission which was used to visualize single cells (127).

By constructing a less stable (and fainter) luciferase with a shorter functional half-life and implanting it into human breast cancer T-47D cells under the control of oestrogen response elements, Leclerc et al. (2000) were able to observe real-time gene expression in single living human breast cancer T-47D cells (128).

Increases in Ca\(^{2+}\) concentrations in HeLa cells and skeletal myotube cells caused by agonist addition have been shown to raise levels of ATP production (129). Ainscow and Rutter (2001) have shown the significance of these results for understanding the role of ATP in muscle contraction.
of this by quantifying (via continuous photomultiplier monitoring and intensified CCD imaging of aequorin and firefly luciferase) the expression introduced by adenoviral vectors in rat pancreatic islet (MIN6) cells and stimulated by the addition of glucose (130). This further indicated that the expression of aequorin and Pluc is dependent on $\text{Ca}^{2+}$ and ATP concentrations.

Synthetic esters of luciferin, a number of which were substrates for purified esterases, apparently hydrolysed into luciferin in intact mammalian cells, where they caused peaks in expression at levels six-fold higher than wild-type luciferin (131). This suggests that such esters may be used to assay for luciferase activity in mammalian cells where the concentration of luciferin would be the rate-limiting factor.

Although imaging luciferase in individual plant cell lines and cultures is more difficult, and therefore has not been done as often as in animal cell lines, several reports are available. Polyadenylated luciferase mRNA electro- porated into tobacco protoplasts has been imaged by video at a wide range of levels of expression (132).

Another advance was the transformation of mouse embryonic stem (ES) cells with a cDNA construct expressing the Ruc–GFP fusion protein, and its visualization (Fig. 6) (69).

Recently a $gfp$–luxABCDE reporter construct, under the control of the XylA promoter, was used to monitor the expression and temporal induction of the quorum-sensing accessory gene regulator ($agr$) in $S. aureus$ infecting bovine mammary epithelial MAC-T cells (133). The reporter gene expression was occasioned by the virulence factor-mediated escape of $S. aureus$ from the host endosome and its ensuing intracytoplasmic growth.

**A further note on low-light imaging of luciferase expression in single cells**

A most exciting feature of luciferase expression imaging is the ability to visualize gene expression in individual cells in real time. Wood and DeLuca (1987) showed that vectors can be checked for functional coding sequences by including a firefly luciferase gene probe, expressing it in $E. coli$, and detecting it by photographic film after the appropriate luciferin is added (134). Back in 1990, Hooper et al. reviewed low-light imaging with particular emphasis on charge couple device (CCD) imaging of single cells expressing luciferase (135). Recombinant Vaccinia virus bearing firefly luciferase were added to cell culture. Virally transduced cells could be detected by imaging of Luc expression at a level of one infected cell per million. Hooper et al. further suggested that imaging of luciferase-expressing viruses could be used to detect virus deletion mutants. Photon-counting CCD imaging of firefly luciferase activity was used by Kennedy et al. to visualize glucose L-pyruvate kinase (L-PK) promoter activity in single living pancreatic islet $\beta$-cells at different glucose levels (136). Detection of the L-PK promoter-driven firefly luciferase activity was standardized using CMV promoter-controlled Renilla reniformis luciferase activity. High intensity real-time photon-counting imaging was able to
detect firefly luciferase and aequorin reporter genes activated by insulin-induced MAP kinase signalling in single CHO.T cells, even when under the control of weak promoters, according to the findings of Rutter et al. (1995) (137). Rutter et al. (1996) also used CCD camera imaging to visualize intramitochondrial Ca\(^{2+}\) concentrations in CHO.T cells, using recombinant, Ca\(^{2+}\)-sensitive aequorin (138). Regulation of human cytomegalovirus (hCMV) and human immunodeficiency virus (hHIV) gene expression in individual, intact HeLa cells has been imaged using constructs with a firefly luciferase reporter gene downstream of viral promoters (139).

Single bacterial cell microscopic imaging also is an emerging field. Hill et al. (1994) were the first to report the imaging of bioluminescence in individual bacterial cells (140). Using photon-counting, they were not only able to visualize individual bacteria (such as *Pseudomonas fluorescens*) that had been transformed with luxAB-bearing plasmids and transposons, but were also able to image naturally luminescent *Photobacterium phosphorum*. Furthermore, they were able to show that expression varied over time, due to cell cycle-related changes in metabolic activity.

Two low-light imaging systems were compared to assess their efficiency in visualization of single cells of *Vibrio fischeri* (MJ-1) and of a strain of *Pseudomonas putida* both of which were expressing *V. harveyi* luxAB (141). The authors found that a slow scan liquid N\(_2\) cooled CCD (C-CCD) camera was preferable for higher resolution of single cell signal at longer exposure times, but that a photon-counting CCD (PC-CCD) camera was to be preferred for living cells at shorter exposure times, even though the resolution was somewhat lower. Phiefer and colleagues (1999) quantified relative photon flux from individual cells of *Vibrio fischeri* and *V. harveyi* using photon-counting microscopy (142). *Vibrio fischeri* luciferase was found to be more stable in expression, while *V. harveyi* luciferase was found to be much more variable in its light emission.

Even the rapid flashing of individual bioluminescent organelles (scintillons), within individual dinoflagellates of the species *Pyrocystis noctiluca*, has now been successfully imaged using video image intensifier light microscopy (143). The flashing, which occurs with fraction of a second rapidity, was induced by concentrated citric acid stimulation.

In another review (1994), Hooper et al. summarized in general the new improvements in low-light imaging technology. They discussed the hardware and software available, noted the rapid non-invasive advantages of low-light imaging in reporter (*lux, lux*) gene expression, in intracellular expression and in analysis of tissue sections, as well as immunoassays, gels and blots (144). Also in 1994, Nicolas reviewed the varied applications in the biological sciences of low-light photon-counting imaging, from the large-scale (immunoassays, DNA probes and *in vivo* imaging of expression and promoter activity) to the small scale (*in situ* hybridization and cellular luciferase expression) (145).

**IMAGING OF LUCIFERASE EXPRESSION IN TISSUES AND ORGANISMS**

In some ways, the goal of luciferase imaging has always been to monitor processes in living, multicellular organisms non-invasively.

**Imaging luciferase expression in animals**

As in cell and tissue cultures, a very wide variety of applications have been made of luciferase expression imaging in multicellular animals—both living and post-mortem.

In 1989, Hohn-Berlage et al. showed that ATP in intact brain cryosections can be imaged using firefly luciferin–luciferase, and that lactate concentrations can be likewise imaged using *V. fischeri* luciferase and lactate dehydrogenase (146) (see also Paschen, 1985) (147). Luciferin–luciferase assays can also be used to image distributions and abundances of respiratory metabolites such as glucose, ATP, and lactate in tissue sections (148).

Following liposome-mediated transformation of luciferase expression vectors, luciferase expression has been monitored in normal and atherosclerotic external iliac rabbit arteries (149). Replication-deficient adenovirus-bearing firefly luciferase and β-galactosidase genes were successfully used to test the comparative efficiencies of transgenic gene delivery to cultured Sprague–Dawley rat thoracic artery and aortic artery smooth muscle tissue cultures (150). Luminometer readings confirmed the luciferase assay differential.

In 1993, Mueller-Klieser and Walenta showed the spatial distribution and concentration of respiratory metabolites (ATP, glucose and lactate) in rapidly frozen tissue in absolute unit concentrations and at single-cell level resolution, using photon-counting visualization of luciferase expression coupled with any particular enzyme of interest (151). Single-photon count imaging of luciferase assays was used to spatially quantify the distribution of respiratory metabolites (ATP, glucose, and lactate) in cryosections of tumours and normal tissue (152). Luciferase light emission was proportional to metabolite concentration. These results were confirmed using other metabolite quantification methods.

Rembold et al. (1997) used a replication-deficient adenoviral vector carrying an apo-aequorin cDNA with a sarcoplasmic reticulum (SR) targeting sequence to infect intact rat tail arteries (153). In this way the authors were able to measure fluctuation in the presence of free Ca\(^{2+}\) in the SR in the presence of coelenterazine, the apo-aequorin luciferin substrate.
Using a luciferase reporter, Thierry et al. (1997) showed that when a lipopolyamine, a neutral lipid and a plasmid DNA are associated in the formation of lamellar vesicles, they can be used to compare in vitro and in vivo transfection efficiencies in mouse tissues (154). These lamellar vesicle–DNA complexes have a higher in vitro transfection efficiency than that of previously reported liposome transgenic delivery systems.

Making use of the fact that muscles, injected with a promoter–firefly luciferase cDNA fusion construct, retain Luc activity for up to 60 days, Davis et al. (1997) showed that, when a luciferase construct was co-injected with a hepatitis B virus surface antigen (HBsAg)-expressing DNA, the luciferase expression time is shortened to about 5 days of strong expression and a cut-off after 20 days (155). These findings suggest that luciferase can be used to image indirectly the progress of immune-mediated destruction of muscle myofibre tissue.

The subcutaneous injection of a lipid gadolinium contrast complex containing Luc was imaged by Wisner et al. (1997) using MRI (156). The Luc was also imaged. The successful insertion of Luc with the complex into the cell provided a possible method for the visualizing of a transfection event. MRI imaging provided an independent check on the luciferase imaging.

In 1997, Contag et al. successfully monitored in vivo transient expression of SV40 promoter/enhancer–Luc fusion constructs, using intensified CCD imaging on the lungs of neonatal rats (157). Cationic liposome delivery of the vectors was used. The expression vector was also induced in mice and in human T-cells.

Endogenous ATP was measured simultaneously with the release of acetylcholine from the isolated superior cervical ganglion of a rat using firefly luciferin–luciferase (158). Using imaging of luciferase light emission, March et al. (1999) showed the feasibility of catheter-based pericardial local delivery of adenoviral vectors for gene therapy in dogs (159). One of the recombinant adenoviral expression vectors encoded luciferase in the cytoplasm. This was visualized post mortem in pericardial tissues in sacrificed animals.

Similar imaging precision has been possible in living animals. In a landmark study by Edinger et al. (1999), HeLa cells stably expressing firefly luciferase were introduced via subcutaneous, intraperitoneal and intravenous injection into SCID mice (160). Tumour cell kinetics and growth were monitored by whole-body photon-counting visualization. Immediately postinjection, the following tumour cell numbers could be observed by low-light imaging: 1 × 10³ cells in the peritoneal cavity; 1 × 10⁴ cells at subcutaneous sites; and 1 × 10⁵ circulating cells.


Sugihara et al. (2000) transfected the chloramphenicol acetyltransferase (CAT) gene and firefly luciferase (luc) expression cassettes by electroporation into the testicles of living chickens (163). The authors were able to image luciferase expression in and around the injection site. A self-replicating sequence of the Epstein–Barr virus was added to stabilize luc expression in vivo.

Using a tetracycline-inducible promoter construct, Hasan et al. (2001) have shown that transgenic firefly luciferase is suitable as a visualizing marker to monitor induction of the expression of a second transgene in living mice, in this case, Cre recombinase (164).

Recently, Lipshutz et al. (2001) tested prenatal, in utero delivery of recombinant adeno-associated virus (rAAV) vectors carrying firefly luciferase via intraperitoneal injection in mice (165). Luciferase expression was visualized by whole-body imaging in all injected animals. At birth, the highest Luc expression was in the peritoneum and liver, with lower expression levels in the heart, brain and lung. Expression persisted for as long as 18 months in the peritoneum. No antibodies against Luc or rAAV were detected and no liver cell damage was reported. These data suggest that in utero DNA delivery is a safe and effective method of prenatal gene therapy in animal models.

Muramatsu et al. (2001) have demonstrated nutritionally-regulated transgene expression in mouse liver using a liver-type phosphoeholpyruvate carboxykinase (PEPCK) gene promoter driving firefly luciferase expression (166). Fasting induced a PEPCK-driven 13-fold increase of luciferase expression in the liver, but no similar induction was found in muscle tissue for either the PEPCK promoter or a control SV40 promoter.

Yu et al. used the ‘Gene Switch’ progesterone antagonist (RU486)-inducible system co-transformed with a Renilla luciferase–gfp fusion construct (CMV–ruc–gfp) to visualize inducible gene expression in COS cell culture, and also intramuscularly in male nude mice (167). RU486-induced Ruc–GFP expression was visualized using low-light imaging.

In short, numerous studies have shown the efficacy of luciferase imaging in modelling and observing complex gene activation events taking place in vivo in live animals and in tissue sections.

**IMAGING OF LUCIFERASE EXPRESSION IN TRANSGENIC ORGANISMS**

In many ways, the ultimate goal of luciferase imaging is...
not only to monitor gene expression non-invasively and in real time in living, multicellular organisms, but also to reveal and document spatial, tissue and cell type-specific expression in genetically altered (transgenic) organisms. Such applications of luciferase imaging have advanced rapidly especially in plants. By 1994, Langridge et al. had addressed the usefulness and convenience of using the bacterial luciferase (luxAB) system in a variety of eukaryotic transgenic organisms and reporter gene applications (97).

Imaging luciferase in transgenic plants

Global imaging of luciferase-expressing transgenic plants has served further research in many gene expression studies, from simple expression of luciferases in transgenic plants, to regulation of developmental genes to the gene expression of wound response, and to the detection of expression in response to thermo-osmotic stress. Photographic film was used by Ow et al. (1986) to visualize transient and stable firefly luciferase (Pluc) expression in Nicotiana tabacum (168). In 1986, Koncz and Schell imaged tissue-specific chimaeric LuxA and LuxB expression in Daucus carota and Nicotiana tabacum (169). By 1987, Koncz et al. (170) had demonstrated the successful assembly and expression of LuxA and LuxB from Vibrio harveyi in transgenic carrot (Daucus carota) and tobacco (Nicotiana tabacum) plants via Agrobacterium-mediated T-DNA or direct DNA transformation. Furthermore, Koncz et al. (1990) discussed the advantages and disadvantages of expressing bacterial (Lux) and firefly (Luc) luciferases as reporter genes in transgenic plants (171).

A promoterless luxA gene was fused to the 5’ end of a T-DNA, adjacent to a cauliflower mosaic virus 35S promoter-driven selectable marker, and inserted into tobacco leaf explants to generate transgenic tobacco plants. Some of these transformed plants were found, by low-light imaging, to express luciferase in only one organ, e.g. a floral corolla. This system thus served as a promoter search assay to find organ-specific promoters (172, cf. 96) (Fig. 7a, b). Sequencing of linked genomic DNA from these plants allowed for the isolation of developmental genes and their regulatory elements.

By 1994, Mayerhofer et al. had visualized stable Renilla luciferase expression in transgenic tobacco leaves, tomato fruit, and potato tubers (173). In a follow-up study, Mayerhofer et al. (1995) used Agrobacterium-mediated transfection to create transgenic alfalfa (Medicago sativa), tomato (Lycopersicon esculentum), tobacco (Nicotiana tabacum) and potato (Solanum tuberosum) plants (Fig. 8) with high levels of Renilla luciferase (Ruc) (60). The authors found that Ruc expression levels are substantially higher than that of either firefly (luc) or bacterial (Lux) luciferase.

Firefly luciferase (luc) was fused with an Arabidopsis circadian regulator, cab2 (chlorophyll binding protein 2) promoter, and the activity of Cab2 was inferred by visualizing luc expression both spatially and temporally in seedlings by low-light video-imaging (1992) (174). A luc fusion construct carried by transgenic Arabidopsis plants was later (1995) used to identify plants with mutant long- and short-period circadian cycle genotypes, namely plants with mutations in toc1, a gene involved in the timing of Cab regulation (175). Another group (1998) used luciferase Cab promoter–luciferase fusion vectors and video-imaging to visualize transgenic expression in
tobacco seedlings, to determine how the photoreceptor phytochrome circadian oscillator regulates expression of \( \textit{cab} \) genes, which drive early seedling development (176). Recently, Schutz and Furuya (2001) monitored \( \text{Cab} \) signalling in the cotyledons of \textit{Nicotiana tabacum} using \( \text{cab} \)-luciferase reporter genes (177).

Luciferase expression in transgenic plants has been used to evaluate imaging equipment and technology. Mutants of transgenic seedlings of \textit{Arabidopsis thaliana}, containing native promoter-inserted luciferase constructs, were monitored by low-light imaging (178). When compared, they found that a cooled CCD camera was more efficient than an intensified CCD camera in detecting mutants in the screen.

Firefly luciferase (\( \text{luc} \)) under the control of the stress-responsive RD29A promoter was introduced into \textit{Arabidopsis} plants by Ishitani \textit{et al.} (179, 180). Induction of the endogenous RD29A gene was visualized using high throughput \textit{in vivo} luminescence imaging. Under different conditions, expression of the various stress/osmotic response pathways was monitored. These pathways included those that are both phytohormone abscisic acid (ABA)-dependent and ABA-independent. Further work by Ishitani \textit{et al.} (1998) established that inducible luciferase expression in \textit{Arabidopsis} could be used to find a temperature cold-response gene (HOS-1) mutant (181).

The general utility of imaging luciferase reporters in transgenic plants has been amply demonstrated, not only for eukaryotic LUC but also for bacterial Lux. Langridge and Szalay (1998) used low-light intensified photon-counting imaging to visualize bacterial luciferase (\textit{Vibrio harveyi}) and eukaryotic luciferase (\textit{Renilla reniformis}) light emissions as markers for transformation and reporters of gene expression in transgenic \textit{Arabidopsis thaliana} and \textit{Nicotiana tabacum}. Both \( \text{luc} \) and \( \text{ruc} \) served as promoter search reporter genes (182). Langridge \textit{et al.} have also imaged bacterial luciferase expression in the tomato, \textit{Lycopersicon esculentum} (see Fig. 5a, b), and in the potato, \textit{Solanum tuberosum} (97).

The success of luciferase expression in plants has been extended to unicellular algae. Anthozoan \textit{Renilla reniformis} luciferase (\text{Ruc}) has been successfully expressed in the chloroplast of the alga \textit{Chlamydomonas reinhardtii} (183). A cryogenic CCD camera was used to image the light-emitting transgenic algal colonies.

Firefly luciferase (\text{luc}) imaging has been used in evolutionary studies in \textit{Arabidopsis}. Jelesko \textit{et al.} (1999) documented unequal meiotic crossing over in \textit{Arabidopsis thaliana} plants with a synthetic \( \text{rbcs} \) gene cluster composed of \( \Delta \text{rbcs1b::luc–rbcs2b–rbcs3b} \), instead of the wild-type \( \text{rbcs1b–rbcs2b–rbcs3b} \) (184). Over 1 million \( F_2 \) generation seedlings screened by low-light photon-counting imaging yielded three light-emitting seedlings with a chimeric \( \Delta \text{rbcs1b::luc–rbcs3b} \) gene cluster, expressing luciferase and a predicted \( \text{rbcs26} \) duplication. These results were confirmed by molecular methods. Luciferase imaging was thus used to assay directly the frequency of evolutionary gene conversion in \textit{A. thaliana} (\( \approx 3 \times 10^{-6} \)).

Xiong \textit{et al.} (1999a, b) utilized an efficient method of high throughput genetic screening of hormone and environmental stress signal transduction mutants of \textit{Arabidopsis thaliana}, using the firefly luciferase (\text{luc}) gene regulated by a cold, osmotic stress and ABA-responsive promoter (185, 186). A thermoelectrically-cooled CCD camera was used to image the plants under addition of ABA and change of temperature conditions. The system allowed screening and recognition of high- and low-expression mutants.

Luciferase imaging, in addition to being used to visualize plant promoter–gene response to thermosomic pressures, has also been used to visualize plant gene response to infection. In 1996, Giacomin and Szalay utilized \textit{Pseudomonas} infection of \textit{Arabidopsis thaliana} to induce expression of the phenylalanine ammonia-lyase (\text{PAL1}) promoter fused to the \text{lux}F gene (187). In another study, transgenic \textit{Arabidopsis} carrying a \text{gst}::\text{luc} transgene were used to image the spatial and temporal concentrations of reactive oxygen intermediates (ROIs) in response to an assault by infectious \textit{Pseudomonas syringae pv. tomato} (188) (for more details, see the section on Imaging of host–pathogen interactions, below).

Transgenic seedlings were engineered by Urwin \textit{et al.} (2000) to carry a bicistronic gene with both GFP and firefly luciferase ORFs linked by the encephalomyocarditis (ECM) IRES element and regulated by the CaMV 35S promoter (189). Both GFP and Luc were expressed...
and detected by in vivo imaging, indicating that the ECM IRES element facilitates the translation of the second ORF in vivo. Northern blot analysis also confirmed the presence of both GFP and luciferase products.

Transgenic tobacco plants (Nicotiana tabacum L.) expressing firefly luciferase (luc) driven by the Arabidopsis phenylalanine ammonia lyase 1 (PALI) promoter have been imaged in vivo for up to 24 h after the addition of exogenous luciferin (190). Wounding enhanced the luc expression in these plants, suggesting that there are barriers in plants to ordinary luciferin uptake in transgenic luciferase assays.

Using a fusion of the GA5 promoter (growth phytohormone gibberellin) and firefly luciferase (ga5–luc) cDNA, Meier et al. (2001) used imaging to show that GA5 promoter regulation of GA occurs at the level of transcription (191). Imaging allowed the investigators to identify recessive mutants with high Pluc expression.

Recently, van Leeuwen and colleagues (2001), using the CaMV 35S, modified CaMV 35S and the Arabidopsis thaliana lipid transfer protein gene promoters in conjunction with the firefly luciferase (luc) gene, were able to show that variant levels of transgene promoter activation result not only from the integration site (position effect) but also from spatial and temporal promoter regulation (192). These patterns are inherited by the next generation. Expression levels were monitored in leaves of individual transgenic plants during a 50 day period, both by imaging and by assaying local mRNA levels. Further work has shown that matrix-associated regions (MAR) elements in proximity to the transgene cause a varying effect on the temporal regulation of the transgene expression between individual plant transformants (193).

In summary, luciferase imaging of transgenic plants has thus been used in the study of whole plant gene expression and regulation, as well as in the screening of mutants.

**Imaging luciferase in transgenic animals**

Whole-body imaging of luciferase expression in transgenic animals has not proceeded as rapidly as in plants, largely because of the greater difficulty of generating transgenic animals. However, during the past decade, significant work has been carried out, particularly in imaging of gene expression during development in transgenic embryos.

In 1990, Tamiya et al. first imaged the distribution of firefly luciferase expression in transgenic zebrafish (Brachydanio rerio) (194). Zebrafish have been a favourite transgenic model because zebrafish eggs are easily accessible to DNA injection. Mayerhofer et al. visualized the spatial distribution of firefly luciferase expression in transgenic zebrafish using low-light video-image analysis (195).

Using photon-counting imaging, Matsumoto et al. (1994) found that mouse embryos from transgenic parents carrying the paternally inherited chicken β-actin promoter–luc construct emitted detectable light at the two-cell stage (196). Luciferase mRNA was found even at the one-cell stage. Thus, luciferase imaging helped to demonstrate the very early onset of embryonic gene expression.

Expression of secreted Vargula luciferase (Vuc) was imaged in live pre-implantation mouse embryos from homozygous transgenic mice containing luc and vuc cDNAs, using image intensifiers connected to a CCD camera (197). In this way, developmental gene expression modulation could be observed and assessed in individual embryos by two different luciferases.

Transgenic mice (adults and neonates) with HIV LTR-luc (firefly luciferase) constructs were found to express luc luminescence after intraperitoneal, topical absorption, or topical electroporative delivery of D-luciferin substrate in DMSO (198). Both near the surface and deeper, visualization of luc expression was possible using intensified CCD (iCCD) and cooled CCD (cCCD) imaging.

Transgene integration efficiency was determined by bioluminescent visualization screening in microinjected bovine embryos (199). A murine HSP70.1 promoter was linked to a firefly luciferase (luc) cDNA and microinjected into zygote pronuclei produced in vitro. Pluc expression was then visualized in the resulting embryos.

Various promotet/enhancer–Pluc constructs were microinjected into pre-implantation bovine embryos and their expression assayed by luminometer and photon-imaging at 2 and 6 days postinjection (200). These experiments tended to document the persistence of early somatic cell promoter activation during embryonic development.

Utilizing transgenic mice that carry an integrated murine heme-oxygenase 1 (HO-1)-luc cistron, Zhang et al. (1999) were able to visualize levels of tissue oxygenation in real time using intensified CCD camera imaging (201). The changing O2 concentrations were triggered by intraperitoneal injections of CdCl2. Both hypoxic and hyperoxic tissue conditions altered HO-1 promoted luciferase expression.

Taking advantage of the recently discovered fact that the Cytomegalovirus immediate–early gene 1 (CMV IE-1) enhancer–promoter is selectively expressed in only certain brain cells, Sigworth et al. imaged Pluc expression in brain sections from two lines of transgenic C57/B6 mice, using a liquid nitrogen-cooled CCD camera (202). One line of mice contained the human CMV::luc firefly luciferase construct and the other contained the human c-fos::luc firefly luciferase construct. The CMV::luc mice brain sections maintained at 30°C or 36°C showed discrete expression patterns, especially in the dorsal suprachiasmatic nucleus circadian pacemaker of the hypothalamus.
Making use of an E1-deletion adenovirus expressing Pluc under the control of a cytomegalovirus promoter, Wu et al. (2001), utilizing cooled CCD imaging, were able to visualize the location, magnitude and persistence of Pluc expression in Swiss Webster mice (203).

**IMAGING OF TUMORS IN VIVO VIA LUCIFERASE EXPRESSION**

Using photoproteins such as luciferase and GFP to image the progression of tumour growth in vivo is an important and rapidly advancing field (204–206). We summarize selected papers published recently. Contag et al. (2000) have discussed the use of GFP and firefly luciferase as reporter in real time, in vivo imaging of tumours. They also discussed the use of these reporter genes in studying cellular and molecular aspects of neoplastic disease, growth and regression under therapy (207). The authors show that photon imaging is sensitive enough now to detect 1000 luciferase-labelled tumour cells spread throughout a mouse peritoneal cavity. The authors further note that, while GFP is adequate for high-resolution analyses after tumour localization in vivo, luciferase-labelling is superior in tracing the progress of neoplastic growth from a few cells to extensive metastases. In future, luciferase reporter monitoring of cancer gene therapy is a suggested application.

In a recent tumour imaging study, Rehemtulla et al. (2000) used 9L rat gliosarcoma cells stably transfected with firefly luciferase (9L_luc) to produce orthotopic brain tumours (208). Other luciferase-transformed tumour lines have been used in intraperitoneal, subcutaneous and intravascular models to visualize the kinetics of tumour growth and response to therapy. Cooled CCD camera and magnetic resonance imaging (MRI) showed an excellent 0.91 correspondence between imaged photon emission and MRI-measured tumour volume. In evaluating chemotherapeutic treatment modalities, CCD and MRI confirmed each other to a p = 0.951 confidence level. It is significant and promising that luciferase in vivo imaging compared so favourably with MRI as a tool for assessing the spatial extent of in vivo tumours.

In a recent important luciferase imaging study, Honigman et al. tested a number of parameters of in vivo imaging in mice and rats (209). After injecting various plasmid vectors, recombinant viruses and transfected tumour cell-lines (see Table 2), the authors imaged bladder, bone, dermis, liver, muscle, peritoneum, prostate, salivary glands, teeth and testis in mice and rats. They used visualization of Luc expression to check organ specificity, efficiency of substrate delivery, long-term monitoring of tumour growth, promoter specificity, and efficiency of injection methods using image-intensified (ICCD) and cooled (CCCD) charge-couple device cameras. Location, magnitude and duration of Luc expression were simply and reproducibly determined by CCCD photon-counting methods. Luminometry served to monitor Luc activity within organ and cell extracts.

In spite of the remarkable progress made, much more remains to be done with luciferase visualization of tumours in vivo. Tumour imaging in general is an important interface between basic research and the clinical applications. Luciferase imaging promises a significant role in this burgeoning field, perhaps eventually in the visualization of tumours in humans.

**IMAGING OF HOST–PATHOGEN INTERACTIONS IN HOST ORGANISMS USING LUCIFERASE EXPRESSION**

Many of the marine bioluminescent organisms are found to exist in symbiotic metazoan–bacterial interactions and so are involved in natural bioluminescent in vivo ‘visualization’ (3). However, most of the applied scientific uses of luciferase imaging directed at such interactions have tended to study host–pathogen relationships.

One of the earliest and most exciting areas of luciferase in vivo imaging has been the real-time visualization of bacterial infection in living organisms, both plants and animals. Back in 1986, Legocki and co-workers used transformed *Bradyrhizobium japonicum* carrying luxAB (V. harveyi) under the control of the B. japonicum nitrogenase *nifD* promoter to monitor the presence of N-fixing bacteria in soybean root nodules, and so indirectly to visualize N-fixation (Glycine max var. Wilkin) (210). Cell extracts of root nodules grown on plants without N₂ were assayed for LuxAB activity by luminometer, indicating *nif*-driven LuxAB synthesis. LuxAB-transformed *E. coli* bacteria were used as a negative control. LuxAB fusion expression in transformed *B. japonicum*-infected soybean root nodules was successfully visualized using photographic film (Fig. 9a, b) and low-light intensified video microscopy (211). Using *N. tabacum* transformed with an auxin-stimulated bidirectional mas promoter–luxAB gene fusion construct, it was possible to visualize the spread of *Pseudomonas syringae* infection in *N. tabacum* leaves (Fig. 10) and of *Agrobacterium* induced crown gall in *N. tabacum* stem sections (Fig. 11) (97).

In 1993 Wang et al. imaged the expression of luxAB-bearing *Bacillus thuringiensis* within sixth instar larvae of *Manduca sextans* (tobacco hornworm) 10 min post-injection and also after larval ingestion of the bacteria (Figure 3a, b) (95). The *B. thuringiensis* bacteria were transformed with a plasmid containing luxAB under the control of the XylA promoter (*B. megaterium*). A recombinant baculovirus (polyhedrin promoter–luc) was developed for use as a rapid luminescent plaque assay to optimize concentrations of recombinant baculoviral infection in insect cell cultures and larvae (93).
Figure 9. (A) Visualization of transformed Bradyrhizobium-infected soybean plant cells \((Glycine\ max)\) in cross-sections of root nodules through LuxAB light emission (211). (B) Visualization of light emission in root nodules of soybean plants \(Glycine\ max\) grown in the absence of \(N_2\) in the gross medium. The bacterial inoculant was \(Bradyrhizobium\ japonicum\), stably transformed with the \(nifD\) promoter–luxAB fusion gene construct (211, cf. 210).
Figure 10. Visualization of the spread of pathogens, *Pseudomonas syringiae*, in *N. tabacum* carrying the bidirectional *mas* promoter-*luxAB* gene fusion construct (97, 170).
Expression of Pluc within invading recombinant vaccinia virus has been imaged in African green monkey BCS-40 kidney cells (212). Time exposure with sensitive film was used to capture the images of the light-emitting viral plaques in cell culture. Although not imaged, the recombinant luc-bearing vaccinia virus could be assayed in target organs in BALB/c mice.

Luciferase expression in transgenic *Arabidopsis thaliana* transformed with a phenylalanine ammonia-lyase promoter (PAL1)–luxF fusion was used to image localized activation of PAL1 by infection of *Pseudomonas syringae* pathovar *tomato*, using photon counting (187). The PAL gene encodes phenylalanine ammonia lyase, which catalyses the first step in a plant development pathway responding to environmental stresses, such as infectious invasion.

In 1995, Contag et al. showed that bacterial infections could be traced non-invasively in vivo in mice using *Staphylococcus typhimurium* transformed with a vector carrying constitutively expressed bacterial luciferase (lux) (213). Recently, Francis et al. (2000) showed that *Staphylococcus aureus* transformed with the rearranged *Photorhabdus luminescens lux* operon (luxABCDE) can be used to visualize the presence of infection and the effectiveness of antibiotic treatment by direct whole-body imaging of mice after intramuscular injection of the recombinant bacteria (214). A similar method for *Streptococcus pneumoniae* has since been developed by Francis et al. (2001), using a Gram-negative transposon bearing the luxABCDE and the kanamycin resistance gene (kan) in one promoterless operon allowing transformed, luminescent, kanamycin-resistant bacteria to be non-invasively visualized in vivo in murine models (215). Rocchetta et al. (2000) have used clinical *E. coli* EC14 transformed with the *P. luminescens lux* operon to visualize bacterial infection in rat thigh muscles, using an intensified CCD camera system (ICCD) (216). The imaging system was sensitive enough to achieve good statistical correlation between luminescence and viable bacterial cell numbers, both with and without the presence of antimicrobial agents. Rocchetta et al. propose the use of this technology for in vivo, therapeutic testing of antimicrobial agents.

Viral infection has also been visualized by luciferase imaging. By using recombinant herpes/pseudorabies virus bearing luc, it is possible with ultra-high-sensitivity...
photon-counting enhanced video imaging to visualize in real-time the progress of viral infection and destruction of mammalian cells in cell culture, even down to single cell resolution, according to Mettenleiter and Gräwe (1996) (217) who used recombinant herpes/pseudorabies virus in African green monkey kidney (VERO) cells. They suggest that in vivo monitoring of the spread of viral infection in a living animal is now feasible. This has in fact been done in insects. Langridge et al. (1996) successfully imaged not only recombinant baculovirus expression of firefly luciferase in insect cell culture (Trichoplusia ni) 386 cells and Bombyx mori N-4 and SF 9 cells, but also tracked in vivo the progress of a Pluc-transformed baculovirus infection in cabbage looper larvae (Trichoplusia ni), using low light photon-counting video imaging (218).

Digital imaging microscopy has also been used (in 2001) to determine the difference in transduction efficiencies on human A549 cells between two recombinant adenoviral vectors, AdCMVLuc and Ad5LucRGD (219).

Another recent development has been the direct in vivo imaging of bioluminescent CD4$^+$ T cells in a murine model (220). In a murine analogue of multiple sclerosis, experimentally-induced autoimmune encephalomyelitis, T lymphocytes transduced to express luciferase by pGC retroviral vectors were visually tracked by low-light imaging cameras into the central nervous system. Long-term transgene expression in the central nervous system was confirmed by histology.

Recent work has shown that injected bacterial infections of Vibrio, Salmonella and E. coli transformed with an expression construct containing the P. luminescens luxCDABE operon can be visualized over many days in C57 mice (Fig. 12) and Sprague–Dawley rats (221). Low-light imaging was used to visualize bacterial infections in muscles, in specific organs such as the kidney and liver in vivo, and in excised hearts. Luminescent bacteria could also be observed through the mouse skull in vivo and through excised rat tibia.

Recombinant vaccinia virus (rVV-RG, LIVP strain), bearing a Renilla luciferase–gfp fusion gene under the control of the vaccinia strong synthetic early–late promoter (rVV–PE/L–ruc–gfp), have been used to image rVV infection in CV-1 African green monkey kidney cells and in athymic nu/nu mice (222).

**SUMMARY AND CONCLUSIONS**

Natural in vivo luciferase ‘visualization’ has taken place in marine organisms since time immemorial in the oceans of the world. Scientific instrumental imaging of luciferase expression in living cells, tissues and organisms has made significant advances over the last few decades. This progress was only possible because of fundamental
research. The mechanisms of the luciferin–luciferase bioluminescence systems are being elucidated and their phylogenetic relationships are being worked out. Various luciferase genes and cDNAs have been isolated and cloned. Advancement in visualization of luciferase expression in individual cells, in somatic plant and animal tissues and in transgenic plants and animals has been made possible because of: (a) recombinant DNA construction of various promoter–luciferase gene constructs and fusion gene products; (b) more efficient and precise delivery of transgenic DNA and exogenous substrate; and (c) development of highly sensitive and versatile imaging technologies. Luciferase has been a useful reporter gene for imaging singly and in concert with other photoproteins, notably green fluorescence protein (GFP). Luciferase imaging has also begun to play a crucial role in imaging of tumours and metastases. Other possible applications include the imaging of LRET between luciferases and GFPs in documentation of intracellular protein–protein interactions. It seems likely that the most significant step forward, although still in its infancy, is the ability of luciferase imaging technology to resolve real-time gene expression in individual cells. Perhaps the imaging of real-time gene expression within individual cells within living organisms may be a wave of the future.

A perspective

One chimaeric dream of modern science has been to observe the world without disturbing it. In a small way, in vivo, non-invasive, real-time imaging of luciferase expression and light emission in living cells and organisms is at least in the spirit of that dream.

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