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Abstracts are in alphabetical sequence of first author

Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications

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Luciferase from the North American firefly *Photinus pyralis* (λ_{max} = 557 nm) is widely used for monitoring gene expression. Previously, we developed a set of red (S284T)- and green (V241I/G246A/F250S)-emitting luciferase mutants with spectral characteristics suitable for bioluminescence reporter applications. We undertook a site-directed mutagenesis approach to improve the thermostability of these enzymes at 37°C. Inspired by the reports of Tisi and co-workers that several mutations combine to significantly improve the thermostability of wild-type *P. pyralis* luciferase, we made variants of the red- and green-emitting enzymes, each containing the additional changes T214A, A215L, I232A, F295L and E354K. The new proteins retained the favourable spectral properties and had enhanced thermostability. The green-emitting mutant (λ_{max} = 546 nm) had a half-life at 37°C of 10.5 h, a 46-fold increase over V241I/G246A/F250S, and became resistant to red-shifting at low pH. The red-emitting mutant (λ_{max} = 610 nm) had a half-life at 37°C of 8.8 h, an 11-fold increase over S284T. Moreover, the additional mutations improved the 37°C expression yields of both thermostable variants ~10-fold. Additionally, model single-colour reporter assays demonstrated that the thermostable mutants could be detected at the attomol level. These results demonstrate the feasibility of using the thermostable enzymes in mammalian cell reporter gene assays.

Evaluation of a bioluminescent bacterial biosensor for rapid assay of cytotoxic drug activity in cell lines and clinical samples from leukaemic patients

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The nucleoside analogue cytosine arabinoside (Ara-C) is the cornerstone of acute myeloid leukaemia (AML) therapy, but approximately 70% of patients fail to achieve complete remission with Ara-C. Current methods of testing drug sensitivity of AML are time consuming and unsuitable for routine screening. This project has developed a bioluminescent bacterial biosensor for use in a simple and rapid assay to determine the Ara-C sensitivity of patient cells, enabling clinicians to consider drug efficacy prior to treatment. An Ara-C-sensitive *Escherichia coli* was constructed and made self-bioluminescent by introducing the pBBR1MCS-2 plasmid carrying the *lux* operon (*lux*CDABE). The biosensor was evaluated with AML cell lines exposed to therapeutic levels of Ara-C. A significant increase in light level was observed in cell-lines known to be Ara-C sensitive. An Ara-C-resistant cell line showed no significant increase in light production in the presence of Ara-C compared to untreated controls. The assay was completed within 8–10 h and correlated well with a standard cytotoxicity assay taking 3–4 days. Samples of bone marrow and peripheral blood from AML patients were tested by both methods and the rapid bacterial biosensor assay correlated well with standard tests and also patient outcomes, indicating potential for clinical use.

Quantitative luminescence yield spectra of firefly bioluminescence

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We have developed a quantitative bio/chemiluminescence measurement system to determine the luminescence yield spectrum, which is the spectrum of total luminescence intensity emitted by the reaction in absolute light units of photons/nm or photons/eV. To quantify the intriguing colour change in firefly bioluminescence from yellow-green to red with decreasing pH, we measured the luminescence yield spectra for *Photinus pyralis* at various pH values. The spectral shapes were well reproduced by one pH-sensitive and two pH-insensitive components with Gaussian energy broadening, peaking around 560, 620 and 670 nm. We found that the pH-sensitive 560 nm peak component contributes mostly to the change in the spectral shape and quantum yield with pH.

Development of bioluminescent pyrophosphate assay using pyruvate phosphate dikinase and its application to SNPs analysis

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DNA analysis is an important technology with respect to diagnosis of infectious disease and tailored medication. In this study, we developed a novel bioluminescent assay for pyrophosphate and it was applied to SNPs analysis using single base extension reaction. The principle of this method is as follows. A specific primer within each aliquot possessing a short 3′ end of the base of interest was hybridized to the single-stranded DNA template; subsequently, one of either α-S-dATP, dTTP, dGTP and dCTP, or (exo-) Klenow DNA polymerase was added and incubated for 1 min. Pyrophosphate released by DNA polymerase is converted to ATP by pyruvate phosphate dikinase (PPDK), and the concentration of ATP is determined using the firefly luciferase reaction. This method, which does not require expensive equipment, can be utilized to rapidly monitor one point mutation in a gene.

Demonstration of tri-coloured reporter assay using a filter luminometer 'Sirius C'

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A reporter assay system to monitor the expression of several genes simultaneously is of great benefit for the investigation of a cascade of transcriptional regulations, which is the molecular basis of various biological functions. Recently, we have developed a novel reporter assay system, a tricolour reporter *in vitro* assay system, in which the expression of three genes can be monitored simultaneously by splitting the emissions from green-, orange- and red-emitting luciferases (green, orange, and red luciferases) with a optical filters (1). In this study, we confirmed this novel system to analyse a transcription–translation feedback loop of the *c-fos* promoter, using by a filter luminometer 'Sirius C'. We have successfully measured simultaneously the activities of the green, orange and red luciferases in a mixture by splitting their emissions with two filters of 575 nm path and 610 nm path, respectively. This system could be utilized for detailed analysis in the new fields of transcriptome and promoterome, as well as in pharmacology or toxicology for the screening of new drugs or the detection of harmful chemicals.

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Direct observation of a radical-ion intermediate in the chemi-excitation step of peroxyoxalate chemiluminescence

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The chemi-excitation mechanism in the peroxyoxalate reaction, one of the most efficient chemiluminescence (CL) transformations known, is thought to involve the CIEEL sequence. However, up to now experimental evidence has been found only for the initial electron transfer step (1). In this work we report the detection of the carbon dioxide radical anion in the peroxyoxalate reaction, using EPR with N-*tert*-butyl-α-phenylnitrone (PBN) as spin-trap. The EPR spectrum obtained from the peroxyoxalate system in the presence of PBN corresponds to the spectrum reported in the literature for the PBN–carbon dioxide radical anion adduct. Furthermore, this adduct has been detected by mass spectrometry of the reaction mixture. These results constitute the first experimental evidence on a reaction step after the initial electron transfer in the CIEEL sequence of the peroxyoxalate chemiluminescence.

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A luminometer specifically designed for Industrial use

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We designed a novel type microplate luminometer, called XS4, for principal use in an industrial environment, responding to major issues with currently used instrumentation, which had been, frequently developed for research use. These issues include difficulties in avoiding or cleaning off reagent spills, insufficient mixing of samples and reagents and the necessity of working with unskilled operators. In the XS4 instrument, an active on-board vortexer, based upon a new design principle, allows highly effective mixing, e.g. for high-fat milk samples to be mixed with extraction and counting reagents in microbial determinations. The system has four high-precision reagent injectors, allowing freely programmable and individual timing and volume settings. Each reagent addition is followed immediately by intense shaking as the new state of the art, replacing injection mixing. This results in high reproducibility, optimum

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signal:blank ratio, and avoids liquid spill-over. Minimum concentrations of luminescent analytes are detected, even in extremely inhomogeneous samples. At the end of a working period, the entire injection system can be cleaned automatically. All parts of the instrument's interior are directly accessible to the user, allowing regular inspection and cleaning. All electronic modules are situated in an enclosed compartment, not accessible for the user and protected from reagents.

Obelin mutants with altered colour of light emission as labels for dual-wavelength immunoassay

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The successful application of Ca^{2+}-activated photoprotein obelin as a label for a highly sensitive rapid immunoassay has been demonstrated. Based on the three-dimensional structure as well as on the proposed mechanism for Ca^{2+} triggering and emitter molecule formation, obelin mutants with altered colour of light emission were produced using a site-directed mutagenesis. Among those are 'greenish' and 'violet' mutants, having a small overlap of bioluminescence spectra. This offers prospects for the development of dual-wavelength immunoassays with these mutants as labels for simultaneous detection of two analytes in the same sample by measuring bioluminescence through optical filters transmitting violet or green light. Several 'colour' mutants have been purified and studied. Two mutants that exhibited high specific activity and stability were selected for dual-wavelength bioluminescent immunoassay of follicle-stimulating and luteinizing hormones. The sensitivity of simultaneous assay of these hormones is closely comparable to that obtained by RIA. Supported by the RAS programme for Molecular and Cellular Biology and the SB RAS Lavrentiev Grant for Young Scientists.

Refolding of the recombinant luciferases of *Metridia longa*

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Metridia longa is a marine luminous copepod. Its bioluminescence is conditioned by secreted coelenterazine-dependent luciferases. Using functional screening the three cDNAs encoding different luciferases were cloned from the expression cDNA library of *Metridia*. One of them, MLuc164, was successfully applied as a secreted reporter enzyme in mammalian cells. Two luciferases, MLuc164 and MLuc39, without signal peptides for secretion, were expressed in *Escherichia coli*. In *E. coli* cells, most of the synthesized protein is accumulated in insoluble inclusion bodies. Both luciferases contain 10 Cys residues, which probably form disulphide bonds in active luciferase conformation. Refolding of proteins with the properly formed disulphide bonds is a tough task. Here we report the results of testing various approaches for the solubilization and refolding of these luciferases. Despite the high identity of luciferases (82%), under the tested conditions MLuc39 gave a better yield of active monomeric protein than MLuc164. Supported by RFBR Grant 05-04-48271, the RAS programme for Molecular and Cellular Biology, the SB RAS Lavrentiev Grant for Young Scientists, and Bayer HealthCare AG (Germany).

Bioluminescent method for the detection of protease activity in UHT milk

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Thermostable proteases from *Pseudomonas* spp. are an important source of spoilage of UHT dairy products. These enzymes destroy casein micelles, resulting in milk gelation. Thus, the level of protease activity in UHT milk is a good indicator of product shelf-life. Several methods to assay protease in milk have been described but they are not sufficiently sensitive, rapid or easy to perform to meet the requirements of the dairy industry. Recently, a homogeneous one-step bioluminescent method was proposed for the detection of the therapeutically important protease caspase-3 (1). This method is based on the cleavage of a synthetic peptide conjugated with aminoluciferin and subsequent release and detection of the latter by firefly luciferase. Although very sensitive (LOD ≈ 2–3 mU/mL) and rapid (1 h), this method is too specific for the detection of proteases in milk. The peptide conjugated with luciferin has a highly conservative recognition site for caspase-3. To apply this approach for the detection of proteases in milk, several different substrates should be synthesized that are specific to each group of milk proteases. This may significantly increase the cost of the analysis. We propose the use of firefly luciferase itself as a substrate for proteases. It is known that even limited proteolysis of the luciferase molecule results in a significant decrease in enzymatic activity (2). Reagent formulation was optimized to ensure the most sensitive detection of changes in luciferase activity/concentration. Two different formats were used, two-step and one-step. The effect of sterilized and UHT milk on bioluminescent protease assay was investigated, using milk samples spiked with different classes of proteases. The results of the developed assay were compared with a commercial protease assay kit based on FITC-casein. It was shown that the bioluminescent milk protease assay is more sensitive than the commercial fluorescent assay and can be performed within 1 h. The one-step assay format can be easily adapted to achieve high-throughput analysis.

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Detection and control of *Salmonella typhimurium* growth using specific bacteriophage

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The main measure to prevent food-borne infections is adherance to strict hygiene standards. However, some pathogens that cause such infections have a high level of resistance to disinfectant treatments. It was shown recently that adaptation of microorganisms to sanitizers commonly used in the food industry can occur and that use of disinfectants may also result in enhanced resistance to antibiotics (1). These emerging resistant pathogens are a serious concern because they are more difficult to treat. Among them are methicillin-resistant *Staphyloccocus aureus* (MRSA), *Salmonella typhimurium* DT104, vancomycin-resistant enterococci (VRE) and naturally antibiotic-resistant *Pseudomonas aeruginosa*. Accordingly, there is an urgent need for novel methods to detect and control growth of pathogens in food-processing environments that can reveal and eliminate both sensitive and resistant strains of bacterial pathogens. A promising approach to eliminate a target pathogen is to use specific lytic bacteriophages. It was shown previously (2) that certain bacteriophages can lyse both antibiotic-sensitive and -resistant bacterial strains effectively; thus providing the means for both detection and elimination of pathogens. Two bacteriophages from Biohage-Pharma Inc. that were shown to be specific to *S. typhimurium* were tested for their ability to lyse and thus to control the growth of *Salmonella*. Bioluminescent ATP-metry was used to monitor both lysis and growth of bacteria. The time-course of changes in both extracellular and intracellular ATP concentration was investigated. An increase of extracellular ATP was observed 15–20 min after infection and it reached a maximum after 100 min postinfection. The amount of released ATP correlated with the efficiency of cell lysis. There was no effect of bacteriophage on the growth of *S. hadar*. The selectivity of bacteriophage-mediated lysis of *S. typhimurium* was used to develop a specific method for its detection. It was shown that, by monitoring extracellular ATP, *S. typhimurium* can be detected in the presence of a five-fold excess of different *Salmonella* serovars without compromising the sensitivity of the bioluminescent assay. There was a 1:2 log reduction in cell counts at 2 h postinfection, when a multiplicity of infection (MOI) of >1000 was used. Longer incubation resulted in significant growth of *S. typhimurium*. At lower MOI, a lesser degree of control of *Salmonella* growth by phage was achieved; followed by fast multiplication of cells after 6 h postinfection. However, bacteriophage-treated *Salmonella* cells were significantly more susceptible to quaternary ammonium compounds—positively charged surfactants used as intra-cellular ATP releasing agents. This difference was detectable 8 min after infection and did not depend on cell concentration or time postinfection. Adsorption of phage on the bacterial cell wall may result in structural changes that allowed the surfactant to disrupt the cell wall more rapidly. Use of bioluminescent ATP-metry allowed us to characterize the lytic ability of bacteriophage and assess their potential for specific bacterial detection and growth control more easily than by conventional plating methods.

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The recombinant Ca^{2+}-regulated photoprotein berovin from *Beroe abyssicola* displays *in vitro* both luciferase and photoprotein activities

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At cloning, the recombinant berovin was defined as a typical Ca^{2+}-regulated photoprotein similar to obelin or aequorin because it contained three 'EF-hand' Ca^{2+}-binding sequences and the apo-berovin produced a photoprotein complex whose light emission can be triggered by Ca^{2+}. Here we report that the purified recombinant apo-berovin also exhibits capability to act *in vitro* as a coelenterazine-dependent luciferase without Ca^{2+}. The highest rate and quantum yield of the reaction catalysed by apo-berovin are observed at neutral pH, 12–14°C, 0.2 mol/L NaCl and 1:100 apo-protein:coelenterazine ratio. The apo-berovin bioluminescence has λ_{max} = 500 and 490 nm at neutral and alkaline pH, respectively. These conditions differ from those that are the best to form a stable photoprotein. The highest yield of a photoprotein is at pH 9.0, 4°C, 0.5 mol/L NaCl, and 1:10 apo-protein:coelenterazine ratio. The yield of a charged berovin is only 47%. However, the berovin purified from discharged photoprotein and apoprotein has a specific activity similar to those for obelin or aequorin. Supported by Bayer HealthCare AG, Germany, RFBR Grant 05-04-48271, and the RAS programme for Molecular and Cellular Biology.

The isospecies of Ca^{2+}-regulated photoprotein bolinopsin from *Bolinopsis infundibulum*

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The seven cDNAs encoding different isospecies of bolinopsin were altogether isolated from the expression cDNA libraries of *B. infundibulum* using functional and PCR screening methods. The specimens belonging to *B. infundibulum* was confirmed by sequence of the 18S rRNA gene. The open reading frames of each cDNA encode 206–207 amino acid proteins with calculated molecular weights of 24 461–24 633. The sequence analysis revealed high homology of bolinopsin isospecies (identity 85.3–99.5%). The bolinopsin isospecies were expressed in *Escherichia coli*, purified and characterized. All isospecies were sensitive to light. Unlike berovin from the ctenophore *Beroe abyssicola*, the sequences of bolinopsin isospecies contain one or two Cys residues. Therefore, in contrast to berovin, the addition of a reducing agent, e.g. DTT, increases the yield of a charged photoprotein. Despite high identity, bolinopsin isospecies differ in some properties. For instance, five isospecies have the bioluminescence maximum at 490 nm, whereas two display green bioluminescence (λ_{max} = 500 nm). Supported by Bayer HealthCare AG (Germany), RFBR Grant 05-04-48271 and the RAS programme for Molecular and Cellular Biology.

Kinetic observation of the chemi-excitation step in peroxyoxalate chemiluminescence

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The peroxyoxalate system, one of the most efficient chemiluminescence (CL) transformations, is composed of a variety of consecutive and parallel reaction steps. Although the kinetics of this reaction have been intensively studied, to date no kinetic information has been obtained with respect to the excitation step, where the 'chemical energy' is transformed into 'electronic excitation energy'. In this work we report for the first time the measurement of rate constants for the interaction of a 'high-energy intermediate' (HEI) formed in the peroxyoxalate reaction and several aromatic hydrocarbons used as activators (ACT). We were able to find experimental conditions in which a HEI is accumulated in the absence of an ACT; delayed ACT addition leads to the observation of a flash of light emission. The observed rate constants show linear dependence with the [ACT] and catalytic rate constants can be correlated with the oxidation potential of the ACT.

Synthesis and studies of fenchon-derived 1,2-dioxetanes

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A new series of 1,2-dioxetanes bearing a fenchon-derived group was synthesized and its thermal stability measured and compared with the corresponding adamantan-stabilized 1,2-dioxetanes. The fenchyl group is a bulky bicyclic [2.2.1] terpene derivative, and its rigid structure provides remarkably stability to a directly bound 1,2-dioxetane ring. The stability of fenchyl-substituted 1,2-dioxetanes is as high as the stability of adamantyl-substituted derivatives, hitherto the most stable 1,2-dioxetanes known. The thermal stability is investigated by common photochemical methods and with the help of differential thermo calorimetry (DSC). Three fenchyl-substituted 1,2-dioxetanes were prepared, including two new derivatives containing protected phenyloxy substituents whose decomposition can be induced by deprotection using appropriate reagents. The synthesis of the olefins was achieved by Barton–Kellogg olefination in yields of 70–95%, whereas using Wittig and Horner–Wadsworth–Emmons (HWE) olefinations maximum yields of 15% were achieved. The photooxygenation of those olefins produced 1,2-dioxetanes in yields of 70–90%, and these compounds showed high thermal stability, important for possible analytical applications. In contrast to adamantan-stabilized 1,2-dioxetanes, the fenchyl compounds form mixtures of diastereomeres. The results of the decomposition experiments suggest that different diastereomeres of the same compound may have slightly different thermal stabilities.

Use of bioluminescent reporter bacteria to study invasion and survival within mammalian cell lines

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Neisseria meningitidis is the major causative agent of bacterial meningitis, with over 500 000 cases occurring globally each year. The lack of any animal models for this human specific disease has hampered our knowledge of exactly how this pathogen enters endothelial cells and breaches the blood–brain barrier to cause fulminating meningitis. Bacterial bioluminescence has been shown to be an accurate real-time reporter of the metabolic activity of bacteria. A strain of *N. meningitidis* C751 has been genetically modified with *luxCDABE* from *Photorhabdus luminescencens* on the plasmid pGLITE to give a self-bioluminescent reporter construct. This self-bioluminescent strain of *Neisseria meningitidis* was used in the development of an assay to study invasion and survival within the endothelial

cell line ECV-304. In addition to this, systems where internalization is known to occur, such as that of *Escherichia coli DH5α* pLITE within the monocytic cell line THP-1, were used as positive controls. Once optimum conditions for this assay have been identified, this method could be utilized to study real-time *in situ* bacterial passage across the blood–brain barrier, with the potential to influence antibiotic studies and also reduce animal testing in this area of research.

Luminous brittlestars to assess sub-lethal toxicity: a metazoan version of Microtox®?

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Brittlestars (Ophiuroidea, Echinodermata) are common benthic invertebrates found in a wide diversity of marine habitats. Living in/on sediment and feeding on it, brittlestars are often on first-line exposure to sediment-associated contamination. Many brittlestar species are bioluminescent and the light production, originating from specialized cells, is under nervous control. Under experimental conditions, bioluminescence can be chemically stimulated with acetylcholine (Ach) to trigger neuro-mediated bioluminescence, or potassium chloride (KCl) to trigger total chemical reaction of bioluminescence. Upon exposure to contaminants, a change in Ach bioluminescence is therefore indicative of neuro-toxicity, while a change in KCl bioluminescence results from deleterious effects directly on the reagents involved in light production. In contaminated brittlestars, Ach bioluminescence intensity can either be increased or decreased, depending on the contaminants, indicating the diversity of action paths by which contaminants affect the nervous system. In contrast, KCl bioluminescence generally decreases in intensity upon exposure to contaminants, indicating that only brittlestars from pristine environmental condition have light-producing reagents with optimal chemical structure allowing for maximum bioluminescence output. Recent data from laboratory experiments on the effect of trace metals on the Ach and KCl bioluminescence capacity of brittlestars is presented, with comparison to toxicity assessment using bioluminescence from the microbial bioassay Microtox®.

Bioluminescence characteristics of the marine worm *Odontosyllis phosphorea*

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This is the first report on bioluminescence of the worm *Odontosyllis phosphorea* analysed in the laboratory. The light production appeared as an intense glow after stimulation with potassium chloride, which was associated with secretion of slime. The slime was viscous, blue-coloured and produced spontaneous light that was greatly intensified by the addition of peroxidase or ammonium persulphate. The spectrum of bioluminescence was unimodal and peaking at 494–504 nm, depending on the chemical stimulation. The fluorescence spectrum was similar, yet always weaker unless from slime that already had produced light, indicating that the oxidized product of the light production is the fluorescent one. Light could not be reconstituted by mixing hot and cold extracts, suggesting that the bioluminescence involves a photoprotein and not a luciferin–luciferase reaction. Accordingly, samples exposed to oxygen depletion with nitrogen were still able to produce intense bioluminescence. The luminous compound was functional at various temperatures, as low as –20°C, while it was degraded above 40°C. Light was produced from individuals as small as 0.5–1.0 mm, in which case the light remained mainly internal and was not secreted as slime. This early occurrence of bioluminescence in the life cycle of *O. phosphorea* supports the hypothesis that bioluminescence is innate and is used for purposes other than attracting mates.

Anthozoa red fluorescent protein in biosensing

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A variety of bioluminescent and fluorescent reporters have been employed in bioanalysis, enabling genetic encoding and multicolour detection. The characterization of proteins that emit light in the red region of the spectrum has brought a new excitement in the luminescence research field, e.g. a red fluorescent protein (DsRed) has become a popular fusion tag because of lower cellular autofluorescence in the red wavelength range, high quantum yield, and spectral separation from GFP enabling multicolour detection. We have employed red fluorescent proteins including DsRed tetramer, DsRed monomer and HcRed as labels for a variety of bioanalytical applications. We have developed a one-step purification method for these proteins, using their intrinsic ability to bind copper. Further, we have genetically fused DsRed monomer to a peptide to demonstrate its application as an affinity tag and as a label in the development of an assay for the peptide of interest. In addition, we have employed DsRed as a quantitative label in the development of assays for small molecules analytes. In this presentation, the assays developed in our laboratory using red fluorescent proteins will be discussed.

Improved sensitivity method for rapid hygiene monitoring using ATP bioluminescence

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ATP bioluminescence is a widely accepted tool for hygiene monitoring in the food and drink industry, providing rapid results. The level of ATP on the surface of food production facilities is a good indicator of bacterial contamination as well as residual food residues. Poorly cleaned/sterilized surfaces will

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provide excellent growth conditions, e.g. for airborne microorganisms. The simple and quick (<1 min) assay procedure promotes routine use and has several advantages over conventional microbiology techniques. For example, traditional techniques produce CFU results after 2–5 days and are unable to detect non-culturable microorganisms. As well as food manufacturers, other markets for surface hygiene are important, such as hospitals, clinics and pharmaceutical companies. Whilst ATP bioluminescence is sufficiently sensitive, at around 10^3 CFU/mL (1), for most applications, clients requiring sterility-level sensitivity have had to continue with more traditional methods. To serve these markets, we propose using an enhanced sensitivity method for rapid hygiene monitoring using adenylate kinase (AK) as a cell marker (2). This method makes use of the fact that AK is ubiquitous in virtually all living cells. Supplementing the ATP bioluminescence reagents with an excess of highly purified ADP and magnesium ions allows the sensitive assay of AK in a coupled reaction linked to the detection of ATP by luciferase/luciferin:

$$2\mathrm{ADP} \xleftrightarrow[\mathrm{AK}]{\mathrm{Mg}^{2+}} \mathrm{AMP} + \mathrm{ATP}$$

Using this method, linear amplification of ATP over time can be achieved. It was shown that for *Escherichia coli* 1 CFU/swab could be detected on a stainless steel food-grade surface, using a 30 min assay. Since low-AK residual organic residues can also indicate poor hygiene, it is required that the improved rapid hygiene test should be able to detect both ATP and AK sensitively. The main problem with developing an AK rapid hygiene assay until now has been the high ATP backgrounds of the purified ADP reagent. This meant that although low levels of bacteria could be detected, this was at the expense of ATP detection, which was not equivalent to current ATP bioluminescence; e.g. 1–10 fmol ATP can typically be detected using ATP bioluminescence, but with AK bioluminescence around 100–200 fmol ATP can be detected, due to the high ADP backgrounds. Further reduction of ADP backgrounds, already purified by column chromatography, has been achieved using apyrase with a high ratio of ATPase:ADPase activity, stabilized by ultrafiltration to remove the apyrase. Detection of 2.5 fmol ATP was achieved on a food-grade stainless steel surface, equivalent to current ATP bioluminescence reagents, whilst 1 CFU/swab of *E. coli* was detected using the same reagents in 30 min, a 100-fold improvement on traditional rapid hygiene methods. It is hoped that these improved reagents will meet the sensitivity needs of the pharmaceutical and hospital markets. It also brings considerable benefit to hygiene monitoring in the food industry, as some types of food residue samples can have little or no ATP but are high in AK.

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Determination of antioxidant capacity and reactivity using hemin-catalysed luminol chemiluminescence

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The determination of the antioxidant potential of plant extracts and isolated natural products constitutes a simple tool to evaluate the potential biological activity of plant constituents. In this work we describe the use of the luminol chemiluminescence antioxidant assay, developed in our laboratory (1), for the determination of the antioxidant activity of several naturally occurring flavonoids. Kinetic analysis of the CL emission curves in the presence of different antioxidant concentrations allows the determination of the antioxidant reactivity. In order to establish a structure–reactivity relationship for flavonoids and related compounds, we have also studied the antiradical capacity and reactivity of several substituted phenols as model compounds.

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The main function of His175, Trp179, and Tyr190 residues of the obelin-binding site is to stabilize the hydroperoxycoelenterazine intermediate

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The Ca^{2+}-regulated photoprotein obelin is responsible for bioluminescence of the marine hydroid *Obelia*. The obelin consists of a single polypeptide chain (~22 kDa), having a hydrophobic cavity in which is bound the 2-hydroperoxycoelenterazine, 'pre-activated' coelenterazine. According to the obelin spatial structure, the side chains of His175, Trp179 and Tyr190 form hydrogen bonds with peroxy and carbonyl groups of a substrate, thereby presumably stabilizing 2-hydroperoxycoelenterazine within a molecule. We substituted these amino acids by residues with different donor–acceptor hydrogen bond properties of side chains. All mutants exhibited luciferase-like bioluminescence activity and a very low (or no) photoprotein activity, except the W179Y and Y190F mutants. They retained 23% and 14% WT obelin activity, respectively. The results indicate that the main function of His175, Trp179 and Tyr190 is rather to stabilize the 2-hydroperoxycoelenterazine intermediate than to participate in catalysing the

bioluminescent reaction, because all mutants revealed luciferase bioluminescent activity. Supported by Grant 05-04-48271 from RFBR and RAS for Molecular and Cellular Biology.

Applications of adenylate kinase detection using bioluminescence

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Microbial adenylate kinase (AK) can be driven to produce ATP by the reaction:

$$2ADP \leftrightarrow AMP + ATP$$

Measuring the resulting ATP by means of firefly luciferase bioluminescence provides a highly sensitive assay for the presence of micro-organisms, e.g. in clinical specimens or as surface contaminants in a hospital environment. The fact that the level of AK per cell for a particular bacterial species remains essentially constant, regardless of physiological state, allows the technique to be used in a range of further applications where the need is to enumerate organisms rapidly or to measure the rate of microbial growth. We have demonstrated the use of bioluminescent AK detection for measuring the susceptibility of bacteria by exposing them to test antibiotics and measuring AK activity over a period of a few hours. A similar approach has allowed the development of a rapid growth-based assay for MRSA directly from clinical swabs, using culture in a mixture of antibiotics, antibody-mediated capture on magnetic microparticles and specific lysis to release AK. The same technique can potentially be applied to other antibiotic-resistant target organisms.

The origin of the luciferases from head and lateral lanterns of railroadworms (Coleoptera: Phengodidae: Mastinocerini)

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Among railroadworms (Phengodidae), those from the tribe Mastinocerini display head lanterns in addition to the usual lateral lanterns found in the whole family, producing the widest range of colours among beetle luciferases. Previously, we cloned the green-emitting luciferase from the body lateral lanterns of *Phrixotrix viviani* and the red-emitting luciferase from the head lantern of *Phrixotrix hirtus*. However, the luciferases from both lanterns of the same species were not cloned, and the evolutionary origin of these enzymes remains unknown. Using PCR techniques, we cloned new luciferases from the body of other railroadworm species within the tribe Mastinocerini: *P. hirtus, Brasilocerus* sp. and *Taximastinocerus* sp. These luciferases have 545–547 residues and display the highest identity (81–99%) with the green-emitting luciferase from the body fo *P. viviani*, and only 69–71% with the red luciferase from the head lantern of *P. hirtus*. A parsimony analysis suggests that the luciferases from the body and the head lanterns of railroadworms are coded by paralogous genes that were duplicated before the divergence of the main Mastinocerini genera. (Financial support: FAPESP)

Bivalent fluorescent reporters for gene expression studies in Gram-positive bacteria

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Listeria monocytogenes is a Gram-positive, aerobic, facultative intracellular pathogen. It is ubiquitous, can grow over a wide range of pH (5.2–9.6) and temperatures (2.5–45°C) and also at high salt concentration. Over the last decade it has emerged as a major food-borne pathogen. Infection results in meningitis, meningo-encephalitis, septicaemia, abortions in pregnant women and disease in newborns, infants, elderly and immunocompromised individuals, with an overall mortality rate of 30%. Strains of serotypes 4b, 1/2a, 1/2b have been responsible for most of the major outbreaks reported. Infection by *Listeria* involves internalization, intracellular replication and cell-to-cell spread and requires the virulence genes *inlA*, *inlB*, *hly*, *plcA*, *plcB* and *actA*. These genes are temporally regulated during the infectious cycle. The central virulence transcriptional activator is encoded by the *prfA* gene. Although many of the virulence genes have been identified and characterized, the actual mechanism of their regulation *in vivo* is not yet fully understood. In this study we have constructed a bivalent reporter gene plasmid in which two different fluorescent proteins (YFP and CFP) are located on one plasmid and regulated by divergent promoters. Overlap PCR and gateway cloning was used to create the final plasmid construct and demonstrates the enormous potential application of this rapid cloning technique. The constructs are stable in *E. coli* and *Listeria*, despite the extensive homology of the two reporter genes, and the signal from both reporter genes can be differentiated by the use of appropriate filter sets. Using the bivalent reporters located on the same plasmid will allow both promoters to be monitored simultaneously, removing the effect of variation in plasmid copy number, and therefore the difference in the intensities of the CFP and YFP will give us real-time analysis of spatial and functional regulation of the Pr_{plcA} and Pr_{hly} promoters and their post-transcriptional regulation by PrfA. The plasmid-based construct can also be rapidly moved into different strains of *L. monocytogenes* to understand differences in gene expression seen between strains.

Bioluminescent alternative to real-time PCR (BART)

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The bioluminescent assay in real time (BART) detects the exponential increase of inorganic pyrophosphate (PP_i) produced when a specific nucleic acid target is amplified. ATP-sulphurylase converts PPi into ATP, which is then detected by a thermostable firefly luciferase. BART operates in a closed-tube no-addition format in which all reagents necessary for nucleic acid amplification, ATP production and the simultaneous production of light from luciferase are present. BART can be used with existing nucleic acid amplification technologies (NAAT), of which around 20 do not involve 'thermocycling' at high temperatures (>65°C). Here we show, using several isothermal NAATs, that PPi accumulates concomitantly with DNA synthesis and that its detection in the BART assay produces quantitative, highly sensitive and specific results that directly reflect the amount of DNA synthesized. Hardware requirements are exceptionally simple, making BART a cheap and simple format for portable devices compared to real-time PCR. We also report here a pilot clinical evaluation of BART using isothermal loop-mediated amplification for the detection of *Chlamydia trachomatis* in human urine samples. The results demonstrate high specificity and sensitivity in assays completed within 1 h. This has significant implications for the development of non-invasive, clinic-based devices for the rapid detection of this pathogen.

Application of luminescence microscope with novel optical system for detection of gene expression pattern of individual cells

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Luciferase is particularly useful as a reporter to monitor gene expression in living cells and organisms because of its simplicity, good S:N ratio and high quantitativity. In luciferase promoter assay, a luminometer has been generally used for detection of the luciferase activity, with the capability of measuring only the total luminescence from the cell population. We therefore developed a luminescence microscope with a novel optical system, which makes it possible not only to measure the light intensity but also to obtain detailed information on the morphogenesis and gene expression patterns of individual cells. We applied the present system for detection of the HSP70 promoter activity on HeLa cells using GL3 luciferase (Promega) as a reporter, and found differences of the promoter activity patterns among individual cells. Furthermore, time-lapse bioluminescence imaging enabled us to detect circadian rhythmicity of a clock gene (Per2) expression in each cell in cultured suprachiasmatic nucleus slices. Thus, our luminescence imaging system can be applied for studies on the dynamics of gene expression in living cells and tissues expressing luciferase.

Advancing the development of dual-luciferase assays

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Bioluminescent cell-based assays are well suited for biological research because of their high sensitivity, wide dynamic range and minimal interference by investigative treatments. In automated screening applications, dual assays can be useful for reducing false positives resulting from treatment-induced cytotoxicity. In these assays, firefly luciferase typically serves as the primary reporter and *Renilia* luciferase is used as an internal control. Unfortunately, such dual luciferase assays can not be configured simply by placing the two luciferase genes on one vector because of cross-interference between promoters and response elements. We have developed a method for constructing stable dual luciferase cell lines using two plasmids. One plasmid expresses firefly luciferase genes under the control of a response element (e.g. CRE or NFAT associated with intracellular signalling by G-protein couple receptors) and a hygromycin-selectable marker. The second plasmid expresses a target receptor (e.g. dopamine receptor D1 or muscarinic 3 receptor M3R) and a *Renilia* luciferase–neomycin seleotable marker fusion. Faster signal responses and shorter assay times were achieved by using destabilized luciferases. Data acquisition was simplified by using optimized proprietary assay chemistries. The combined effect of these enhancements yielded dual-luciferase GPCR assays having improved data quality.

Profiles of pelagic bioluminescence on the mid-Atlantic ridge

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The abundance of pelagic bioluminescent organisms was investigated along and across the Mid-Atlantic Ridge from the sub-surface layer to the sea floor, using a low-light ISIT camera looking down onto an impact screen. This ISIT profiling system was mounted on a water sampling rosette and lowered on a wire at 0.8 m/s to depths from 975 m over a sea mount to 3005 m off the ridge axis, to estimate the number of stimulable sources of bioluminescence in the water column. Fourteen stations were sampled between 42°56′N and 53°18′N in the central north Atlantic ocean. Light emissions were observed at all depths, decreasing from typical values of $46/m^3$ at 300 m depth to $5/m^3$ at 2800 m. High abundance was observed over the Faraday Sea mount $>30/m^3$ at 900 m depth.

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At the southern-most station, high abundance (>40/m^3) occurred down to 1500 m. North of the sub-polar front there was little evidence for an effect of the presence of the Mid-Atlantic Ridge, but in the frontal zone, in the vicinity of the Charlie–Gibbs fracture zone, patches of enhanced abundance occurred within an anticyclonic eddy.

Novel tools for quantification of luminescence and fluorescence

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Powerful techniques based on luminescence and fluorescence are now widely used in research; however, there is a requirement for accurate quantification of data and universal standards that allow researchers to compare results. With such a broad range of instrumentation, it is difficult to evaluate sensitivity, e.g. comparison of 'relative light units' values between luminometers. LUX Biotechnology Ltd is a company dedicated to the development of novel luminescence and fluorescence technologies, ranging from calibration standards to live cell assays based on luminous fungi. We introduce Glowell™, a range of unique devices for quantification of luminescence for both microplate readers and low-light imaging systems, e.g. CCD cameras. The practical applications of Glowell™ standards are discussed, including methods for optimizing fluorescence and luminescence imaging techniques.

Luminescent and fluorescent proteins are lighting up cell biology and education

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We describe the use of a novel marine luciferase from the copepod *Gaussia princeps*, expressed in living fungal cells as an indicator of toxicity. We also outline development of an aequorin-based assay, investigating the role of calcium signalling in response to mechanical perturbation in filamentous fungi. Finally, we discuss the use of luminescence and fluorescence in education, e.g. naturally glowing mushrooms and purified recombinant proteins, such as GFP and aequorin. We believe that these visually stunning and scientifically relevant materials help to excite and inspire students during practical demonstrations and lectures.

Development and characterization of highly efficient intramolecular BRET probe

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Bioluminescence energy resonance transfer (BRET) is an intrinsic process that can be observed in sea creatures, such as the jellyfish *Aequorea victoria* and the sea pansy *Renilla reniformis*. It involves non-radiative energy transfer from a donor luciferase to a suitable acceptor fluorescent protein after substrate oxidation. Resonance energy transfer efficiency depends on several factors, such as distance between donor and acceptor molecules, relative orientation and degree of spectral overlap. A conventional BRET method has been developed with an emphasis on separation of the emission peak of fluorescent protein from that of luciferase. This approach results in a striking decrease of emission intensity. From the viewpoint of the nature of the intrinsic BRET phenomenon, we attempted to develop intramolecular BRET proteins consisting of *Renilla* luciferase and GFP variants from the jellyfish *A. victoria*, in order to make fluorescent protein brighter and increase the S:N ratio. We have succeeded in producing highly efficient intramolecular BRET probes and discuss their possibilities.

Mechanistic study of the *Cypridina* (*Vargula*) bioluminescence reaction

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The luciferin–luciferase reaction for the luminous ostraced *Cypridina* (*Vargula*) produces blue light with a high Φ_{BL} (ca. 0.3). In the reaction, *Cypridina* luciferin is oxidized by molecular oxygen (O_2) to give the singlet-excited state of the neutral amide form of oxyluciferin. The reaction consists of two important parts: (a) oxygenation to make a high-energy dioxetanone intermediate; and (b) chemiexcitation from the dioxetanone to the excited molecule with a high efficiency (Φ_S). To establish the reaction mechanism for these parts, we have investigated chemiluminescence reactions of a series of substituted imidazopyrazinones as a bioluminescence model. Especially, we focused on the kinetics and a solvent effect on the chemiluminescent property. From these results, we can explain the oxygenation mechanism including the rate-determining single electron-transfer from imidazopyrazinone anion to O_2, which was predicted by Goto. We will also show the important factor to produce the singlet-excited oxyluciferin with a high Φ_S by the thermal decomposition of the neutral dioxetanone intermediate. In particular, we clarify that the electron-donating indolyl group of the luciferin plays important roles to accelerate the oxygenation and to increase the Φ_S value for the chemiexcitation.

Expression and characterization of luciferases from the glow worm *Lampyris turkestanicus* and the firefly *Photinus pyralis*

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Expression and purification of luciferases from the glow-worm *L. turkestanicus* and firefly *P. pyralis* was carried out using Ni-NTA Sepharose. Kinetic properties of a luciferase from *L. turkestanicus* have been compared with luciferase from *P. pyralis*. cDNA encoding *L. turkestanicus* luciferase was isolated by RT–PCR, cloned and functionally expressed in *Escherichia coli*. Amino acid differences in its primary structures in relation to *P. pyralis* luciferase brought about changes in the kinetic properties of the enzyme, as evident by substantial lowering of K_m for ATP, stability of luminescence signal and more thermosensitivity. The K_m values for ATP and optimum pH were different for both *Photinus* and *Lampyris* luciferases, although their light emission spectra were similar. Enhancement of tryptophan fluorescence was observed to be higher for *P. pyralis* than *L. turkestanicus* luciferase. Luciferase from the glow worm *L. turkestanicus* followed Michaelis–Menten kinetics, with a K_m of 182 µmol/L for ATP and 29 µmol/L for luciferin, with a maximum activity at pH 9.0, which can make it as a suitable reporter in molecular biology and microbial screening. Structural characterization using CD and IR spectroscopy will be reported.

Time-resolved measurement of firefly bioluminescence using photolysis of caged ATP

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A new method to study dynamic processes of bioluminescence has been developed. Caged ATP was added with luciferin, luciferase and $MgSO_4$ to HEPES buffer (pH 7.8) in the dark and the solution was irradiated by a Q-switched laser pulse (355 nm). ATP released from caged ATP instantly triggered the reaction. Temporal variation of the luminescence intensity in the range 530–610 nm was observed using a photon-counting system. A mechanical shutter was closed for 2 ms after the laser shot, so as to prevent output saturation due to the luminescence of luciferin itself. A very fast peak and a monotonous decay were clearly observed with a resolution of 3 ms. The time course is well reproduced by a sum of two exponential functions. The fast and the slow decay constants are about 5 ms and about 20 ms, respectively. The origin of these two kinds of decay will be discussed.

Simultaneous bioluminescent immunoassay using aequorin-labelled Fab fragment and biotinylated firefly luciferase

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We have developed the rapid and sensitive simultaneous bioluminescent immunoassay (BLIA) using aequorin (Aq)-labelled Fab fragment and biotinylated firefly luciferase (b-Luc). We had previously established a simultaneous bioluminescent assay using Aq and b-Luc. The minimum detection limits of Aq and b-Luc were 9.4×10^{-21} mol/L/assay (blank + 3SD) and 3.6×10^{-19} mol/L/assay (blank + 3SD), respectively. The measurements of two photoproteins were completed in 4 s with a single assay medium. In this study, we selected prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) as the analytes, and applied simultaneous BLIA for measurement of these analytes. PSA and PAP were detected by b-Luc–streptavidin complex and Aq-labelled anti-digoxigenin Fab fragment, respectively. The measurable ranges of PSA and PAP were 0.2–200 ng/mL and 0.04–100 ng/mL, respectively. The concentrations of PAP and PSA in human serum could be accurately measured by the proposed BLIA. Good correlations were observed between the results obtained by the proposed BLIA and those by commercial kits.

Chemical approaches for enhanced firefly bioluminescence

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Although firefly bioluminescence has attracted people for thousands of years, the precise chemical mechanism of the light production has not been clearly understood. The light-producing luciferin–luciferase reaction of the firefly consists of two sequential steps; the adenylylation of D-luciferin (D-LH_2) followed the oxygenation of the resulting D-luciferyl adenylate (D-LH_2-AMP) with molecular oxygen. To clarify the oxygenation step in detail, we investigated the light production kinetics by using the key intermediates D- and L-LH_2-AMP. Diastereomerically pure D- and L-LH_2-AMPs were prepared by the condensation of pure D- or L-LH_2 and AMP with DCC, followed by HPLC purification. The natural isomer, D-LH_2-AMP, strongly emitted yellowish green light with luciferase without Mg^{2+} and ATP. Its luminescence was more accelerated than the conventional bioluminescence reaction using D-LH_2 and Mg-ATP. On the other hand, the diastereomeric L-LH_2-AMP produced no light, but served as a strong competitive inhibitor for the light production from D-LH_2-AMP. These results clearly indicated that firefly luciferase strictly recognizes the chirality of LH_2-AMP for bioluminescence.

Bioluminescence in the spaceflight and life science training programme

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Bioluminescence was used for the 6 week Spaceflight and Life Science Training Program (STSTP) to attract the 'best and brightest' US students to careers in space science and

engineering. The students worked with the research projects 'Bioluminescent biosensors for space biotechnology', which had two components. One was to conduct experiments to understand the plant metabolic changes that may become stressed from low pressure/low temperature and other conditions, growing in closed environmental chambers and the Mars greenhouse, located at Kennedy Space Center. Another was to detect contaminants in the air, water, and soil taken from environmental growth chambers. We provided research projects, lecture courses on luminous organisms, biochemistry of bioluminescence, bioluminescent analysis and biosensors, and experience while mentoring the trainees in the laboratory, evaluated the performance of the trainee each week using attendance, performance levels in laboratory projects, and oral/written presentations of laboratory findings. The advantages of using bioluminescence in scientific and educational programmes for Russian and US universities have been discussed. Research funded by the Fulbright Program, a grant of the Russian Humanitarian Scientific Foundation, and a Grant of CRDF and the Russian Ministry of Education and Science (No. KY-002-X1).

Do muscular sphincters control oxygen supply to photocytes in northern krill, *Meganyctiphanes norvegica*?

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The beautiful bell-shaped photophores of the northern krill are innervated by serotonin (5-HT) nerves and richly supplied by vessels carrying oxygenated haemolymph, both crucial for bioluminescence. Filamentous tissue forms a cuff-like structure ('sphincter') around the vessels where they open to the photocytes (1). We investigated whether these 'sphincters' are contractile and able to control light production by changing oxygen delivery to the bioluminescent tissue. Histochemical labelling of photophore sections, using a marker for filamentous actin (rhodamin phalloidin), reveals a ring-shaped structure around vessels where they empty next to the photocytes, indicating that the filaments of the 'sphincter' might be contractile. A muscle-relaxing drug, papaverine (0.1 mmol/L), stimulated light production in a living specimen of northern krill, while the muscle-contracting substance *S*-(–)-BayK8644 (0.01 mmol/L) did not affect light production. Surprisingly, both papaverine (0.1 mmol/L) and *S*-(–)-BayK8644 (0.01 mmol/L) increased the bioluminescence response to 5-HT (0.03 mmol/L). Immunoreactivities against 5-HT and nitric oxide synthase (NOS) were also detected in the 'sphincter' area, indicating that both 5-HT and nitric oxide might be involved in the control of oxygen to the photocytes. The results suggest that photophores of Northern Krill contain functional muscular sphincters involved in control of bioluminescence in this species.

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Non-invasive biosensors for the evaluation of bacterial quorum sensing in GI disorders

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Bacteria are thought to be involved in various disorders of the gastrointestinal (GI) tract, ranging from infection to chronic inflammation. Therefore, bacterial load may play a crucial role in pathogenesis and disease course. Bacteria are known to communicate with each other by producing and responding to signal molecules, such as N-acyl-homoserine lactones (AHLs), whose concentration is proportional to the cell population density. Expression of genes responsible for bacterial pathogenicity and other functions occurs when a critical concentration of signal molecules is reached. This phenomenon is known as quorum sensing, and allows bacteria to exhibit group-based behaviour. In our study, we are investigating the association of AHLs present in human saliva and stool samples with GI disorders, using whole-cell-based sensing systems that employ bacterial luciferase as the reporter protein. The intensity of the bioluminescence produced is directly related to AHL levels in the sample. Our method of detection is non-invasive, cost-effective and fast, while requiring simple sample preparation and providing high-throughput results. We envision that this novel biosensing system could serve as an important tool for monitoring disease activity and managing bacteria-related GI disorders, thus paving the way to the use of quorum-sensing molecules as biomarkers of disease.

Calcium dependence of aequorin bioluminescence dissected by random mutagenesis

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Aequorin bioluminescence is emitted as a rapidly decaying flash upon calcium binding. Random mutagenesis and functional screening were used to isolate aequorin mutants showing a slow decay rate of luminescence. Calcium sensitivity curves were shifted in all mutants, and an intrinsic link between calcium sensitivity and decay rate was suggested by the position of all mutations in or near EF-hand calcium-binding sites. From these results, a low calcium affinity was assigned to the N-terminal EF hand and a high affinity to the C-terminal EF-hand pair. In wild-type aequorin, the increase of the decay rate with calcium occurred at constant total photon yield and thus determined a corresponding increase of light intensity. Increase of the decay rate was underlain by variations of a fast and a slow component and required the contribution of all three EF hands. Conversely, analyses of double EF-hand mutants suggested that single EF hands are sufficient to trigger luminescence at a

slow rate. Finally, a model postulating that proportions of a fast and a slow light-emitting state depend on calcium concentration adequately described the calcium dependence of aequorin bioluminescence. Our results suggest that variations of luminescence kinetics, which depend on three EF hands endowed with different calcium affinities, critically determine the amplitude of aequorin responses to biological calcium signals.

Cholinergic modulation of the cerebral cortical network monitored by bioluminescent aequorin imaging and patch clamp

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Neuronal network activities show precise spatio-temporal patterns which involve heterogeneous populations. These activities result from interactions between the intrinsic properties of neurons and their synaptic connections, which can be modified by neuromodulators. Among these neuromodulators, acetylcholine acts in many higher brain functions in which the neocortex plays a key role. The cholinergic modulation of the neocortical network was studied in rat acute brain slices by optical imaging, using the bioluminescent protein aequorin as a calcium sensor and whole-cell patch-clamp recordings. We found that the cholinergic agonist carbachol (CCH) increased the spatial and temporal range of bioluminescent responses (i.e. calcium signals) evoked by electrical stimulations. The CCH effects involved pyramidal cell assemblies, mainly localized in layer V. In the presence of glutamatergic antagonists, the effects of CCH were attenuated, suggesting an involvement of intra-cortical excitatory connections in the spatial and temporal increase of the response. We sought for electrophysiological activities that could underly the CCH effects on calcium dynamics. Current-clamp recordings of layer V pyramidal neurons revealed that CCH enhances and prolongs the spike discharge and the afterdepolarization evoked by electrical stimulations. Voltage-clamp recordings showed that in the presence of CCH, electrical stimulations resulted in a sustained EPSC increase. Effects of CCH were mimicked by muscarine but not by nicotinic agonists. These results indicate that, in the presence of muscarinic agonists, transient stimuli induce long-lasting recurrent network activities involving layer V pyramidal neurons.

Optimizing multilabel luminometric assays with luminescence spectral scanning

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Using multible luminescent labels in the same assay is a new emerging technology which is increasingly interesting researchers. Commonly two or more labels are measured, using filters to separate the signal from each label. In this paper we show the principle and practical examples of how the monochromator-based spectral scanning microplate reader can be used in easy optimization of the multilabel assays. Optimization is based on the measurement of luminescence spectra from each label and from the label mixture. Mathematical analysis of the resulting spectra reveals the optimal wavelengths to be used for the best possible separation of the individual signals from a multilabel sample. Optimal spectral bandwidths for the measurements are also analysed, based on the luminescence spectra. Two different multilabel combinations were used as examples of the efficiency of the optimization. One was the commonly used combination of firefly and *Renilla* luciferase and the other was the combination of green and red click beetle luciferase used in Promega Corporation's Chroma-Glo technology. When optimal wavelength parameters for both assays were revealed with luminescence spectral scanning, special filters were produced, based on the existing data. The performance of the assays with optimal filter constructs was evaluated against standard filters used with these labels. All spectral scanning measurements were performed with Thermo Electron's new Varioskan Flash multitechnology reader, using a 384-well plate format and the instrument's luminometric spectral scanning mode. Filter system evaluations were done with the same Varioskan Flash instrument, using its filter measurement mode. All luciferase samples used in the measurement were kindly provided by Promega Corp.

One luciferase gene with two different domains in the heterotrophic dinoflagellate *Noctiluca miliaris* has evolved into two separate genes in photosynthetic species

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In seven species studied earlier, the luciferase (*lcf*) gene codes for a single protein (~138 kDa), with an N-terminal ~100 amino acids (aa) of unknown function and three contiguous homologous domains, each with a luciferase active site. In some, there is a second gene coding for a luciferin-binding protein (LBP). In the heterotrophic species *N. miliaris*, the organization of the luciferase gene is strikingly different, coding for a single polypeptide with two contiguous distinct domains. The N-terminal portion (where the ~100 aa sequence is absent) is shorter than, but homologous to, the individual domains of the Luciferases (LCF) of the seven species. The C-terminal part has sequence similarity to the LBP, encoded by a separate gene in *Lingulodinium polyedrum*. The native protein has the same size on Western blots (~100 kDa) as the heterologously expressed polypeptide, ruling out the possibility of its being a polyprotein. Thus, sequences found in two proteins in the *L. polyedrum* luminous system are present in a single polypeptide in *Noctiluca*. Unlike the seven other species, the activity of the expressed luciferase domain of *Noctiluca* is not pH-dependent, but the reaction in cell extracts is.

Evolution of luciferase in seven photosynthetic dinoflagellates: coding sequences are conserved but intergenic regions between tandem copies are not

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We have examined the structures, sequences and evolutionary relationships of seven luciferases. All have the same unique structure: three homologous domains, each with catalytic activity, with an N-terminal region of unknown function. Coding sequences are highly conserved, but the regions between tandem copies are strikingly different and contain no identifiable canonical promoter sequences, thus bearing similarities to ribosomal genes. Individual domains are more similar between species than between domains within species, and trees constructed from each of the domains are congruent with the tree of the full coding sequence. Luciferase and ribosomal DNA trees both indicate that the luciferase of the prototypical species *Lingulodinium polyedrum* diverged early from the other six. The very strong conservation of the amino acid sequence in the central regions of the domains indicates this as the catalytic site, this being confirmed by the X-ray structure. There is also a striking and unexplained nucleotide conservation in this region in some species; it correlates inversely with the content of GC_3, which can be accounted for by the usage biased toward C-ending codons at the degenerate sites.

Stabilization of *Luciola mingrelica* firefly luciferase by mutation of non-conservative cysteine residues

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Luciola mingrelica firefly luciferases with point mutations Cys86Ser, Cys146Ser and Cys146Ile were prepared. The catalytic properties of all mutants were similar to those of the wild-type recombinant enzyme. Cys146Ile and Cys146Ser enzyme mutants were two- and three-fold more stable than wild-type luciferase at 37°C and the Cys146Ser mutant showed the higher resistance to organic solvent. 50% of the mutant enzyme activity was lost in the presence of 20% DMSO, while more than 75% of the wild-type enzyme was denatured. The stabilization effect was higher when the Cys146 located on the surface of enzyme molecule was replaced by a hydrophilic Ser residue, but not by hydrophobic Ile.

An innovative chemiluminescent flow-assisted immunoassay for whole-cell detection

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Field-flow fractionation (FFF) is a family of flow-through separation techniques able to fractionate macromolecules and nano- and micro-sized particles, based on their different morphological properties, such as size, density, shape and surface features (1). This study describes the implementation of FFF into innovative chemiluminescent (CL) enzyme immunoassays for whole-cell detection. Such immunoassays combine the sensitivity of CL detection with the separation capabilities of gravitational FFF (GrFFF). In particular, peroxidase-labelled monoclonal antibodies produced against cellular surface antigens are directly added to the cell sample and the mixture is immediately injected into the GrFFF channel, where the immunological reaction takes place. After incubation, the flow is started and the free and bound antibody fractions are separated by GrFFF. The activity of the peroxidase is measured on line by a flow-through chemiluminometer, following post-channel infusion of a properly formulated H_2O_2–luminol-based enhanced CL substrate. The free and bound antibody portions are then quantified by voidpeak and retained-peak area measurements. Bacterial cells were chosen as the model analyte. This new, flow-assisted immunoassay format shows many advantages over conventional microtitre plate sandwich-type immunoassays: (a) antibody immobilization is not required; (b) only a single monoclonal antibody is employed for cell detection, thus avoiding antigen cross-reaction or matrix interferences; (c) no washing steps are needed to separate free and bound antibodies; (d) analysis time and reagent consumption are reduced. In addition, the method is suitable for automation and multiplexed immunoassays for simultaneous analysis, because cells present in the same sample with different morphological properties can be selectively fractionated by GrFFF before CL detection.

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Chemiluminescent sensor to assess circulating phagocyte activity

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We describe a chemiluminescent sensor based on a novel computerized multi-sample temperature-controlled lumino-

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meter for a fibre array-based biosensor to monitor circulating phagocyte activity. It can simultaneously perform integral measurements of chemiluminescence emitted from samples containing less than 0.5 μL whole blood, while the samples and detector do not change their position during the measurement cycle. The optical fibres in this luminometer are used as both light guides and solid phase sample holders. The latter feature of the instrument design simplifies the assessment process of both the extra-cellular and the intra-cellular parts of the phagocyte-emitted chemiluminescence, using the same system. The effect of cuvette geometry, temperature and some priming agents were tested to prove the system. Several groups of patients were tested: healthy dialysis, healthy dialysis with diabetes, peritonitis, tunnel infections, and suppressed after transplantation, to build a multidimensional description of respiratory burst pattern of CAPD-related clinical groups. Up to four clinical groups were separated with classification error 0. Good correlation was observed between the clinical and respiratory burst status of several peritonitis patients during their sequential 'blindly performed' measurements. The work describes a chemiluminescent system with potential for an automated system for assessment and monitoring of circulating phagocyte functional status by the chemiluminescence emitted during stimulated respiratory burst.

Echinoderm bioluminescence: new observations

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Within the phylum Echinodermata, bioluminescence is not uniformly distributed across the classes: ophiuroids (38 species) and holothurians (30 species) represent 78% of luminous echinoderm species, while asteroids and crinoids contribute 23% and 3%, respectively. Field surveys in Australia in 2005, using scuba-divers along the Victorian and Queensland coasts and a deep sea expedition around south-western Australia, provided unique opportunities to study echinoderm bioluminescence in different biotopes. Among the 125 echinoderm species tested, 32 were found to be luminescent. More than 20 new bioluminescent ophiuroids, six holothurians and one crinoid were recorded. Important findings include: (a) bioluminescence is not uniformly distributed across the different biotopes; (b) the light emitted can be green or blue; (c) the intensities and patterns of light emission are highly variable between species. It is suggested that bioluminescence in echinoderms is more widespread than initially thought. New surveys will be conducted to develop this research programme in order to understand why echinoderms are so bright.

Recombinant GFP of the jellyfish *Clytia gregarium*: bioluminescent resonance energy transfer (BRET) between photoprotein clytin and GFP

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Many marine coelenterates exhibit a green bioluminescence *in vivo* but a blue bioluminescence from the *in vitro* reaction of the purified proteins. This phenomenon is called BRET because it is believed to occur by a non-radiative coupling of electronic excited states of a bioluminescent donor and a fluorescent acceptor, analogous to the Förster mechanism described in homogenous solution. Although the difference for *in vivo* and *in vitro* spectra is observed for many coelenterates, only the *Aequorea* and *Renilla* systems have received any detailed study. Here we report cloning, sequence analysis of the cDNA encoding *Clytia gregarium* GFP (cgGFP), cgGFP cDNA expression in *Escherichia coli* and mammalian cells, and its spectral properties. We demonstrate also that, unlike aequorin and *Aequorea* GFP, the mixture of recombinant photoprotein clytin and cgGFP displays a green bioluminescence *in vitro* at low protein concentrations, similar to that reported for *Renilla* luciferase GFP. This presumes non-radiative energy transfer between clytin and cgGFP and protein–protein complex formation. Supported by Bayer Healthcare AG (Germany), RFBR Grant 05-04-48271 and the RAS programme for Molecular and Cellular Biology.

Improved Ca^{2+}-activated photoproteins for HTS applications

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The use of a Ca^{2+}-activated photoprotein, with its inherent property of flash-type luminescence, represents a valid tool for analysing all aspects of Ca^{2+}-mediated signal transduction processes in mammalian cells. While the miniaturization of the HTS process for testing chemical compound libraries has allowed scaling up of the number of wells/plate from 96 to 384 and up to 1536, and a saving on the volumes of compounds used, the lower number of cells/well means that the reporter system must necessarily generate a strong and reproducible signal that can be accurately detected. To face these challenges, we are engaged in finding and developing new photoproteins with novel or improved properties, such as different Ca^{2+} affinity, higher light release and slower kinetics. In order to select a pool of very sensitive photoproteins, several approaches, including construction of chimeric molecules, site-directed and random mutagenesis were evaluated. Considering both the intensity of light release and sensitivity to low Ca^{2+} stimulation as selecting criteria, a high-throughput functional screening for new photoproteins was developed, taking advantage of a CCD camera-based plate reader for the detection of flash luminescence. The most interesting new photoproteins were further characterized and used in cell-based assays for the screening of chemical compounds.

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High-throughput detection of hydrogen peroxide. Validation of homogeneous chemiluminescent assays for choline in human plasma and whole blood

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Choline is a fascinating molecule whose metabolism/function is intertwined with that of phospholipid synthesis and intercellular signalling, cholinergic neurotransmission and homocysteine biosynthesis. The concentration of free choline is highly regulated in humans and can be perturbed by diet, pregnancy and pathological conditions such as ischaemia and renal failure. Additionally, a number of enzymes of current interest as biomarkers/drug targets lead directly (lysophospholipase D/autotaxin) or indirectly (phospholipase A_2) to choline. Thus, a rapid high-throughput method for choline analysis was highly desirable. We developed a method of quantitation of choline in neat human plasma and ultrafiltered whole blood, based on the enzymatic generation of hydrogen peroxide from choline (0–150 µmol/L), using choline oxidase and acridinium-9-carboxamide chemiluminescent detection. The assays run on an open platform microplate luminometer with a throughput of 250 analyses/h with excellent analytical sensitivity (LOD 0.1 µmol/L), total imprecision (1.4–4.3%), recovery (90–106%) and dilution linearity ($R^2 > 0.99$). Specificity (<15% interference) was shown by testing with a series of endogenous substances (bilirubin, triglycerides, protein, haemoglobin) and common drugs (ascorbic acid, aspirin, heparin, etc.) The methods were further validated by correlation to LC–MS/MS (slope, 0.91–0.94; *R*, 0.971–0.989).

Elevation of choline concentration in cardiac troponin-I positive human plasma

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Choline has gained recent attention as a cardiac marker, particularly in patients with suspected acute coronary syndrome (ACS). The relationship between choline and other established cardiac markers, such as cardiac troponin T or I, is of current interest. In a recent study on reperfusion injury and ischaemic pre-conditioning, it was reported that the efflux of choline paralleled that of cardiac troponin during experimentally induced global ischaemia/reperfusion in the isolated rat heart model. Here our method of quantitation of choline in neat human plasma, based on the enzymatic generation of hydrogen peroxide from choline (0–150 µmol/L) using choline oxidase and acridinium-9-carboxamide chemiluminescent detection, was used to evaluate the level of choline in normal donor and cardiac troponin-I positive human plasma. In the normal donor population the median choline concentration was 11.28 µmol/L (25th–75th percentiles, 9.73–13.13), while in the troponin-positive plasma samples the median choline concentration was 20.6 µmol/L (14.60–26.80), almost double that seen in the normal donor population. 75% of the troponin positive samples exceeded 14.51 µmol/L (90th percentile of the normal donor population) and 60% exceeded 18.42 µmol/L (97.5th percentile).

Oceanic biofluorescence: coral reefs, open ocean and the deep sea

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The fluorescence of non-bioluminescent marine organisms remains poorly characterized. Even in reef-building corals, which have been extensively mined for GFP-like fluorescent proteins in recent years, the function of fluorescence is still enigmatic. Recent data indicate that the fluorescent colour diversity in corals is a product of adaptive evolution that appeared independently several times, and that the ancestral colour was green, as in bioluminescent jellyfish. It is plausible that this diversity evolved as part of the mechanism regulating coral–alga symbiosis. Another possibility is, quite unexpectedly, camouflage: our recent analysis suggests that fluorescent proteins may conceal the colour of symbiotic algae (zooxanthellae) from the eyes of the reef fishes, which can be a form of protection against herbivory. Other reef inhabitants (crustaceans) may use fluorescence as visual communication signals or as camouflage against the coral background. Recently we were able to take the first look at biofluorescence in the open ocean and the deep sea during two research cruises involving submersible diving ('Operation Deep Scope', http://www.oceanexplorer.noaa.gov/explorations/05deepscope/welcome.html). In the open ocean, which provides a perfect blue light field and a contrasting background for viewing fluorescent signals, fluorescence is very common in prey-capturing appendages of non-bioluminescent hydroids, e.g. jellyfish and siphonophores, suggesting a prey attraction/retention function. A hydroid jellyfish was also found that possesses green fluorescent nodules containing zooxanthellae. GFP-like proteins are used by planktonic copepods (family Pontellidae) most likely as a means of visual communication. Several shrimp species of the *Sargassum* weed community possess fluorescent chromatophores, sometimes of diverse colours, possibly for the same purpose. Perhaps surprisingly, several prominent examples of fluorescence have been observed in the benthic fauna at 600–800 m depth, well below the photic zone. These examples include zoanthids, tube anemones and some fish species. It is tempting to speculate that deep-sea biofluorescence utilizes community bioluminescence, rather than downwelling light, as a primary excitation source.

Development of an electrochemiluminescence assay for detection and quantification of *Clostridium perfringens* α-toxin

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A direct sandwich electrochemiluminescence (ECL) assay was developed for identification and quantification of *Clostridium perfringens* α-toxin (phospholipase C). Biotinylated antibodies to *C. perfringens* α-toxin bound with high affinity to streptavidin paramagnetic beads were used to immunoadsorb soluble sample α-toxin, which subsequently immunoadsorbed ruthenium-labelled detection antibodies to *C. perfringens* α-toxin. The ruthenium (Ru) chelate of the detection antibodies chemically reacted in the presence of tripropylamine (TPA), such that, upon electronic stimulation, photon emission (electrochemiluminescence) resulted that was detected by a photodiode detector. Elevated toxin concentrations increased toxin immuno-adsorption and the subsequent immunoadsorption of Ru-labelled antibodies to α-toxin, which resulted in proportionally increased toxin-dependent electrochemiluminescent, signals. The standardized assay was rapid (single 2.5 h coincubation of all reagents), required no wash steps, and had a sensitivity of about 1 ng/mL toxin. The assay had excellent accuracy and precision when validated in buffer, serumand urine with no apparent matrix effects.

Development of a multiplexed bioluminescent cell-based assay with *luc* gene from *Luciola italica* for high-throughput screening of cholesterol-lowering drugs

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The cDNA encoding the luciferase from the Italian firefly *Luciola italica* has been recently cloned and preliminary expressed in *Escherichia coli* (1). The bioluminescence emission of the Italian firefly luciferase has a maximum at 566 nm at pH 7.8 and shows extended light emission decay kinetics. This new luciferase has been cloned in suitable vectors and expressed in *E. coli*, *S. cerevisiae* and mammalian cells (HepG2) to explore its applicability as a reporter protein in whole-cell biosensors, non-invasive *in vivo* imaging and multiplexed assays. Diverse mutants were also obtained by site-directed or random mutagenesis. In the present study, a new cell-based assay with two luciferases emitting at different wavelengths has been developed to monitor *in vivo* the two pathways of bile acid biosynthesis. Bile acid biosynthesis is in fact a key step of intracellular cholesterol homeostasis and, in turn, cholesterol synthesis rate in hepatocytes. Bile acids are synthesized via the classic pathway initiated by cholesterol 7α-hydroxylase (CYP7A1), and via alternative pathways, one of which is initiated by sterol 27-hydroxylase (CYP27). We evaluated the ability of natural and synthetic bile acids and other compounds to activate or hamper the two bile acid synthesis pathways, using a recombinant HepG2 cell-based luciferase multiplexed assay. Cells were stably transfected with the expression vector pcDNA3.1-FXR, containing the cDNA encoding human Farnesoid X Receptor, in order to obtain clones that stably expressed the receptor. The clones were transiently transfected with reporter plasmids containing the CYP7A1 promoter driving the expression of a wild-type *Photinus pyralis* luciferase and the CYP27A1 promoter regulating the expression of a red-emitting thermostable *L. italica* luciferase. The developed multiplexed cell-based assay allows the simultaneous monitoring of the two pathways in a high-throughput format (96-well microtitre plate), being thus suitable for the screening of new cholesterol-lowering drugs.

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Construction of transgenic mice expressing novel beetle luciferases

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A variety of luciferase genes are employed for long-term and non-invading monitoring of gene expression, because their products catalyse the emission of light without requiring exogenous illumination. Although firefly luciferase is the most frequently used as a reporter gene, other types of beetle luciferase genes have not been utilized, so far as we know. In this study, we have generated two kinds of transgenic mice expressing novel green light-emitting beetle luciferases from *R. ohbai* and *P. termitilluminans* under the control of murine clock gene promoters *Per2* and *Bmal1*, respectively. After intraperitoneal injection of D-luciferin into both mice, significant bioluminescent signal could be detected using a CCD camera. Furthermore, a robust circadian rhythm of bioluminescence from cultured peripheral tissues, such as lung, isolated from both mice, was also detected using a dish-type luminometer (ATTO Co.). These results suggest that the two beetle luciferases are useful bioluminescent reporters for *in vivo* imaging and real-time monitoring of cultured tissues, and that transgenic mice created in this study will help determine the molecular mechanisms of the circadian clock.

Detection of a bioluminescent milky sea from space

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Some of the most fascinating displays of bioluminescence are the legendary 'milky seas', where the surface of the ocean in all directions produces a uniform and sustained glow. Although a full explanation is still debated, this phenomenon is hypothesized to be caused by large numbers of bacteria, because those are the only known organisms whose emission properties match with the characteristics reported for these displays. Because of their unpredictable nature and the scarcity of scientific observations, full explanation of milky seas has remained elusive. Here we report the first satellite observations of a bioluminescent milky sea. A ~15 400 km^2 area of the Indian Ocean, roughly the size of the state of Connecticut, was observed to glow over four consecutive nights. The event was simultaneously observed on the first night by a ship passing through the waters. Using the detector's sensitivity and the area of the event, we calculate the approximate number of bacteria involved in the event, giving a number comparable to estimates of total bacteria in the coastal oceans of the world.

Real-time and simultaneous measurement of clock gene expressions by multicolour reporter assay system

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To analyse the dynamics or persistence of clock gene expression, the real-time monitoring system using firefly luciferase is widely used. Although this system enables longitudinal and quantitative monitoring of gene expression in living cells, more than one gene expression cannot be monitored. To overcome the limitations of the current system, we have developed a real-time monitoring system in which two gene expressions can be monitored simultaneously by splitting the emissions from green- and red-emitting beetle luciferases. To verify the system, promoter regions of *mPer2* and *mBmal1* were connected to the red and green luciferases, respectively, and the constructs were transiently co-transfected into NIH3T3 cells. The bioluminescence emission from the cells was continuously measured with a luminometer equipped with an optical filter (ATTO Co.). The circadian rhythms of bioluminescence of *Per2* and *Bmal1* expressions were almost 180° out of phase, consistenting with endogenous mRNA expression patterns. The result clearly indicates the system can accurately monitor respective gene expression profiles in the same cell populations, even when these were measured simultaneously.

cDNA cloning, expression and homology modelling of a luciferase form the firefly *Lampyroidea maculata*

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The cDNA of a firefly luciferase from lantern mRNA or *Lampyroidea maculata* has been cloned, sequenced and functionally expressed. The cDNA has an open reading frame of 1647 bp and codes for a 548 residue polypeptide. Sequence analysis and homology modelling showed the highest degree of similarity with *Hotaria unmunsana* and *Luciola mingrelica* luciferases, suggesting a close phylogenetic relationship despite the geographical distance separation. The deduced amino acid sequence of the luciferase gene of the firefly *L. maculata* showed 93% identity to *H. unmunsana*. Superposition of the three-dimensional model of *L. maculata* luciferase (generated by homology modelling) and the three-dimensional structure of *Photinus pyralis* luciferase revealed that the spatial arrangements of luciferin and ATP-binding residues are very similar. The putative signature of the AMP-binding domain among the various firefly species and *L. maculata* was compared and a striking similarity was found. Different motifs and sites have been identified in *L. maculata* by sequence analysis. Expression and purification of luciferase from *L. maculata* was carried out using Ni-NTA sepharose.

Biosynthesis of firefly D-luciferin

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In the course of determining the structure of firefly luciferin, both D- and L-luciferin were synthesized from corresponding D- and L-cysteine. Because only D-luciferin showed bioluminescence activity *in vitro*, it was concluded that the D-isomer is the natural product. However, D-cysteine, from which D-luciferin can be obtained, is not the usual stereoisomer as a natural α-amino acid. Here we show that luciferase has enzymatic activity on L-luciferin, which is the stereoisomer of D-luciferin. Thus, L-luciferin was converted into luciferyl-CoA in the presence of luciferase, ATP, Mg^{2+} and co-enzyme A. Subsequent enzymatic hydrolysis of the resulting luciferyl-CoA with porcine liver esterase yielded L-luciferin as well as D-luciferin that could be used for the bioluminescence reaction. These results indicate that the biosynthesis of firefly D-luciferin is not from D-cysteine but from L-cysteine via L-luciferin. Luciferase is the enzyme for bioluminescence but it could also be responsible for stereoisomeric bioinversion of L-luciferin into D-luciferin.

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Enzyme-embedded organic monolith sensor for flow injection-chemiluminescence

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A photopolymerized organic monolith produces a reaction field for chemiluminescence (CL) in an ultra-small space (e.g. a channel of a microchip). A novel enzyme-embedded organic monolith by photopolymerization has been developed for CL reactors in this study. The photopolymerization was conducted in a capillary glass tube (18 mm length, 0.85 mm i.d.) with 3-methacryl oxypropyl trimethoxysilane, DAROCUR1173 and enzymes (peroxidase, blend of glucose oxidase and peroxidase) under UV irradiation for 12 h to make reactors for the assay of hydrogen peroxidase (H_2O_2) and glucose, respectively. The reactors were coupled in a flow injection–horseradish peroxidase (HRP) catalysed imidazole CL system. A microscopic observation of the reactor showed a hollow in middle of the capillary tube, resulting in no back pressure to the flow injection system. The durability of the HRP-embedded reactor was tested by continuous assay of H_2O_2 for 11.2 h, resulting in stability for 2 h. The reproducibility of assaying H_2O_2 (970, 485, 122 μmol/L) was 3.87% ($n = 20$), 2.19% ($n = 7$), 14.3% ($n = 6$), respectively. A calibration equation for H_2O_2 (0, 122, 243, 485, 970, 9700 μmol/L) was $y = -0.147x^2 + 1992x - 1.64 \times 10^5$, $R^2 = 0.993$ (y = light intensity in RLU; $x = H_2O_2$ in μmol/L), and the detection limit was 25.5 μmol/L (S:N = 2). A calibration equation for glucose (0, 62.5, 125, 250, 500 mg/dL) was $y = -4.70x^{-4}x^2 + 0.424x + 13.9$ ($R^2 = 0.954$) (y = light intensity in RLU; x = glucose in mg/dL), and the detection limit was 6.8 mg/dL (S:N = 2). These results showed that the enzyme-embedded organic monoliths assayed hydrogen peroxide and glucose, producing good quantitative results. The method is applicable for making an enzymatic reactor in the ultra-small space of microchips.

Homologous genes of firefly luciferase in Japanese firefly *Luciola cruciata* and non-luminous beetle *Tenebrio molitor*

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Previously, we reported that firefly luciferase is a bifunctional enzyme, having luminescence activity and synthetic activity of fatty acyl-CoA from various long-chain and medium-chain fatty acids in the presence of ATP, Mg^{2+} and CoA. Recently, we found two homologous genes of firefly luciferase (*LcLL1* and *LcLL2*) in the Japanese firefly *Luciola cruciata*. The gene product of *LcLL1* had long-chain fatty acyl-CoA synthetic activity, but not luciferase activity with firefly luciferin. The other product of *LcLL2* showed neither enzymatic activities of acyl-CoA synthetase nor luciferase. We also found three homologous genes of firefly luciferase (*TmLL1*, *TmLL2* and *TmLL3*) in the non-luminous beetle *Tenebrio molitor*. These three gene products showed fatty acyl-CoA synthetic activity, but not luciferase activity. These results suggest that firefly luciferase arose from a fatty acyl-CoA synthetase by gene duplications during insect evolution.

Biogeography and biodiversity of a luminous marine ostracod driven irreversibly by the Japan Current

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The biogeography of the luminous marine ostracod *Vargula hilgendorfii*, also called 'Umihotaru', shows that this organism may have arrived relatively recently on the Japanese islands during the final glacial period, approximately 10 000 years ago. Phylogenetic relationships also strongly indicate that the Japan Current drove the Umihotaru ostracod northwards. It is evident that the Umihotaru ostracod spread rapidly to the major Japanese islands 3000 km north, whereas its spread was slow in the south-west of the Japanese islands, covering a distance of 400 km. The meandering of the Japan Current, where it passes by the Tokara Gap at 28° N latitude, may be a barrier to Umihotaru ostracod extension. We also discuss the diversity of the marine luminous species based on the luciferase genes. Our experimental results will contribute to marine evolutional studies.

Development of a yeast high-thoughput reporter assay using secretory luciferase, CLuc

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The yeast reporter assay is widely used for detection of transcription activation. One of the most useful applications of the yeast reporter assay is a two-hybrid system. However, β-galactosidase, the most common reporter enzyme, is not suitable for a high-throughput analysis, since collection and lysis of yeast cells are required prior to determination of enzymatic activity. We present here a novel yeast reporter assay suitable for high-throughput analysis. In the reporter assay, we employed secretory luciferase derived from *Cypridina noctiluca*, named CLuc, as a reporter enzyme. CLuc was effectively secreted to a culture medium and emitted a light by the addition of CLuc luciferin to the medium. We constructed a reporter plasmid harbouring DNA encoding CLuc. When

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the activities of several yeast promoters were measured, the result was comparable to that from a parallel experiment using β-galactosidase. In addition, truncation assays of promoters revealed the existence of *cis*-elements in the promoters, as in literature reports. The CLuc reporter assay could be semi-automated with a laboratory automation system. In conclusion, the CLuc reporter assay can be applied to not only comprehensive promoter analysis but also high-throughput two-hybrid analyses. A part of this study was supported by NEDO, Japan.

Efficiency of electron-transfer induced chemi-excitation: a comparison of inter and intramolecular processes

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Electron-transfer processes play an essential role in efficient singlet excited state formation in bioluminescence and chemiluminescence (1). Intramolecular electron-transfer-catalysed decomposition of 1,2-dioxetanes, containing electron-rich substituents, leads to efficient singlet-excited state formation. Contrarily, decomposition of peroxides catalysed by intermolecular electron transfer from appropriate activators was shown to occur with low excitation efficiency (1). Furthermore, it has been suggested that chemi-excitation is achieved by intermolecular back-electron transfer (BET) in induced 1,2-dioxetane decomposition, a surprising fact due to the high efficiency of this transformation. Based on these facts, we have studied the decomposition of diphenoyl peroxide and 1,2-dioxetanones, catalysed by several phenolates, as model systems for the comparison of intra- and intermolecular BET chemi-excitation. Although phenolates showed to be highly reactive as activators for peroxide decomposition, the quantum yields obtained in these processes are extremely low. Therefore, the highly efficient induced 1,2-dioxetane decomposition should proceed by an intramolecular BET process.

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A comparison of bioluminescence and chlorophyll fields of the World Ocean

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On the global scale, bioluminescence intensity acts as a sensitive indicator of plankton biomass, toxicity in the marine environment and the functional state of planktonic ecosystems. Plankton bioluminescence, however, has never been amalgamated or analysed in a form of global distribution patterns. The synthesis of a global map of bioluminescence, based on 30 years of field surveys carried out by Russian and Ukrainian research vessels in the World Ocean, is given. Data included about 25 000 oceanographic stations with bioluminescence profiles obtained in the upper 100 m layer in the Atlantic, Pacific and Indian Oceans, the Mediterranean Sea basin and the Norwegian and Barents Seas. The relationships between bioluminescence intensity, remotely sensed chlorophyll *a* and the abundance of phyto- and zooplankton species are discussed.

Bioluminescent biosensors for space biotechnology

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Pyridine and flavine nucleotides mediate hundreds of redox reactions, and thus impact on virtually every metabolic pathway in the cell. Bioluminescent biosensors based on the bacterial coupled enzyme system NADH:FMN-oxidoreductase–luciferase can be used to detect different levels of the metabolites for plants grown in the Mars greenhouse, located at Kennedy Space Center, under stress conditions. Onion and radish grown at CO_2 levels of 350–4000 ppm in environmental chambers were obtained for sampling. The results show that the plants' Flavine Mononucleotide (FMN) levels varied depending on plant type and environmental stress type. Radish extracts indicated FMN levels four times higher than those of onion extracts. The concentration of FMN in radishes is not uniformly distributed throughout the plant: tubers appear to contain five times more FMN than leaves; stems contain three times more. Increase in environmental CO_2 levels by a factor of five increases FMN production by 20%. The sensitivity of the bioluminescent assay for FMN, NAD^+ and NADH was equal about 10 nmol, 5 nmol and 10 pmol, respectively. Research funded by CRDF and Russian Ministry of Education and Science Grants REC 002 KY-002-X1, Y1-B-02-11, Y2-B-02-19, N 02.444.11.7261, N 02.442.11.7364, the Fulbright Program and the National Aeronautics and Space Administration.

Bioluminescence as an alternative method of bacterial cell protection

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A biological role is suggested for bacterial bioluminescence as an alternative method of bacterial cell protection against the influence of reactive oxygen species (ROS) during oxidizing stress. The basic species of luminous bacteria, recombinant strains and their dark mutants [*Vibrio harveyi* (1212), *V. harveyi* (178), *hotobacterium phosphoreum* (1764), *P. phosphoreum* (1764), *Escherichia coli* (Lux^+)] from various ecological niches have been affected under a series of oxidants and oxygen derivatives. Bacterial growth, changes of the kinetic and spectral characteristics of bioluminescence, and activity changes of antioxidant enzymes and luciferase *in vivo* and *in vitro* have been studied. The mechanism of ROS participation in the genetic regulation of bioluminescence is supported by the results of these investigations. On the other hand, the participation of aldehyde-type metabolites in the luminescent reaction was studied on an example of malonic aldehyde. It is known that high ROS concentrations usually result in lipid peroxidation of bacterial membranes. Thus, the lipid peroxidation process is considered as an alternative mechanism of aldehyde substrate formation for the luminescent reaction. Research funded by CRDF and Russian Ministry of Education and Science Grants Y2-B-02-19 and N 02.444.11.7261 and Russian Foundation for Basic Research Grant N105-04-49316-a.

Female responses to complex luminescent male displays in a Caribbean ostracod

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Although bioluminescence is much more prevalent in marine environments than terrestrial ones, describing female response behaviour in mating systems where males utilize distinct, species-specific luminescent courtship displays to attract females has been restricted to a few terrestrial species of fireflies. Male myodocopid ostracods (<2 mm) utilize luminescence in the most unique and complex displays known to date in marine environments, and females are attracted to, and exhibit choice between, these signals. Previous attempts to observe female behaviour have been confounded because displaying males in the same tank intercept the females before she can exhibit choice, and try (unsuccessfully) to copulate with the female. Here, by using a light-emitting-diode array to mimic a male display and infra-red (IR) cameras to record behaviour, we give evidence that females are indeed responding to luminescence and approach a signal in such a way as to intercept a signalling male. We hypothesize that luminescence is the primary signal to attract receptive females, and that secondary chemical cues may prove to be vital for the final homing in on a displaying male.

The use of *lux* genes for monitoring the uptake of *Pseudomonas aeruginosa* by respiratory epithelial cells

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Pseudomonas aeruginosa is found ubiquitously in the environment and is an important cause of respiratory infections in individuals with impaired immunity. Increasing antibiotic resistance in *P. aeruginosa*, coupled with the danger of nosocomial transmission to susceptible individuals, illustrates the importance of fully understanding the means by which the organism persists within the human lung. Numerous bacterial virulence determinants have been implicated in respiratory adhesion and a number of host cell receptors have been identified as potential targets. It has been suggested that a defective host-cell receptor mechanism leads to uptake of bacteria without clearance from the lung, and that such an intracellular reservoir might contribute to the persistence of *P. aeruginosa* lung infections. We have used bioluminescent *P. aeruginosa* to observe bacterial uptake by respiratory epithelial cells and showed that light from intracellular bacteria remained clearly distinguishable 24 h post-inoculation. Internalization was mitigated by temperatures of 10°C, indicating that the process of bacterial uptake was mediated by the human cell-line rather than by the bacteria, and we have begun receptor blocking experiments in order to identify key host-cell receptors. Bioluminescent *P. aeruginosa* has the potential to resolve key difficulties in the understanding of how *P. aeruginosa* persists in susceptible individuals.

Novel BRET-based biosensors with a new bioluminescent donor, *Gaussia* luciferase, for oestrogen and androgen receptor ligands

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Bioluminescence resonance energy transfer (BRET) is a natural non-radiative energy transfer phenomenon occurring in marine organisms between a bioluminescent donor protein and a fluorescent acceptor protein. In order to study an interaction between two target proteins, labelled proteins are generated by genetic fusion with either a bioluminescent donor or fluorescent acceptor. Recently, several *in vivo* and *in vitro* applications of BRET assays have been reported, ranging from membrane-bound and cytosolic receptors dimerization studies to nuclear factor interactions monitoring (1). The majority of BRET assays use *Renilla* luciferase (Rluc) as the energy donor; in the presence of coelenterazine Rluc, instead of emitting blue light, emits blue light which is able to excite a variant of the green fluorescent protein (enhanced yellow fluorescent protein, EYFP) acting as fluorescent acceptor. The recent cloning of several reporter proteins with different spectral properties has enlarged the number of possible bioluminescent donor proteins to be used in BRET assays. In particular, the use of a new bioluminescent donor, *Gaussia* luciferase, has been investigated as an alternative to Rluc. This protein has the advantages of smaller size, higher bioluminescent signal emission when it is codon-humanized, and a flash kinetic (λ_{max} 480 nm), thus suitable for efficient energy transfer to EYFP. Novel BRET homogeneous assays for oestrogen- and androgen-like compounds have been developed and optimized, based on oestrogen and androgen receptor homo- and hetero-dimerization. The developed assays fulfil all the standard requirements of accuracy and intra-assay and

inter-assay precision, with a limit of detection comparable to those of whole-cell biosensors or analogue bioassays, with the peculiar advantages of homogeneous assays.

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Quantitative immunohistochemistry and *in situ* hybridization with luminescence detection: a new potential diagnostic tool

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The ultrasensitive localization and quantification of target molecules inside tissues and cells is fundamental for both the understanding of physiopathological events and the early diagnosis of pathologies. Luminescence imaging techniques allow the performance of multiplexed detection via the combination of different detection principles and/or labels, while chemiluminescence imaging of enzyme-labelled biospecific probes joins high sensitivity with easy and reliable evaluation of the amount of the probe, thus enabling quantitative analysis of the target molecules. Here we report applications of luminescence immunohistochemistry (IHC) and *in situ* hybridization (ISH) procedures for the detection and quantification of target proteins and nucleic acids, including the quantitative detection of the multidrug resistance protein 2 (MRP2) in liver biopsies, the evaluation of the progression risk of cervical intraepithelial neoplastic lesions by analysis of the $p16^{INK4A}$ neoplastic marker concentration profile, and the co-localization of mucosal human papillomaviruses (HPVs) and the HMB-45 melanoma antigen marker, using a combined ISH/IHC method with fluorescence and chemiluminescence detection.

Molecular tuning of aequorin for bioanalytical applications

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Aequorin is a bioluminescent photoprotein that exists as a complex of apoprotein, an imidazopyrazine chromophore and molecular oxygen. Aequorin emits light following the addition of calcium, and has been utilized for *in vivo* calcium detection and as an ultra-sensitive label in a variety of assays. Our research involves tuning both the inherent and analyte-specific bioluminescent properties of aequorin. The inherent bioluminescent properties of aequorin, such as its emission maximum and half-life, have been rationally manipulated by incorporating non-natural amino acids into the primary structure of aequorin and by using various chromophore analogues in place of the native chromophore, coelenterazine. The analyte-specific properties of aequorin are being tuned by creating analyte-specific molecular switches. These molecular switches function by splitting aequorin into two halves and connecting these halves with either a protease-specific sequence or a conformationally flexible protein. The addition of a protease or an analyte that changes the conformation of the inserted protein will then result in the decrease or increase, respectively, of aequorin's bioluminescent signal. We believe that the molecular tuning of aequorin in this manner will both increase the bioanalytical applications of this protein and contribute to a greater understanding of aequorin's photophysical properties and mechanisms.

Molecular phylogenetic analysis of click beetles (family Elateridae) inferred from 28S ribosomal DNA: the origin of bioluminescence in elaterids

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Click beetles (family Elateridae: Coleoptera) include luminous and non-luminous species. In this study, we examined the evolutionary relationships between species of Elateridae based on partial sequences of nuclear 28S ribosomal DNA. The most parsimonious reconstruction of the 'luminous' and 'non-luminous' state, based on the molecular phylogeny, indicates that the ancestral state of Elateridae was non-luminous. This suggests that bioluminescence in the click beetle evolved independently of that of other luminous beetles, such as Lampyridae and Phengodidae, despite their common mechanisms of bioluminescence.

Novel chemiluminescence flow-injection analysis for the determination of sulphide in aqueous samples

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The analysis of sulphide using a chemiluminescence–flow-injection procedure (CL–FI) was described. Acidic potassium permanganate was used as the chemiluminescence agent and the analytical conditions were optimized. The proposed method offers a very sensitive analytical procedure for

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sulphide in aqueous samples, with a limit of detection of 2 μg/L. The method was rapid and precise and the solutions can be analysed at a rate of 150/h. The procedure was applied to the determination of sulphide in several natural spring waters and wastewater in the northern region of Thailand.

Using recombinant antigens for detection of antibodies to *Trypanosoma cruzi* on a fully automated chemiluminescence analyser

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Chagas' disease (American trypanosomiasis), caused by the protozoan *T. cruzi*, is endemic to most regions of Latin America. Transfusion of blood from infected donors is one of the routes for contracting the disease. Currently, Chagas' disease is not endemic in the USA, neither is blood screened there. Concerns on the safety of blood supply in the USA have been raised because of a largely 'silent' reservoir of *T. cruzi* in Latin American immigrants, who represent a growing donor population. The assay uses: (a) microparticles coated with four different multi-epitope recombinant antigens (rAgs) of *T. cruzi* to capture antibodies to *T. cruzi*; (b) an acridinium derivative-labelled mouse anti-human IgG to tag the captured human Ab on the microparticles; and (c) alkaline peroxide to trigger the chemiluminescence. The assay was evaluated with 351 Chagas specimens for sensitivity and 7911 unscreened plasma and sera for specificity. Two ELISAs plus radio-immunoprecipitation assay (RIPA) were used as supplemental tests on repeatedly reactive samples for confirmation. The sensitivity and specificity of the assay was evaluated by testing a number of *T. cruzi*-positive specimens, random donor negative serum and plasma donations to set a provisional cut-off to differentiate positive and negative responses. Preliminary data showed 100% detection of confirmed *T. cruzi*-positive specimens ($n = 351$). Specificity was estimated to be 99.92% ($n = 7911$); among six repeatedly reactive, there was one confirmed and five non-confirmed. A prototype Chagas assay based on rAg and chemiluminescence detection was developed on the fully automated PRISM® instrument and exhibited excellent sensitivity and specificity. The development of this assay into a product for blood screening to prevent transfusion-transmitted Chagas' disease is in progress.

Time-dependent spectral change of chemiluminescence from firefly luciferin in deoxygenated dimethyl sulphoxide

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We have investigated the chemiluminescence reaction process of firefly luciferin (Ln) in dimethyl sulphoxide (DMSO) with added potassium *t*-butoxide (BuOK). This light emitter, an exited oxyluciferin (OxyLn*), is interpreted to be formed from a dioxetanone. We produced Ln intermediates before forming the dioxetanone. The intermediate (called M_{420}) shows 510 and 620 nm light emissions when oxygen gas is introduced into its solutions under some combinations of the water content of DMSO and *t*-BuOK concentration. The products (called N_{420} and P_{535}) formed after these emissions show absorption and fluorescence peaks at 420 and 560 nm, and 535 and 620 nm, respectively. We thus conclude that M_{420} emits light simultaneously at peak 510 nm from an excited unidentified species and 620 nm from an excited P_{535}, respectively.

Cost-competitive bioluminescence-based biosensors: advantages of integrated approach in biosensor design to successful commercialization

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Bioluminescence-based biosensors play an increasing role in environmental control, medical diagnostics and safety monitoring. However, their widespread use may be hindered by some disadvantages, including instability of the enzyme systems during use, limited shelf-life, the need to control ambient conditions, interference from substances in the sample, and manufacturing cost. Bioluminescent enzyme system technology was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for medical, environmental and industrial applications. This technology enables rapid design of polyenzymatic systems that incorporate multiple substrates with the luciferase–oxidoreductase enzymes isolated from a proprietary collection of luminous bacterial strains. A patented stabilization and immobilization process preserves up to 50% of enzymatic activity. Prototype biosensors developed with this technology offer cost advantages, versatility, high sensitivity (up to 10^{-14} mol analyte), rapid response time (less then 3 min), extensive shelf-life (up to 5 years without loss of activity) and flexible storage conditions (up to +25°C). We are aiming to commercialize this technology for markets that require rapid, inexpensive detection methods without the need for time-consuming sample preparation. The long-term goal is to build a platform technology for a range of compounds toxic to human and other organisms. Research funded by a grant of CRDF and the Russian Ministry of Education and Science, No. KY-002-X1.

Luciferase from the Italian firefly *Luciola italica*: molecular cloning and expression

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The cDNA encoding the luciferase from the Italian firefly *Luciola italica* was cloned using reverse transcriptase-PCR and a gene-specific primer set based on the DNA sequence of *Luciola mingrelica*. The cDNA sequence of *Luciola italica* luciferase was determined to be 1647 base pairs in length, with an open reading frame of 548 amino acids. Phylogenetic analysis of the protein sequence demonstrated that this luciferase is closely related to that of other fireflies of the family Lampyridae, particularly within the subfamily Luciolinae, showing 96% homology to luciferases from the fireflies *Hotaria unmunsana* and *H. parvula*. The specific activity of the *Luciola italica* luciferase was 78% of the North American enzyme after correction for emission colour differences. The bioluminescence emission of the Italian firefly is pH-sensitive, with maxima at 566 nm and 614 nm at pH 7.8 and 6.0, respectively. Interestingly, the total bioluminescence output was ~two-fold greater than that of *P. pyralis* luciferase, due to differences in turnover characteristics evidenced by extended light emission decay kinetics. We expect that this newly discovered luciferase will be suitable for a wide range of bioluminescence applications, including *in vivo* imaging and multiplex assays.

Coelenterazine-binding protein of *Renilla muelleri*: cloning and determination of three-dimensional structure

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The bioluminescence of marine coelenterates is initiated by an increase in intracellular Ca^{2+}. In *Renilla*, Ca^{2+}-induced bioluminescence involves two distinct proteins, Ca^{2+}-triggered coelenterazine-binding protein (CBP) and luciferase. CBP contains one molecule of a tightly bound coelenterazine. The coelenterazine of CBP is released for reaction with luciferase and O_2 only subsequent to Ca^{2+} binding. CBP is a member of the EF-hand superfamily of Ca^{2+}-binding proteins and, like Ca^{2+}-regulated photoproteins, contains three 'EF-hand' Ca^{2+}-binding sites. Here we report cloning, CBP cDNA expression in *Escherichia coli*, and CBP crystal structure in two states: CBP with bound coelenterazine and Ca^{2+}-loaded apo-CBP. The CBP contains 186 residues and differs from *R. reniformis* CBP by only four amino acids. Although CBP and photoproteins both bind coelenterazine, there is little sequence homology between them. However, the overall CBP spatial structures approximate a well-known protein scaffold characteristic of Ca^{2+}-regulated photoproteins or sarcoplasmic Ca^{2+}-binding proteins. Supported by RFBR Grant 05-04-48271, the RAS program for Molecular and Cellular Biology, and NIH Grant RF SECSG SUPLMT WANG.

Brazilian species of bioluminescent fungi

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Although luminous fungi have been reported since ancient times, the chemical pathways involved in light emission and the identity of the emitter and its biological function(s) remain unsolved. Likewise, firefly bioluminescence (BL), whose elucidation harnessed the development of a bioassay for food contaminants and the use of *luc* reporter gene as a probe in Molecular Biology, unveiling the BL mechanism in fungi, could also generate analytical tools. Surprisingly, there was no reliable report of luminous fungi in Brazil until 2005, despite the reported large biodiversity of fungi. The first luminous fungus species described by our group was *Gerronema viridilucens*, which is the only known luminescent *Gerronema* and which may have evolved independently amongst euagarics. During 2002–2006 we were able to collect 11 new luminous mushroom species, eight of them found in the Atlantic Forest near the municipality of Iporanga, São Paulo State. These discoveries increased the reported number of taxonomically valid luminous species to 54, representing 22% of all species described in the world. This is the first report on new luminous mushrooms since the publication of Wassink in 1978.

Evaluation of an *in vivo* gene induction system in infected tumour-bearing mice

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Tumour-specific targeting of bacteria can, in theory, be used to diagnose and treat certain types of cancer by expression of suitable genes. Therefore, a tight regulation of (possibly toxic) protein production is desired. Here we describe the evaluation of a gene induction system *in vivo*, monitored through *lux* expression and low light imaging.

Effect of micro-environmental changes on kinetic parameters of steady-state enzyme-induced bacterial bioluminescent reaction

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The effects of methanol, ethanol, acetone, dimethyl sulphoxide, glycerol and formamide on kinetic parameters of steady-state enzyme-induced bacterial bioluminescent reaction were

studied. The modulation of the maximal bioluminescence intensity, the decay constant of light emission, the quantum yield of reaction and the apparent value of the Michaelis constant of luciferase from *Photobacterium leiognathi* by organic solvents were investigated. The kinetic analysis of inhibition types indicated that the molecules of organic solvents could be bound to the active centre of luciferase. The modifications to K_m reflect the nature of binding of an enzyme with substrates; from the represented data it is possible to draw a conclusion about the primary contribution of these (hydrophobic) and other (electrostatic) forces to the formation of an enzyme–substrate complex. The research described in this publication was made possible in part by Award No. RUX0-002-KR-06 of the US Civilian Research and Development Foundation (CRDF) and RF Ministry of Education and Science, BRHE Programme, Grant Nos 2006-PH-19.0/001/454 and 2006-PH-111.0/002/065 of the RF Ministry of Education and Science.

Thermal stability and absorption spectra of bacterial luciferases from *Photabacterium leiognathi* in water-organic solvents

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The effects of organic solvents concentrations which led to higher activity or quantum yield of the bacterial bioluminescent reaction *in vitro* on the thermal stability and absorption spectra of luciferases from *Photobacterium leiognathi* were studied. Luciferase was resistant to heat at small concentrations of methanol, acetone and DMSO, and its activity was little affected by 38°C; but it was unstable in solutions of methanol and DMSO if its concentration was increasing. Luciferase was unstable in all solutions of formamide at 38°C. Qualitatively dissimilar spectral changes were observed for the organic solvents used. The absorption spectra of luciferase produced in buffer were almost identical to those in methanol only. The absorption spectrum of luciferase in DMSO and 3.3% v/v acetone shows two maxima. The research described in this publication was made possible in part by Award No. RUX0-002-KR-06 of the US Civilian Research and Development Foundation (CRDF) and RF Ministry of Education and Science, BRHE Programme, Grant Nos 2006-PH-19.0/001/454 and 2006-PH-111.0/002/065 of the RF Ministry of Education and Science.

Bioluminescence assay for RNA quantitation

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Identification and quantification of nucleic acid molecules is an intrinsic part of genomic research. Various genetic analysis platforms are available, of which microarrays and quantitative RT–PCR methods are the most dominant in the field. Yet each method has its drawback, whether accuracy, high complexity or cost of analysis. In this study we report on a new highly sensitive and cost-effective method of detection and quantification of large (mRNA) and small (microRNA) ribonucleotide molecules, using our high-throughput reverse transcription quantitative bioluminescence assay (RT-qBLA). The RT-qBLA implements a detection concept similar to that of pyrosequencing, but expands it to the highly sensitive quantification of RNA molecules. The RT-qBLA can successfully challenge the real-time quantitative RT-PCR (RT-qPCR) in many applications and can be superior to RT-qPCR for the analysis of small RNAs. The RT-qBLA assay can be implemented with different RNA and DNA replication strategies, including polymerase (PCR) and ligase (LCR) chain reaction, 'constant temperature' PCR and rolling circle amplification. The RT-qBLA zeptomolar sensitivity and 4 logs dynamic range has been demonstrated with photon-counting luminometers manufactured by Berthold Detection Systems GmbH. The assay can be used in a number of applications in life sciences research, drug discovery and clinical diagnosis.

Luminescence microscope for reporter assay of single live cells

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The luciferase reporter assay system is widely used for the study of gene expression, signal transduction and other cellular activities. The luciferase assay is conventionally performed by the photon-counting luminometer method. In this system, light emitted from cells is measured as an integrated value through all cells. At an embryo and cell–cell contact level, it is needed to monitor the expression activity of genes of interest in each cell spatially and chronologically. To obtain a luminescence image of cells and an embryo, ultra-low imaging technology, such as photon-counting CCD and liquid nitrogen cooled CCD cameras, has been required. We have successfully developed a new optical system for bioluminescence imaging of single live cells using a conventional cooled CCD camera. Generally the brightness of the image is positive and negative in relation to numerical aperture (NA) and magnification, respectively. Therefore, some optical parameters (e.g. NA of objective and tube lens, and total magnification) are optimized for luminescence imaging of single cells. Using microscopy with heat shock prompter activity, c-fos and tetracycline repressor were assayed at the single cell level. From the data, it is expected that this luminescence imaging system will provide answers to the outstanding problems in luminometric promoter assays, such as transfection efficiency, physiological condition of each cell (cell cycle) and heterogeneous cell populations in tissues, among others.

Rational design strategy of functional bioluminescence probe

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The bioluminescent assay system using luciferase and a functional bioluminescence probe is known as one of the most highly sensitive systems for detecting target analytes. So far, several functional bioluminescence probes have been developed; however, a limited range of analytes could be assayed by using these probes, due to the design strategy of luciferin recovery. Recently, we have established a novel rational design strategy for fluorescence probes, based on the photoinduced electron transfer (PeT) process. The concept of PeT was applied to develop novel functional bioluminescence probes. For the scaffold of a tunable luminophore, we selected aminoluciferin as the mother structure and synthesized various derivatives bearing a benzene ring in the vicinity. The derivatives were confirmed to act as effective substrates for luciferase. Among them, derivatives bearing the benzene moiety of high electron density did not emit light at all, therefore it was found that luciferase-dependent luminescence could be controlled by the concept of electron transfer. By applying the design strategy of quenching by electron transfer, a novel bioluminescence probe for highly reactive oxygen species (hROS), such as hydroxyl radical, peroxynitrite and hypochloite, was developed and it will be possible to detect the formation of hROS *in vitro* and *in vivo* with our probe.

Red-chemiluminescent probes for detecting superoxide anions

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Some superoxide-induced chemiluminescence probes that emit red light (λ_{max} 610 nm) were synthesized and characterized. These red-chemiluminescent probes consist of a 6-(4-methoxyphenyl)imidazo[1,2-*a*] pyrazin-3(7*H*)-one moiety, which reacts with superoxide anions to generate energy, and a sulphorhodamine 101 moiety, which accepts the energy to emit red light. Using the hypoxanthine–xanthine oxidase system for the generation of the superoxide anions, it was shown that the superoxide-induced chemiluminescence of one probe was more intense than those of the blue- and green-chemiluminescent probes, MCLA and FCLA, which are generally considered to be the most sensitive chemiluminescent probes, and its superoxide specificity was similar to those of MCLA and FCLA. Due to the highly intense superoxide anion-induced chemiluminescence and superoxide-specificity at low probe concentrations, the red-chemiluminescent probe is better than MCLA and FCLA for the measurement of superoxide anions.

Peculiarities of the bioluminescence distribution in the Atlantic sector of the Antarctic Ocean

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Bioluminescent surveys of the upper 100 m layer were carried out in the 7th Antarctic Ukrainian expedition in March 2002. The samples of phyto- and zooplankton were collected simultaneously with bioluminescence vertical profiling, for which a submersible hydrophysical complex, 'Salpa' was used. The investigated region (from 62°44′ to 55°40′W, and from 64°17′ to 62°47′S) was a zone of interaction of the southern limb of the Antarctic Circumpolar Current (with salty waters of the Weddell Sea) confronting desalinated waters of the Bellinsgausen Sea. The northern part of the investigated region was occupied by warm and salty waters of the Pacific Ocean. For the bioluminescence amplitude, a difference between stations was up to three orders of magnitude. Low phyto- and microzooplankton abundances were noted in the cold waters of the Weddell Sea. Productivity of phyto- and microplankton was higher in shallow waters than in deep waters.

Molecular phylogeny of cypridinid ostracods and the evolution of bioluminescence

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The ostracod family Cypridinidae contains approximately 200 species, of which about half are bioluminescent. Cohen and Morin (2003) reconstructed a phylogeny for the Cypridinidae based on morphology. They proposed that bioluminescence evolved just once in the Family Cypridinidae. Bioluminescent signalling for courtship appears to have evolved subsequently and just once in the Caribbean cypridinids, where males of over 60 species display nightly on coral reefs to attract females. Here, we construct a molecular phylogeny to independently test the hypotheses of Cohen and Morin (2003). Sequences from the 16SrRNA and 12SrRNA mitochondrial genes from 25 species, representing several genera, are used to reconstruct relationships within the Cypridinidae. The patterns of evolution of bioluminescence and the major signalling display types are examined by mapping behavioural characters onto the molecular phylogeny. The molecular phylogeny is largely congruent with the morphological phylogeny of Cohen and Morin (2003).

Standardization of bioluminescent raw milk quality assay using the Lumtek test system

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Lumtek test systems for bioluminescent assay of bacteria and the somatic cell count in raw milk have been developed. Lumtek test systems comprise assay protocols, reagents, supplies and a portable luminometer. The test systems proposed were compared with standard microscopy cell count and opto-fluorometric assay for bacteria cell count, as well as with viscosimetric assay for somatic cell count in compliance with the appropriate ISO. Statistical analysis of the data obtained proved a good correlation between the standard and bioluminescent assays. The test system for bacterial cell count in raw milk has been adopted by the State Standard Committee in Russia (GOST 52415-2005).

Bioluminescent testing of redox-active compounds

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The bioluminescent system NAD(P)H:FMN-oxidoreductase–luciferase with benzoquinone was proposed as a specific test system for the detection of redox-active compounds in natural water. As a result of these studies, the bioluminescent system has undergone appropriate modification; also, the qualitative and quantitative composition of the reaction mixture components has been chosen. An analysis procedure was developed. It was established that the most sensitive kinetic parameters of bioluminescence influenced by a reducing agent are the induction period (T) and luminescence delay (t_{max}). It was found that T and t_{max} are strongly correlated with the concentrations and redox potentials of exogenous compounds. The bioluminescent system with benzoquinone was adapted to ecological monitoring of Shira lake salt water. Bioluminescent parameters T and t_{max} were used for construction of a depth and coastline redox heterogeneous map.

Influence of inorganic sulphur compounds on the bioluminescent system NAD(P)H:FMN-oxidoreductase–luciferase

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The influence of inorganic sulphur-containing compounds on the bioluminescent coupled system NAD(P)H:FMN-oxidoreductase–luciferase has been studied. It was shown that the presence of sulphur compounds leads to activation or inhibition of bioluminescence intensity, depending on the concentration of the sulphur compounds. Mechanisms of bioluminescence activation and inhibition by sulphur compounds have been discussed. A decrease in the concentration of oxygen dissolved in the bioluminescent system aquatic medium in the presence of sulphur compounds is followed by inhibition of bioluminescence. Inhibition coefficients and maximum bioluminescence activation depend on the oxygen concentration in the bioluminescent system and the reducing properties of the sulphur compounds. It was found that increasing $FMNH_2$ in the presence of sulphur compounds is the reason for bioluminescence activation. The increase of the $FMNH_2$ reduction rate in the bioluminescent system depends on the concentration and reducing properties of the sulphur compound. Hence, the physicochemical basis for bioluminescent testing of sulphur compounds in natural water has been discovered.

The structural origin of pH-sensitivity in beetle luciferases: influence of the loop between residues 223–235

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Beetle luciferases are divided in two functional groups according to their bioluminescence spectral sensitivity to pH: the pH-sensitive firefly luciferases (Lampyridae) and the pH-insensitive click beetle (Elateridae) and railroadworm (Phengodidae) luciferases. Most studies have focused on the pH-sensitive firefly luciferases. Despite the identification of important residues for bioluminescence colour, the origin of pH sensitivity remains unknown. Through a comparative site-directed mutagenesis study, using the pH-sensitive (*Cratomorphus*, *Macrolampis*) and pH-insensitive (*Phrixotrix* spp, *Pyrearinus termitilluminans*) luciferases cloned in our laboratories, we found that several residues affecting bioluminescence colour in both groups of luciferases are clustered in the loop between residues 223–235 (*P. pyralis* sequence). The position 227 was found to be involved in a labile interaction. Furthermore, among the conserved residues differing between pH-sensitive and pH-insensitive luciferases, the substitution F257L was one that had major effects on pH sensitivity. The results suggest that the loop between residues 223–235, and other interacting residues, play a major structural and dynamic role of in bioluminescence colour and pH-sensitivity determination. Financial support was provided by FAPESP.

Luciferin binding site properties of pH-insensitive luciferases

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The luciferases of the railroad worm *Phrixotrix* (Coleoptera: Phengodidae) are the only beetle luciferases that naturally produce true red bioluminescence. Previously, we cloned the green- (PxGR; λ_{max} = 546 nm) and red-emitting (PxRE: λ_{max} = 623 nm) luciferases of the railroad worms *Phrixotrix*

viviani and *P. hirtus* and the green-emitting luciferase from the larval click beetle *Pyrearinus termitilluminans* (Pyt: λ_{max} = 534 nm), which produce the most green shifted bioluminescence among beetle luciferases. These luciferases were expressed and purified, and their properties were determined. The substrate affinities and catalytic efficiency of PxRE luciferase (K_{MLH2} = 7 μmol/L; K_{MATP} = 130 μmol/L; k_{cat} = 2 s^{-1}) were higher than those of PxGR luciferase (K_{MLH2} = 65 μmol/L; K_{MATP} = 330 μmol/L; k_{cat} = 2 s^{-1}). According to fluorescence of 2,6 TNS and ANS, the luciferin-binding site of PxRE luciferase is more polar than that of PxGR and *Photinus pyralis* luciferases. The results, supported by site-directed mutagenesis and modelling studies, indicate that the luciferin binding site of the red-emitting luciferase is more polar and partially tighter than that of PxGR luciferase. (Financial support: FAPESP and JSPS).

Insights on the origin of beetle luciferase activity from luciferyl–adenylate chemiluminescence

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Luciferyl adenylate, the key intermediate in beetle bioluminescence, is produced through adenylation of D-luciferin by beetle luciferases and also by mealworm luciferase-like enzymes, which produce a weak red chemiluminescence. However, luciferyl adenylate is only weakly chemiluminescent in water at physiological pH and it is unclear how efficient bioluminescence evolved from its weak chemiluminescent properties. We found that bovine serum albumin (BSA) and neutral detergents enhance luciferyl adenylate chemiluminescence by three orders of magnitude, simulating the mealworm luciferase-like enzyme chemiluminescence properties. These results suggest that the beetle protoluciferase activity arose as an enhanced luciferyl adenylate chemiluminescence in the protein environment of the ancestral AMP-ligase. The predominance of luciferyl adenylate chemiluminescence in the red region, under most conditions, suggest that red luminescence is a more primitive condition that characterized the original stages of protobioluminescence, whereas yellow-green bioluminescence may have evolved later through the development of a more structured and hydrophobic active site.

Quantum-chemical approach to the amino analogues of firefly luciferin

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In order to research the molecular mechanism of firefly bioluminescence, White *et al*. (1966) synthesized amino analogues of firefly luciferin (Ln) and found that only aminoluciferin (AmiLn) reacts with firefly luciferase and ATP to produce red light, regardless of pH, contrary to the reaction with Ln, emitting in the yellow-green at neutral pH and in the red at acid pH. In this report, the optical properties of Ln and its amino analogues are described by the ZINDO method. The chemical excitation processes of Ln and AmiLn include decomposition of dioxetanone intermediates, in which the potential energy curves of the ground states for these molecules were estimated by IRC calculation in the AM1 method. The electronic properties and the potential energy curves along the reaction coordinates obtained by these calculations are discussed in relation to the spectroscopic data for these molecules.

Firefly luciferin and its analogues—a source of new luminescence dyes and ligands

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The bioluminescence of beetles has been a topic of investigation for many years. We know that the firefly luciferin–luciferase reaction is the most efficient bioluminescence system. The high quantum yield of the reaction demands a near-100% fluorescence quantum yield of the emitting oxyluciferin. To learn more about the fluorescence properties of oxyluciferin and its tautomeric equilibrium, we have synthesized some more simple derivates. In these derivates we substitute the benzothiazole part of the molecule by other aromatic residues. These new thiazoles as well as the oxyluciferin show a pH dependence of their fluorescence. Between the oxo-form and the hydroxy-form of our thiazoles, a tautomeric equilibrium exists. This equilibrium lies on the side of the aromatic hydroxythiazoles. We have never observed the corresponding, non-aromatic thiazolones. Some of the synthesized molecules are able to form complexes with rhenium and ruthenium. These new compounds show the typical behaviour of such complexes, with a beautiful red emission of ruthenium (II) and a marvellous electroluminescence.

Characterization of a fish symbiont of *Photobacterium phosphoreum* with altered spectral properties

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Bacteria cultured from the photophore of a *Physiculus* spp. fish recovered from the Arabian Sea produced a distinctive blue luminescence that was brighter than other isolates on labora-

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tory culture. Morphological characterization and biochemical profiling suggested that this bacterium belonged to the species *Photobacterium phosphoreum*, and 16S sequence analysis confirmed that this isolate was closely related to this species. Analysis of *luxA* identified only small differences in the gene sequence, but the two organisms had different autoinducer profiles. Spectral analysis confirmed that the luminescence had a different λ_{max} value to that of *P. phosphoreum* and a novel *lumP* (lumazine) gene was identified. This would account for the different spectral properties and suggests that this bacterium represents a new subspecies of *P. phosphoreum*.

Switchable luciferases: the third wave of bioluminescence technology

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Although the history of bioluminescence research is filled with the promise of practical utility, commercial successes were initially few. This has changed dramatically in the life science market, where increased laboratory automation and sample miniaturization are benefited by the high performance of luminescent assays. Development of this market has gone through three phases, beginning in the late 1980s with the cloning of firefly luciferase. This popularized the use of luminescence as a genetic reporter technology and led to the broad availability of luminometers in research laboratories. The second phase ushered in a diverse range of assay designs linking luminescence to reaction substrates. Although this approach had been long represented in the scientific literature, it was not readily adopted until accessibility to instrumentation became widespread. Both of these phases are built on correlating light intensity with the concentration of one of the components in the bioluminescent reaction. The third phase, just beginning, is based on modulation of the luciferase specific activity. Recognizing that luminescent function is critically dependent on enzyme structure, our laboratory is developing chimeric proteins designed to enable conformational modulation. In one model, we can increase luminescent activity by over 2000-fold through proteolytic cleavage. This approach has been shown to be generally effective for detecting and characterizing endoproteases. A second model is based on allosteric modulation through reversible binding to cAMP. These proteins are able to detect changes of cAMP within living cells, and thus are responsive to intracellular signalling. This new strategy for engineering switchable luciferases opens the potential for creating intracellular luminescent sensors of living processes.

Development of the *Cypridina* bioluminescent system for high-throughput screening assays

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The *Cypridina* bioluminescent system is a typical luciferin luciferase reaction. *Cypridina* luciferase, which has high turnover rates and a secretion property, has a big advantage as a bioluminescent reporter in various high-throughput screening assays. *Cypridina* luciferin is an imidazopyrazinone compound with three functional groups at the C2, C6 and C8 positions; it contains an *S* chiral centre in the *sec*-butyl moiety at the C2 position. The first total synthesis of (*S*)-*Cypridina* luciferin was achieved in 1966, but the yield was very low, which contributed to its limited use in various applications. Here we report a convenient synthesis by condensation of ethioluciferin with a chiral ketoacetal to afford (*S*)-*Cypridina* luciferin in a moderate yield. Stabilization and spectral characterization of *Cypridina* luciferin and its analogues are also reported. Our experimental results will contribute to a novel high-throughput promoter assay, BRET assay and immunoassay by using the *Cypridina* bioluminescent system.

A novel multi-channel chemiluminescence immunoassay to detect HIV antibodies as a more specific supplemental test

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AIDS is a worldwide problem of public health. For detection of the infection, the screen test is applied, followed by a more specific supplemental assay. Unfortunately, as a widely used supplemental test, Western-Blot (WB) is high cost, time-consuming and complex to handle. We set up a simple substitutional method that largely reduces the cost, time and expense while showing the same specificity and more sensitivity. In this assay, the seven recombined HIV proteins were coated on polystyrene particles. After they were incubated with diluted sera and HRP–anti-human antibody separately, the particles were washed and the chemiluminescence substrate was added, then the signal was detected sing a luminometer. The results were determined according to the criteria guidelines for WB assay from WHO. After it had passed the tests set by the seroconversion panel for WB quality control from SFDA with a fully correct response, a clinical trial was conducted with 1250 specimens, including 500 normal and 750 individuals who underwent the screen and supplement test. The sensitivity and specificity were 99.2% and 96%, respectively, for 528 WB-positive and 171 WB-negative specimens, while it showed negative results for all normal specimens. It also identified three of 51 WB-indeterminate specimens that were reconfirmed by a follow-up PCR assay.

Chemiluminescence enhancement effect of trace metal elements

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The H_2O_2–gallic acid–$KHCO_3$ in MeCHO system is one of the chemiluminescence (CL) systems with a decomposition of

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H_2O_2. This study focused on the effect of trace metal elements (TMEs) on the H_2O_2–gallic acid–$KHCO_3$ in MeCHO system (CL standard system). TME addition, especially of Mn, Fe, Cu and Zn, showed remarkable enhancement of CL arising from the CL standard system. TME addition also increased H_2O_2 decomposition to 2–14-fold compared with that of the CL standard system. For the CL enhancement effect, TMEs have optimum concentrations (Fe, 0.78–3.12 mmol/L; Zn, 0.19–0.78 mmol/L; Mn, 0.024–0.095 mmol/L; Cu, 0.012–0.095 mmol/L). Emission spectra of the CL standard system (E_{max} 650 nm) were shifted to 530 nm by TME addition, according to the electron populations of the 3d and 4s orbitals of TME. Free radical analysis using ESR showed the generation of gallic acid, CH_3^- and CH_3O^- from the CL standard system. These radicals were increased and decomposed rapidly by TME addition. HO spectra, which are generated from the Fenton reaction, were not observed in any TME addition systems. A correlation of free radical intensity and photon intensity suggested that the CL enhancement effect of TME resulted in the catalytic effect of the CL reaction rate.

Quantitative Western blotting using a GZ-11-based chemiluminogenic signal reagent

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In order to assess the stability of protein vaccine components, a quantitative Western blotting method was developed using GZ-11 as part of the HRP signal reagent. Proteins were separated on gels and blotted on PVDF. Incubation with different monoclonal antibodies directed against different parts of the protein was followed by incubation with detecting antibody (HRP-labelled goat-anti-mouse). The blots were visualized after addition of GZ-11 containing signal reagent, using a CCD-camera, and quantified using software Phoretix 1D quantifier. After normalizing the signal, the amount of degradation was calculated. The method was standardized and showed good reproducibility. The technique was also used to map epitopes on the A-fragment of diphtheria. By this application, mutants were made which were expected to contain the epitope of one of the three monoclonal antibodies. By mutation of the epitope, the binding to the monoclonal antibody will be decreased and this can be visualized by quantitative Western blotting. The mutants were expressed in *Escherichia coli* and isolated by periplasm isolation. The samples of the different mutants and the native protein were separated with SDS–PAGE and blotted onto nitrocellulose membranes. The blots were incubated with the specific monoclonal antibody, followed by incubation with detecting antibody and visualization as indicated above.

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