Abstracts of the 13th International Symposium on Bioluminescence and Chemiluminescence

Homogeneous chemiluminescent assays: selective quenching of acridinium tracers
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Chemiluminescent N-sulphonyl-acridinium-9-carboxamide reporter groups have been successfully commercialized on high-throughput, automated, in vitro diagnostic analysers that utilize a heterogeneous assay format, i.e. the bound and free labelled material is physically separated before the signal is generated. Homogeneous chemiluminescent assays offer significant advantages in terms of assay simplicity, cost and manufacturability of reagents. We describe here a homogeneous chemiluminescent assay concept that exploits an acridinium label designed to achieve signal modulation. Specifically, acridinium labels equipped with hapten-derivatizable linkers were designed to provide tethered or released acridone species upon chemiluminescent triggering. The labels were conjugated to derivatives of biotin, folic acid and vitamin B_{12}. The resulting conjugates were characterized in terms of chemiluminescent output and signal modulation upon specific binding to protein receptors (avidin, folate binding protein and intrinsic factor, respectively). The tethered and released acridone conjugates exhibited markedly different chemiluminescent outputs relative to each other in the presence of a specific binding protein. Signals from conjugates that generated a tethered acridone species upon chemiluminescent triggering were selectively quenched in the presence of a specific binding protein. This signal modulation was exploited in homogeneous chemiluminescent assays for biotin, folic acid and vitamin B_{12}.

Tandem bioluminescent enzyme immunoassay for BDNF and NT-4/5
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We have developed a highly sensitive simultaneous bioluminescent assay of acetate kinase (AK) and pyruvate phosphate dikinase (PPDK) using a firefly luciferase–luciferin reaction, and applied this assay to a tandem bioluminescent enzyme immunoassay (BL-EIA). Recently, Nelson et al. reported that the neonatal blood concentrations of VIP, CGRP, BDNF and NT-4/5 were higher in the autistic spectrum than in control children (1). Therefore, measurements of these four factors in neonatal blood are made possible to diagnose and care for autism at an early stage. In this study, we established a highly sensitive tandem BL-EIA for BDNF and NT-4/5. In the proposed assay, we added 50 µL standard or sample solutions...
to the wells of a microtitre plate which were coated with anti-BDNF and anti-NT-4/5 antibody, and incubated overnight at 4°C. After washing, we added 50 μL FITC-labelled anti-BDNF and biotin-labelled anti-NT-4/5 antibody to the plate. After incubation for 3 h at room temperature and then washing the plate, we added 100 μL AK-labelled anti-FITC and PPDK-labelled anti-biotin Fab antibody to the plate and incubated for 1 h at room temperature. After washing, the plate was assayed using a simultaneous bioluminescent detection method (2). The measurable ranges of BDNF and NT-4/5 were 4.9–40000 and 31.25–2000 pg/mL, the detection limits (at blank + 3 SD) of BDNF and NT-4/5 were 1.2 and 11.4 pg/mL, respectively. The intra-assay coefficients of variation of BDNF and NT-4/5 with each standard point were 1.8–9.8% (n = 8) and 2.3–6.4% (n = 8), respectively.

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Synthesis and application of boronic acid derivatives of firefly luciferin to chemi- and bioluminescent detection of peroxide

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We report the preparation of novel compounds based on the firefly luciferin ring system and related structures as chemiluminescent and bioluminescent reporters of hydrogen peroxide. The compounds contain a boronic acid or boronate ester group which reacts with peroxide to initiate the reaction. Total synthesis of two boronic acid compounds is presented. Reaction of the compounds with peroxide under various protic and aprotic conditions produces easily visible green, orange or red light, depending on reaction conditions. Spectral and kinetic characterization of the reactions are described. We have also coupled the reaction to the well-known luciferin/luciferase bioluminescent system. Reaction of a luciferin boronic acid derivative with peroxide produces native firefly luciferin, which can be detected by means of the luciferin-catalysed bioluminescent process. This reaction can be done either in two discrete steps, by preliminary reaction of substrate with peroxide followed by addition of luciferase and ATP, or in one step, by reaction with peroxide in the presence of luciferase and ATP. Comparative properties of the two modes of reaction are discussed. The new luciferin-catalysed reaction finds utility in detecting luciferase in reporter gene applications and in ATP detection for monitoring bacterial contamination in food and beverages.

Chemiluminescent substrates for phosphatases and peroxidases

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Enzyme labels are widely used for analysis in the clinical setting and in the research laboratory. Use of enzymes allows the highest sensitivity because of catalytic signal amplification while avoiding radioactivity. Chemiluminescent detection of enzyme activity can achieve superior sensitivity compared to colourimetry when care is taken to optimize assay conditions. We have developed a number of chemiluminescent enzyme substrates which are used worldwide in immunoassays and nucleic acid assays. Enzymatically triggered dioxetanes generate chemiluminescence by removing a protecting group with a hydrolytic enzyme such as alkaline phosphatase (AP). Enhanced formulations Lumi-Phos® 530 and Lumi-Phos Plus are used worldwide in automated clinical immunodiagnostic analysers, in life science research and in forensic and human identity analyses. More recently, Lumigen has developed a new chemiluminescent AP substrate, Lumigen® APS-5, which produces maximum intensity emission in seconds at room temperature. The substrate has an acridan ring bound to an enol phosphate group. Reaction with an AP label and O₂ generates a transient dioxetanone intermediate, which undergoes a chemiluminescent decomposition. An enhanced formulation incorporating lucigenin significantly raises peak chemiluminescence intensity, permitting detection of 10⁻²¹ moles of AP. Lumigen has also recently developed a new family of substrates for horseradish peroxidase (HRP), one of the most common label enzymes. These compounds produce bright chemiluminescence, reaching peak intensity in seconds. The exceptional hydrolytic stability of the compounds has permitted development of the first working reagents that can be stored in a single container including the peroxide. Lumigen PS-atto provides ultrasensitive detection of HRP conjugates in solution assays. Lumigen TMA-6 provides ultrasensitive, extended duration detection of HRP conjugates in blotting assay formats.

Bioluminescence: adaptive or defensive mechanism

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This study was research on glowing flies among which fireflies are well known. More than 600 flies were observed at night (9:00–11:00 p.m.) for exactly 7 weeks around Usmanu Danfodiyo University campus garden. The observations were conducted based on two criteria: (a) adaptation of fireflies with their surrounding environment and (b) their defensive mechanism as a response against any suspected predator.

Based on certain updated information, it was verified that most fireflies respond to both of these criteria, depending on the situation that was presented to them. Therefore, throughout the practical part of this work, the fireflies showed intensive light emission when in community moving together, for mating or sexual appeal for mates and for defence purposes in the presence of any imminent attack of a predator.
Construction of a novel bioluminescence bacterial biosensor for real-time monitoring of cytotoxic drugs activity

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A bacterial lux reporter has been developed to measure the activity of cytosine arabinoside (Ara-C), a synthetic pyrimidine nucleoside analogue, that is the mainstay of treatment for acute myeloid leukaemia (AML). Since Ara-C has to be taken up and converted by the cell to its active derivative Ara-CTP, resistance to chemotherapy is common and 30% of patients fail to achieve remission. Current methods for measuring AML response to Ara-C are complicated, expensive and require lengthy incubation times. The bioluminescent reporter was constructed by the addition of constitutively expressed lux genes to E. coli strain (SO5110) defective in its pyrimidine salvage pathway. This strain is sensitive to Ara-C due to the expression of the human deoxycytidine kinase gene (dCK) under the control of the lac promoter. In the presence of IPTG, the growth of E. coli SO5110 after 4–5 h was reduced by 48% when 20 µmol/L Ara-C was added. E. coli SO5110 was made constitutively bioluminescent when transformed with pAL2 carrying the modified lux ABCDE operon from Photorhabdus luminescens. The activity of Ara-C on E. coli SO510 (pAL2) was determined by monitoring OD600 and light output at concentrations of 25, 50, 75 and 100 µmol/L (clinical dose). As previously reported, the effect of Ara-C was inhibitory to culture growth; however, the bioluminescence of E. coli SO510 (pAL2) showed an increase of 74–172% in a concentration-dependent manner. The biosensor construct did not respond to Ara-C in the absence of IPTG, indicating that the activity of the human dCK gene is essential, and that the biosensor is able to detect Ara-CTP. It therefore has potential for use as a biosensor within human AML cell samples and for rapid and non-invasive screening of cancer cell sensitivity to nucleoside analogues.

Use of bioluminescent Salmonella typhimurium DT104 to monitor uptake and intracellular survival within a human cell line

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Clinically important bacteria, transformed with the luxCDABE operon, emit light as an accurate reporter of their metabolic activity. Bioluminescence as a reporter is used to monitor real-time intracellular survival of bacteria, giving a more accurate picture of microbial–host interactions that occur in vivo than the traditional method of viable counts. *Salmonella typhimurium* is a pathogen that colonizes macrophages, leading to problems in clearance and destruction. This study aimed to establish an assay using bioluminescence to investigate the uptake and intracellular survival of *S. typhimurium* in a macrophage-like cell-line. *S. typhimurium* DT104 was transformed using the broad host range plasmid pBRRMCS-5 containing a Lux cassette. A photon-counting camera was used to select light emitting colonies from the gentamicin-resistant transformants. The resulting recombinant bacteria were internalized within a human monocytic cell line, THP-1. Control cultures of non-bioluminescent internalized *S. typhimurium*, non-internalized Lux+ and Lux−*S. typhimurium* and uninfected cells were also monitored. Varying multiplicities of infection and incubation periods were assessed. Extracellular bacteria were killed using colistin and the internalized location of the bacteria confirmed following the addition of saponin. The internalized bacteria gave a stable light output over 24 h, 2 log10 higher than the negative controls, suggesting intracellular survival of the bacteria. These results indicate that this bioluminescence-based assay can be used as an effective real-time method to monitor uptake and intracellular survival of *S. typhimurium* and potentially the effects of antimicrobial agents *in situ* on this clinically important pathogen.

Toward blue-shifted firefly bioluminescence by the modification of the luciferin structure

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Various beetles produce light with maxima between 536 nm (yellow-green) and 623 nm (red), by utilizing the same luciferin. Various bioluminescent beetles, which emit blue-shifted light (400–500 nm) for excitation of GFP, have been reported. For developing new luciferins having the blue-shifted maxima, we synthesized firefly luciferin analogues with shorter π conjugation or analogues having an oxazine ring instead of a thiazoline one, and evaluated their bioluminescence activities, the results of which are discussed.
A new assay for determining pyrophosphate using pyruvate phosphate dikinase and its application to DNA analysis

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We developed a novel bioluminescent assay for pyrophosphate in PCR product. The principle of this method is as follows. Pyrophosphate released by PCR is converted to ATP by pyruvate phosphate dikinase (PPDK) in the presence of pyruvate phosphate as a substrate and AMP as co-enzyme, and the concentration of ATP is determined by the firefly luciferase reaction. The detection limit for pyrophosphate is 1.5 fmol/assay and the time course of light emission was shown to be stable for more than 10 min. This method is applied to the detection of cariogenic bacteria in the dental plaque as a preventive dental diagnosis. In this study, the dextrans gene (dex) in Streptococcus mutans was selected as a marker gene. Allele-specific PCRs, which consisted of mutant PCR and S. sobrinus PCR, were developed relative to slightly distinct dex genes in S. mutans and S. sobrinus. The pyrophosphate, produced in two allele-specific PCRs, was measured by the proposed bioluminescent assay. This protocol, which does not require expensive equipment, can be utilized to rapidly monitor cariogenic bacteria in the dental plaque.

Importance of firefly luciferase C-terminal domain in binding of luciferyl adenylate

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Recently we reported that the N-terminal domain (N-domain, 1–440 aminoacids) of firefly luciferase can emit light in itself, and the emission increases slowly over several minutes without any flash phase (1). Subsequent analysis of the slow emission profile revealed that the luciferyl adenylate (LH2-AMP) produced by the N-domain was mostly released to the solution rather than incorporated to the following oxidative step, and that the light intensity from the N-domain faithfully reflected the amount of free LH2-AMP in the system. This finding allowed us to estimate the amount of free LH2-AMP produced in the prior reaction, including unreacted substrates without any purification steps, only by taking the aliquot of the reaction mixture to the N-domain solution and measuring the initial light emission. When we compared the amounts of LH2-AMP produced in the reaction of K529A, a mutant lacking the lysine presumed to be involved in adenylation, to that of the N-domain, a smaller amount of LH2-AMP was released from K529A than from the N-domain. Because the specific light emission of K529A was 40–50-fold greater than that of the N-domain, the result suggests that the C-terminal domain is important not only in adenylation, which is a K529-dependent process, but also in the binding and efficient oxidation of LH2-AMP, which may be K529-independent.

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Biololuminescence and mating behaviour in the pony fish, Leiognathus nuchalis

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Some leiognathid species, e.g. Leiognathus elongatus, are well known as luminous fish, possessing light organs containing symbiotic luminous bacteria, and their light emissions have been observed in the field (Sasaki et al. 2003). However, what kind of behavioural and/or ecological function the luminescence has is still uncertain. The luminescence of L. nuchalis, which is a most popular pony fish in the Japanese coastal area, has not been observed with the naked eye or CCD cameras. We attempted to detect their luminescence and to clarify the functions of the bioluminescence in the laboratory. In addition to confirming the existence of the light emission, we focused on the seasonal change and diurnal rhythm of luminescence in this species. The fish were kept in a 500 L transparent circular aquarium under constant dark conditions. The experiments were conducted three times during the breeding season and non-breeding season, respectively. Luminescence was measured by highly sensitive photometer set at the side of aquarium. Luminescence from adult fish was detected only during the breeding season. After breeding season, the fish did not emit light. The frequency of light signals increased at night, especially in the middle of the night (20.00–24.00), which is the spawning period. Little light emission was observed during the daytime. These results supported the hypothesis that there was a relationship between light emission and spawning behaviour.

Application of highly luminescent quantum dot bioconjugates in protein imaging: quantum dot-based immunoblot analysis

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We attempted to detect their luminescence and to clarify the functions of the bioluminescence in the laboratory. In addition to confirming the existence of the light emission, we focused on the seasonal change and diurnal rhythm of luminescence in this species. The fish were kept in a 500 L transparent circular aquarium under constant dark conditions. The experiments were conducted three times during the breeding season and non-breeding season, respectively. Luminescence was measured by highly sensitive photometer set at the side of aquarium. Luminescence from adult fish was detected only during the breeding season. After breeding season, the fish did not emit light. The frequency of light signals increased at night, especially in the middle of the night (20.00–24.00), which is the spawning period. Little light emission was observed during the daytime. These results supported the hypothesis that there was a relationship between light emission and spawning behaviour.

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Highly luminescent semiconductor quantum dots (QDots) have attracted a great interest in life sciences research, because of their potential to be used as new fluorescent probes in cell and protein imaging techniques. The interest to QDots is based on their higher brightness than the conventional fluorophores and privilege for single-source excitation for all colours.

The present study describes a synthesis of highly luminescent Qdot bioconjugates and the development of a method for Qdot-based Western blot analysis. Water-soluble CdSe quantum dots (~2–3 nm) were conjugated with several antibodies (anti-c-abl, anti-lamin A/C and anti-β-actin) and were applied for immunoblot analysis of the respective proteins in leukemia cells (K-562, derived from chronic myelogenous leukaemia; and Jurkat, derived from acute lymphoblastic leukaemia). In Qdot-based Western blot analysis we used only a primary antibody and the photoluminescence of Qdot–antibody conjugates, retained on a PVDF membrane, was detected by ChemiImager. The described procedure avoided the application of a secondary antibody and subsequent HRP-catalysed enzyme reaction with formation of luminescent product, which often compromises the results after ensuring saturation. The sensitivity of Qdot-based immunoblot analysis is about three-fold higher than that of the conventional Western blot procedure. However, the photobleaching of Qdot-labelled blots was faster and the stability of Qdot–antibody blotted membranes was lower than in the conventional immunoblot procedure. To ensure higher efficiency and to guarantee comparatively high stability of Qdot-blot photoluminescence, biotinylated antibodies were covalently conjugated with Qdot and a sandwich-type avidin–biotin assay system was additionally applied, using Qdot–avidin and Qdot–biotin conjugates.

The chemiluminescent measurement of the antioxidant capacity of black and green tea and comparison with their antimicrobial activity

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The lifetime of reactive oxygen species (ROS) is extremely short, and if a physiological acceptor does not immediately neutralize them, ROS can damage biological systems. All aerobic organisms have developed more or less complex systems to neutralize them before their potentially harmful effect is activated. Nutritional elements are also extremely important. Foods that have potential or definite antioxidant capacities are mainly vegetables and fruits, as well as beverages like red wine, tea and beer.

Tea is a source of epigallocatechin gallate in green tea, and theaflavin and the associated thearubigins in black tea. Cancer of the colon, breast, prostate and pancreas may be caused by a new class of carcinogens, the heterocyclic amines, formed during the broiling or frying of creatinine-containing foods, including fish and meats. Their formation and action can be inhibited by antioxidants such as those in soy and tea. Black tea is a powerful chemopreventor of ROS and was found to be more efficient than green tea.

A sensitive and simple chemiluminescent (CL) method for measuring antioxidant activity was developed. The determination of Trolox equivalent antioxidant capacity (TEAC) is based on the inhibition of CL intensity of luminol by an antioxidant. Antimicrobial activity was tested as a minimal inhibitory concentration (MIC) by the broth microdilution method on Gram-positive (Enterococcus faecalis, Staphylococcus aureus) and Gram-negative (Pseudomonas aeruginosa, Escherichia coli) bacterial strains. We also tested the stability of the frozen extracts.

Our results do not confirm a predominance of black tea over green tea or vice versa in their antioxidant capacities or antimicrobial activities.

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Superoxide or singlet oxygen: the chemiluminescence of Cypridina luciferin analogues in photodynamic solutions

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Photodynamic therapy (PDT) is based on the photodynamic effect, which involves the combination of light, an organic dye–photosensitizer and molecular oxygen. Upon irradiation by laser, the photosensitizer is excited to the first excited singlet state, which can react in two ways. A Type I mechanism involves hydrogen atom abstraction or electron transfer reactions to yield free radicals and radical ions. A Type II mechanism results from an energy transfer and generates singlet oxygen.

Various chemiluminescent compounds (e.g. luminol and its derivatives) have been studied in order to find suitable and specific probes for detection of particular reactive oxygen species (ROS). Recently, Cypridina luciferin analogues (CLA and MCLA) have been thought to emit light only when reacting with superoxide anion and singlet oxygen.

Luminol, CLA and MCLA were studied in three ROS-generating chemical systems in vitro (HRP–H2O2, CuSO4–H2O2 and NaClO–H2O2 systems). Measurements were carried out in phosphate buffer (pH 7.4) at 25°C and 37°C, using a Fluoroskan Ascent FL luminometer. Superoxide dismutase and sodium azide were used as a specific scavenger of superoxide anion radical and a quencher of singlet oxygen, respectively. Their effect was compared with that of Trolox (a water-soluble analogue of vitamin E), which should scavenge all reactive species present in the reaction mixture, and tryptophan. As the photodynamic systems, we used phthalocyanines and chlorophyll a, for example.

The declared specificity of Cypridina luciferin analogue chemiluminescence, based on superoxide and singlet oxygen and the suitability of this proof, is discussed.
Acknowledgements

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Method for implementing bioluminescence-based analytical assays in nanolitre volumes

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Bioluminescence-based analytical assays in a miniaturized and stable format, can measure various analytes in nanolitre sample volumes. Nanolitre volumes of multiple bioluminescent analytical assays were deposited in an array format and lyophilized. ATP–firefly luciferase and NADH–bacterial luciferase platform reactions are compared. We achieved parallel sample delivery via sample-hydrated gels. The luminescence was measured by CCD and photodiode arrays. These miniaturized assays and instruments can be prepared as micro-analytical systems to operate in point-of-care (POC) diagnostic devices.

Multi-parametric experimental design of bioluminescence-based analytical assays

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Multiparametric data analysis of bioluminescence-based analytical assays, lyophilized in the nL volume range, offers an inexpensive and efficient assay optimization method. Moreover, the various parameters associated with single or multiple bioluminescence-based analytical assays can be analysed in parallel. Optimization parameters include concentrations of enzymes and reagents, pH, on-board calibrations and references, interference effects, and error analysis. Efficient algorithms for multiparametric experimental design that can be used in these optimizations are presented. These algorithms will accelerate the development of new bioluminescence-based analytical assays for specific diagnostic panels as well as enhancing analytical accuracy and precision.

The first deep-sea observations of natural benthic bioluminescence using an in situ ISIT camera

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An ISIT (intensified silicon intensifying target) camera mounted on an autonomous lander was used to observe bioluminescence produced by organisms on the sea floor of the Porcupine Seabight and Abyssal Plains regions of the north-eastern Atlantic ocean. The camera recorded natural bioluminescence (that occurring without human stimulation) without the need for lights, for up to 3 h after lander touchdown. Eighteen deployments carried out over 3 years, at depths of 970–4000 m, detected natural bioluminescence of organisms living on or near the sea floor. At most sites, 0.7 events/min were observed. However, one location at a depth of 1000 m on the Porcupine Seabight exhibited much higher levels of bioluminescence (on average, more than three bioluminescent events/min) in both spring and autumn. A control experiment without bait at this site elicited little bioluminescence. Large numbers of the cut-throat eel Synaphobranchus kaupi, some sharks and amphipods were observed at the bait. The source of the bioluminescence remains enigmatic, since these species of fish are not known to emit light. A dominant form of light emission appeared to be extrusion of luminescent material from the seafloor, suggesting a benthic faunal source. This 'bioluminescent garden’ is located close to known cold water coral mounds, which are sites of high biodiversity and hence may harbour bioluminescent fauna in greater abundance than elsewhere on the sea floor.

Bioluminescence variability of lux-marked strain Escherichia coli Z905/pPHL7 in aquatic microcosms with different salinity

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The population dynamics and expression variability of the transgenic microorganism (TM) Escherichia coli Z905/pPHL7 (Ap‘Lux’+) bioluminescence genes cloned in a recombinant plasmid have been studied in artificial aquatic ecosystems with different salinity and bacterial species composition. It was shown that, in competition with indigenous microflora, the TM population was 2–4 times lower than in sterile freshwater and brackish microcosms. Higher salt concentration in the medium led to differences in displaying the plasmid genes expression as compared to freshwater microcosms, independent of their complexity. Particularly, in brackish medium the bioluminescence expression reduced to a greater extent and was revealed only at plating into TM accumulative cultures with high content of selective factor (50–200 µg/ml ampicillin). TM clones isolated from freshwater microcosms maintained a higher bioluminescence level; at that for sterile microcosms it was higher than for non-sterile. Many clones could be seen during the plating of microcosm water samples on the selective media.
Bioluminescence colour determination in firefly luciferase

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Beetle luciferases use the same luciferin substrate to naturally display light ranging in colour from green ($\lambda_{\text{max}} \sim 530$ nm) to red ($\lambda_{\text{max}} \sim 635$ nm). We reported (1) that the synthetic adenylate of firefly luciferin analogue 5,5-dimethyl-luciferin was transformed into the emitter 5,5-dimethoxy-luciferin in bioluminescence reactions catalysed by luciferases from Photinus pyralis and the click beetle Pyrophorus plumipes. Green light emission was produced by the firefly enzyme and red light was observed with the click beetle protein. These results provided experimental support for the mechanism of firefly bioluminescence colour that requires only one single keto-form of oxyluciferin. We have conducted mutagenesis studies designed to determine the basis of the observed differences in bioluminescence colour with the analogue adenylate. Mutants of luciferase putative active site residues were constructed and characterized using bioluminescence emission spectroscopy and steady-state kinetics. Based on an analysis of these and additional recently reported data (2), we have developed an alternative mechanism of bioluminescence colour. The basis of the mechanism is that luciferase modulates emission colour by controlling the resonance-based charge delocalization of the anionic keto-form of the oxyluciferin excited state.

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Flashers, glowers and sniffers: sexual selection and signal evolution in fireflies

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Through a phylogenetic analysis using adult morphological characters, a monophyletic concept of the family Lampyridae is established (with modification) and the phylogenetic position of the Lampyridae in relation to closely related families is discussed. The resulting evolutionary pattern suggests that not only does bioluminescence predate the family Lampyridae, but it appears to have arisen at least twice within this lineage of beetles. Within the Lampyridae, pheromonal sexual signals occur basally and are then used in conjunction with, and then subsequently replaced by, photic signals in several lampyrid lineages. In lineages that have lost pheromonal sexual signals, both photic sexual signals and the photic organs used to produce them have become greatly elaborated. In addition, flashed signals and some adult male photic organ morphologies appear to be convergent. A study of lampyrid fossils supports many of these findings, as well as providing new insights into the evolution of the Lampyridae.

Comparison of kinetic properties of firefly luciferase from Photinus pyralis and Luciola mingrelica

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The time-course of bioluminescence was investigated for both Photinus pyralis and Luciola mingrelica firefly luciferases in a wide range of substrate concentrations. It was shown that the kinetic behaviour of the two enzymes was very similar. The integral light output was limited by the amount of active enzyme present in the reaction mixture. Substantial inactivation of enzymes was observed in course of reaction in the stages after enzyme–substrate complex formation.

A previously proposed kinetic model (1) for Luciola mingrelica firefly luciferase was applied, with some modifications for the data analysis, and kinetic constants for both enzymes were calculated.

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Bacterial rainbow for microbial ecology studies

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The growth and survival of bacteria in food are of major importance for food safety specialists. Existing methods of tracking bacteria in food matrices are time- and labour-consuming. Cultural methods provide information only on numbers of bacteria, without any knowledge on their spatial distribution.

Recently, genes for fluorescent proteins with a wide range of emission spectra from cyan to red were cloned. Recombinant bacteria, internally labelled with different fluorescent proteins, provide an opportunity of monitoring both the location and growth of bacterial directly in food samples and in real time. However, detection of fluorescent bacteria in vivo in some cases proves to be difficult, due to the autofluorescence of live bacterial cells as well as the sample matrix.

Recombinant bacteria E. coli were constructed that carry genes for fluorescent proteins of different colours (ECFP, EGFP, EFYP and DsRed). Several approaches were employed to image bacteria in pure culture and in mixtures, including
flow cytometry, epifluorescent microscopy, confocal microscopy and spectrofluorometry. The advantages and limitations of each method are discussed.

**Use of the peroxyoxalate chemiluminescent reaction in acetone in the presence of Nile red for the analysis of glucose**

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The peroxyoxalate chemiluminescent reaction has a high emission efficiency in organic solvents. Unfortunately, due to the low solubility and instability of TCPO [bis(2,4,6-trichlorophenyl) oxalate] and other oxalate esters in the presence of water, this chemiluminescent system has limited analytical applications in aqueous media. In previous studies of our laboratory we have demonstrated that the TCPO–H₂O₂ chemiluminescent reaction in acetone can be used for the detection of fluorophore-labelled protein and DNA bands on membranes. In this work, we have developed a procedure to use the peroxyoxalate reaction in acetone for the quantitative analysis of glucose. We have incubated solutions containing different concentrations of glucose with glucose oxidase. This enzymatic reaction was performed in aqueous solutions in the presence of 0.1 mol/L imidazole-HCl, pH 6.4. Small volumes (20 µL) of the resulting solutions, containing different amounts of H₂O₂ produced by the enzymatic reaction, were mixed with a large volume (2 mL) of a solution of TCPO and Nile red in acetone. The high-energy intermediates produced in the reaction of TCPO with H₂O₂ excite the hydrophobic fluorophore Nile red. Our results demonstrate that the light emitted by this fluorophore is linearly proportional to the concentration of glucose in the initial aqueous solution in the range of clinical interest: 10–540 mg/dL. The interference produced by components present in serum precludes the use of this sensitive chemiluminescent method for the direct determination of glucose in blood samples.

**Solvent effects on the chemiluminescence of TCPO in presence of 7-amino-4-trifluoromethylcoumarin**

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The fluorescent probe of 7-amino-4-trifluoromethylcoumarin (ATFMC) shows intense chemiluminescence in the presence of bis(2,4,6-trichlorophenyl)oxalate (TCPO) and hydrogen peroxide. The coumarin derivative acts as a fluorescer in this system. The maximum wavelength of chemiluminescence, fluorescence and absorption spectra are influenced by the polarity of solvents. A red and blue shift in the emission λ<sub>max</sub> is observed in the protic and aprotic solvents, respectively. In the protic solvents, such as MeOH, the light produced is green and in aprotic solvents such as ethyl acetate it is blue.

The rise and fall rates of the chemiluminescence, a theoretical maximum level of intensity, intensity at the maximum, the time to reach the maximum intensity and the total light yield were affected by the solvent polarity and evaluated using the pooled intermediate model by a non-linear least-squares curve-fitting programme, KINFIT. In protic solvents the light intensity is lower than that of aprotic solvents.

**The diversity of bioluminescent beetles in the Atlantic rain forest (Brazil)**

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In the terrestrial environment, bioluminescence is predominantly found among insects. Among beetles, bioluminescent species occur in three main families: Lampyridae (fireflies), Phengodidae (railroad worms) and Elateridae (click beetles). About 2000 species of luminescent beetles are described around the world. Brazil has the richest biodiversity of luminescent insects, with about 20% of the described species in the world, but many more remain to be described. The three main families of luminescent beetles as well as luminescent species of Staphylinidae are found in Brazil. They are distributed among three main ecosystems: the Amazon rain forest, the Atlantic rain forest forest and Cerrado (savannas). During the last two decades we have investigated the fauna of luminescent beetles occurring mainly in the south-eastern domain of the Atlantic rain forest, one of the richest and most threatened tropical forests in the world. Here we compare the diversity of luminescent beetles in two main collecting sites of the São Paulo state Atlantic rain forest (EBB: Estação Biológica de Boracéia, which is a large contiguous reservation; and Faz. Santana, a small remnant of semi-deciduous forest) and one of Goiás State, cerrado ecosystems (Faz. Sta Cruz). An underestimation showed that EBB showed the highest diversity (25 species), followed by Faz. Santana (18 species) and Faz. Sta Cruz (18 species). Comparatively, the Highlands Biological Station, a Neartic biodiversity hot spot in the Appalachian Mountains, showed five species. Besides the beauty, scientific interest and ecological importance of the bioluminescent diversity in the Atlantic rain forest, the biotechnological and biomedical potential of new luciferase genes as biomarkers can not be dismissed. The green and red emitting luciferases of Phrixotrix railroad worms are examples of currently used reporter genes coming from such areas. Unfortunately, this diversity is severely threatened by urban sprawl and increasing levels of artificial illumination. Preservation of such unique biodiversity hot spots is therefore essential.

**Inhibition and enhancement of luminol chemiluminescence by organic compounds**

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Chemiluminescence (CL) has become an important and valuable detection method in analytical chemistry in recent years because of its high sensitivity, wide linear range and simple instrumentation. CL allows for the detection of numerous analytes based on the inhibition or enhancement by them of certain CL reactions. Therefore, much effort has been made to explore CL inhibition and enhancement by a variety of substances of the CL reactions. Luminol in aqueous alkaline solution can be oxidized to undergo CL by a variety of oxidants, such as hydrogen peroxide, permanganate, hypochlorite and iodine. Many organic compounds were observed to increase or decrease luminol CL. However, the mechanism of the inhibition and enhancement is far from being understood. The discovery of new inhibitors and enhancers is usually achieved by trial and error. In this work, the effect of organic compounds, including phenolic compounds, anilines and amino acids, on the chemiluminescence of the systems luminol–H_2O_2–Co^{2+}, luminol–potassium ferricyanide, luminol–dimethylsulphoxide–OH– and luminol–KIO_4–H_2O_2 was systematically studied. It was found that most of the compounds tested could inhibit or enhance CL intensity, dependent upon the medium, the pH of the medium, and the concentration and structure of the compounds. The mechanism for CL inhibition and enhancement has been proposed according to CL spectra, UV-visible absorption spectra, and variation of CL reaction conditions. Finally, the analytical potential of luminol CL reactions for the tested compounds was explored. The results imply that luminol CL reactions are still of great potential for the detection of numerous compounds separated by high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE). Additionally, they provide an insight into the possible interferences in analytical procedures using luminol CL reactions.

**Bacteriophage-based bioluminescent bioreporters for monitoring opportunistic pathogens in the spacecraft environment**

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Bacteriophage-based bioluminescent bioreporters are currently being developed for the detection and identification of bacterial pathogens within the spacecraft environment. Microbes in enclosed environments pose serious threats; with the recent mandate for the USA to establish a lunar base within the next 10 years and reach Mars by 2025, the need arises for early and rapid detection of bacterial pathogens that may compromise astronauts’ immune systems, contaminate food and water supplies, and degrade system materials. Bioluminescent bioreporters are whole-cell biosensors that produce light in response to specific chemical agents via transcriptional activation of lux–reporter gene fusions. Bacteriophage-based bioreporters utilize the pathogen-specific phage infection as a means of inducing bioluminescence through a modified quorum sensing signal. **luxI** is genomically inserted into the phage, resulting in the production of acyl-homoserine lactone (AHL) autoinducer molecules upon host infection. In the proposed overall bioreporter system, the AHL molecule will diffuse into co-located bioreporter cells containing the luxCDABE + luxR operon; these cells produce measurable light in response to the infection. Bioreporter systems are currently being developed for the detection of *Salmonella*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Preliminary data have been collected from the *Salmonella* and *E. coli* systems. In both, a constitutive promoter was fused to **luxI** and incubated with bioreporter cells, producing bioluminescence at 8000 and 14 000 counts/s, respectively. These bioreporter systems are to be coupled with an integrated circuit optical transducer, the end product being a sensitive, low-cost biochip capable of real-time, on-line monitoring of microbial contamination within the spacecraft environment.

**Applications of lumigen PS-atto and TMA-6 chemiluminescent peroxidase substrates**

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Lumigen PS-atto and TMA-6 have been developed as enhanced chemiluminescent detection reagents for the detection of horseradish peroxidase (HRP) conjugates in solution-phase and solid-phase immunodetection assays, respectively. These substrates suffer no deterioration of reagent performance on storage of the working solution for up to 3 weeks at room temperature or 4 months at 4°C. The structurally related compounds exhibit subtle differences in performance properties, tailored to the intended application. Reaction of HRP leads to very rapid generation of peak light intensity. Unlike luminol-based reagents, there is essentially no build-up time to peak emission. The light intensity was sufficiently intense to enable detection of HRP conservatively estimated at 10−20 moles in a 6 min assay with PS-atto and 10−25 moles for TMA-6, with a linear dynamic range of at least four logs of peroxidase concentration. The extreme detection sensitivity allowed the development of highly sensitive enzyme immunoassays. A commercial colourimetric ELISA kit for TSH (Cobas Core, Roche), using an antibody–HRP conjugate, was adapted for chemiluminescent detection by diluting the conjugate two-fold and substituting PS-atto for the detection reagent. The chemiluminescent assay achieved a measurement of 0.003 mIU/L with a signal/blank of 2, compared to a detection limit of 0.05 mIU/L in the colourimetric assay. Lumigen TMA-6, developed for use in blotting applications such as Western blotting, enjoys the advantages of PS-atto in rapid signal generation and reagent stability and permitted pg-level detection of proteins in membrane-based blotting assays. Signal duration is extended on typical blotting membranes to provide sufficient time for optimization of imaging parameters. A Western blot assay of β-galactosidase achieved quantitation of 5 pg–5 ng protein at time points between 10 min and 2 h.
Use of a new chemiluminescent reagent in detection of oxidase enzymes and their substrates by a coupled enzyme reaction

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A recently developed chemiluminescent peroxidase substrate, Lumigen PS-atto®, produces high-intensity rapid-onset light emission in the presence of peroxyde, a peroxidase enzyme and an enhancer. This reaction provides an ultrasensitive method for chemiluminescent detection of peroxidase activity and peroxidase conjugates in enzyme-linked assays for reporting the presence of peroxyde. Since oxidase enzymes and their substrates undergo reaction with molecular oxygen to produce hydrogen peroxyde, we have investigated the use of PS-atto to detect the hydrogen peroxyde generated by various oxidase enzymes in a coupled oxidase/peroxidase reaction scheme. While chemiluminescent detection of oxidase and dehydrogenase enzymatic reactions using a peroxidase-catalysed reporter reaction has been accomplished previously with other substrates, the present method represents an improvement, due to the unique combination of features afforded by PS-atto. Chief among these are the speed of grow-in of light emission and unparalleled storage stability of the working reagent solution. The coupled enzymatic reaction presented here represents a new chemiluminescent method for the detection of oxidase enzymes and their substrates. Assay parameters have been investigated with respect to the amount of oxidase enzyme or substrate, amount of peroxidase, reaction pH, concentration of acridan compound and reaction time. A number of analytes of clinical importance, including glucose and cholesterol, can be quantified with good sensitivity and precision.

Effect of anaesthesia with propofol and remifentanil on whole-blood chemiluminescence: discriminant analysis of the results

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Propofol (2,6-diisopropylphenol) is an intravenous anaesthetic agent increasingly used for the induction and maintenance of general anaesthesia. The action mechanism of propofol is multifaceted, involving, among others, a positive modulation of γ-aminobutyrate, a reduced excitability of sensorimotor neurons, and a decrease of the open time of the acetylcholine receptor channel. Furthermore, propofol decreases cardiac β-adrenoceptor responsiveness and suppresses the activity of t-type calcium channels in the heart. However, in addition to these mechanisms, propofol exerts its effects also by a less specific interaction with membrane lipids, due to its highly hydrophobic structure.

Although the anaesthetic effect of propofol rapidly fades, both in vitro and in vivo results indicate that propofol can also exert some effects on the immune system. In particular, platelet–erythrocyte and platelet–leukocyte interactions are modified, and platelet aggregation is reduced through inhibition of TxB2 synthesis and an increase of NO production. Furthermore, neutrophil respiratory burst and protein phosphorylation by specific kinases are inhibited.

In the present work, the effect of propofol anaesthesia on neutrophil function was studied in a group of young patients undergoing surgery for strabismus correction. Anaesthesia was obtained by intravenous administration of propofol and remifentanil (a specific μ-receptor opioid agonist). Neutrophil function was measured by whole blood luminol- and lucigenin-dependent chemiluminescence (CL) in the presence or absence of zymosan or PMA. Peripheral blood was obtained before and after 45 min of general anaesthesia, and after 2 and 24 h after the end of surgery.

Despite the small number of patients (nine), discriminant analysis of the results clearly indicates that, while no variation of CL response was present after 45 min of anaesthesia, CL specific activity was significantly modified after 2 and 24 h. In particular, modifications of either CL intensity or kinetics were obtained.

The results of this study clearly indicate that propofol (and remifentanil) anaesthesia induces a modification of neutrophil function that is long-lasting when compared to the short-lasting anaesthetic effect.

Induction of differentiation in HL-60 leukaemia cells by HEMA, TEGDMA and bis-GMA

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A number of chemically distinct compounds induce differentiation of the HL-60 human promyelocytic cell line, all trans-retinoic acid, DMSO, 1,25-dihydroxy-vitamin D3, anthracyclines and bezafibrate. During differentiation, HL-60 cells stop growing, and acquire the ability to form reactive oxygen species, as shown by the appearance of a chemiluminescence (CL) response dependent on PMA-stimulated respiratory burst.

In endodontic and restorative materials, molecules such as 2-hydroxyethylmethacrylate (HEMA), triethyleneglycol dimethacrylate (TEGDMA) and 2,2-bis[4-(2-hydroxy-3-methacryloxy)-phenyl] propane (bis-GMA) are present. In clinical use, these compounds are polymerized by chemical or photochemical reactions but a certain amount of monomers is released in the pulp cavity, where they can induce inflammatory reactions and cellular damage. The aim of the present study was to determine the differentiating effect of these monomers on a HL-60 cell line.

HL-60 cell line cultures were incubated for 5 days with different concentrations of monomers and after incubation the cells were processed for CL. The results obtained show that all the monomers considered inhibit the growth of the cell culture.
and induce a significant expression of CL while no response was obtained in the absence of the monomers. These results indicate that the monomers considered inhibit the proliferation of the HL-60 cell line and induce its differentiation.

Application of enhanced chemiluminescence for immunochromatographic rapid assay of fatty acid binding protein (FABP)
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Acute myocardial infarction is a significant global public health problem, and the development of effective diagnostic tests is an actual problem in modern cardiology. Much effort by many investigators has been undertaken to improve the specificity and sensitivity of such tests. Fatty acid binding protein (FABP) is a soluble cytoplasmic protein which was recently found to be one of the specific markers for myocardial decomposition. We have developed a one-step immunochromatographic assay combined with enhanced chemiluminescence detection for rapid determination of FABP in human serum, with the use of recombinant FABP and its fusion with horseradish Peroxidase (HRP). The method was based on a competitive scheme of immunoassay. Nylon modified with p-azidobenzoic acid was used as porous support for covalent immobilization of specific antibodies. The developed assay allows the determination of FABP in a concentration range of 10–500 ng/mL, which is adequate for the purposes of myocardial infarction diagnostics.

Chemiluminescent detection of peroxide using novel stabilized 1,2-dioxetane boronic acids and boronate esters
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We describe the development of a novel class of thermally stable 1,2-dioxetanes bearing boronic acid or boronate ester moieties for the detection of hydrogen peroxide. These dioxetanes are converted to the corresponding phenols through reaction with hydrogen peroxide. The hydroxy-substituted dioxetanes decompose under alkaline conditions, generating chemiluminescence. Solutions of the boronic acid-substituted dioxetanes and various monomeric and polymeric cationic surfactants provided enhanced levels of light emission. Enhanced formulations were prepared and used to detect peroxide concentrations over several orders of magnitude. Application of this peroxide detection system to peroxide-generating systems was also examined. As a proof of principle, detection of glucose concentrations over several orders of magnitude was achieved. Reaction of glucose with glucose oxidase generated peroxide, which then further reacted with the boronic acid-substituted dioxetane under alkaline conditions to generate a chemiluminescent signal proportional to the quantity of glucose initially present. Kinetic and spectroscopic studies of this new reaction will be presented.

A spectrophotometry of luminescence for quantitative spectral analysis
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Bioluminescence and chemiluminescence are widely used for visualizing molecular dynamics. In recent years, new tools such as luciferases emitting different colour and bioluminescence resonance energy transfer (BRET) are also being developed. Applying these new technologies is important to analyse spectral information; however, it was difficult for existing spectrophotometers to detect the feeble light. We have developed a highly sensitive spectrophotometer for this purpose. In order to achieve high sensitivity, we adopted a cooled CCD instead of a conventional photomultiplier tube.

On the other hand, comparison between the measurement results from different spectrophotometers was not possible, because the measured value of luminescent intensity is expressed in relative terms. We also have developed a new method for measuring luminescent intensity by introducing a coefficient to convert the relative light units (RLU) to the photon number in each wavelength. The value of the coefficient is determined in consideration of detection efficiency and spectral sensitivity characteristics of the new spectrometer.

In this study, we present an example of spectral measurement of luciferase using this method. Quantum efficiency is determined from the number of molecules and amount of photons at each wavelength. The result shows that this method is effective for quantitative spectral analysis.

Thermostabilization of firefly luciferase by using osmolytes
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A method for thermostabilization of firefly luciferase by using osmolytes is presented. In the presence of an osmolyte the firefly luciferase retained most of its activity for more than 60 min at 22–40°C. The stabilizing effect was concentration-dependent and, on average, with a remaining activity of over 80%. Practical aspects of the use of osmolytes in bioluminescence are discussed.

The osmolyte strategy is applied to real-time bioluminescence-based assays and compared with the strategy of using thermostable firefly luciferase mutants. A second
application for the osmolyte technique is the Pyrosequencing technology. The advantage of performing DNA sequencing with the Pyrosequencing method at 37°C is shown, with several real-time sequencing examples.

**Immobilization of bioluminescent systems and their applications**

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A systematic review of immobilization of different luminous organisms and their bioluminescent enzymatic reactions is presented. The main sections are focused on the methods of immobilization of bacterial and firefly luciferases on sepharose 4B, agarose, nylon, collagen films, modified glass beads, polyacrylic acid, albumin gel and others. The chemical methods of immobilization give better yields of active immobilized luciferases than the physical ones, and agarose, collagen, epoxymethacrylate and nylon have proved to be the most effective of the different solid supports that have been investigated.

The applications of immobilized bioluminescent systems as the chemicals for bioluminescent analysis and as the models for *in vitro* and *in vivo* bioluminescent processes are discussed. The results of studies of the basic properties of the different immobilized luminescent enzyme systems and their analytical applications are described. The stability, sensitivity, precision, and the effects of interfering substances and the microenvironment of the immobilized enzymes have been examined. The use of immobilized enzymes for analytical purposes adds advantages to bioluminescent assays, including: greater stability of the immobilized enzymes over the soluble forms; increased sensitivity of detection relative to the soluble forms; and reusability of the immobilized enzymes. It is likely that future developments of bioluminescent assays will be with immobilized enzymes using an automated instrument. The continuous-flow system adopted is simple; it requires only minor modifications of a commercial detector, and allows the analysis of about 20–30 samples/h. The analytical performances can be further improved by the use of multichannel systems.

This review shows a great possibility of application of immobilized bioluminescent systems as biosensors and for different researches in the fields of biology, molecular biology, enzymology, biotechnology and others, due to the peculiarities of bioluminescent systems.

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**A homogenous bioluminescence assay for rapid microbial detection**

Braeden Butler, Keith Wood and Frank Fan

ATP-based detection of microbial cells represents a key application of the luciferase/luciferin bioluminescence assay. Conventional methods require two steps: application of a lysis reagent to release microbial ATP, followed by a detection reagent to elicit bioluminescence. We have developed an assay that combines the lytic reagent with luciferase/luciferin, thus allowing sensitive detection of microbial cells in a single step. This has been made possible by using a novel luciferase (Ultra-Glow™), developed through directed evolution to be robust to lytic agents. In addition, the composition of the reagent has been adjusted to achieve fast, efficient and uniform release of microbial ATP and to inhibit endogenous ATPase. The assay involves the addition of a single reagent directly to microbial cells in medium, which results in the release of ATP and the generation of a stable ‘glow’ luminescent signal with a typical half-life of over 30 min. The assay has been validated on a variety of bacteria, e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Yersinia enterocolitica*, *Francisella philomiragia*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Bacillus cereus*. The luminescence is proportional to ATP concentration and correlates to the number of viable cells, typically over four orders of magnitude. The sensitivity of the assay is sufficient to detect as few as 100 bacterial cells, depending on the species. The simplicity and robustness of this assay allow easy adaptation to a variety of applications, such as high-throughput screening of antimicrobial compounds and the detection of microbial contamination.

**Calcium-regulated photoprotein obelin as a label in immunoassay: an outlook for application**

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Obelin is one of the Ca²⁺-regulated photoproteins deriving from the marine bioluminescent hydroid Obelia longissima. This is a comparatively small (22.2 kDa) single polypeptide chain protein that forms a stable complex with hydroperoxycelenterazine. The binding of Ca²⁺ ions initiates conformational changes in a molecule, resulting in substrate decarboxylation with the yield of CO₂, celenteramide and blue light. In our laboratory the cDNA encoding obelin was cloned, the *E. coli* overproducing strain was constructed, and the simple and effective technology to obtain over 40 mg highly purified apo-obelin from 1 g raw cell paste was developed. Apoprotein is effectively activated by incubation with synthetic celenterazine under reduction conditions. Among the obvious advantages of the obelin label for immunoassay, as compared to others, are: the availability of recombinant obelin; an unlimited linear range of its bioluminescence response; the simplicity with which the reaction is triggered (no need for any other co-factor or substrate as well as incubation period); no radiation hazard and wastes; sensitivity improvement to amol level due to the high quantum yield of the photoprotein bioluminescent reaction; and the lack of the background. Furthermore, the
protein is stable in solution, under lyophilization and chemical and genetic modifications. The number of obelin conjugates was synthesized and the derivatives obtained were applied to protein, hormone and toxin immunoassays. The construction of obelin colour mutants broadens the scope of photoprotein application in immunoassay for simultaneous measurement of several analytes at different wavelengths becomes possible.

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Bioluminescent assay of the hygiene quality of ground beef
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Bioluminescent assay of total bacterial contamination (TBC) was applied for assessment of hygiene quality of ground beef. For assay, bacteria cells were washed away from ground beef particles to saline by shaking. Non-bacterial ATP was eliminated by filtration of bacterial suspension obtained through a Filtravette™ (special luminometric microcuvette, with the bottom made of bacterial membrane filter). ATP from bacterial cells concentrated in the Filtravette™ was released by dimethylsulphoxide and ATP reagent designed in our laboratory was added to the same Filtravette™. The bioluminescent signal from Filtravette™ was measured using a portable luminometer 3550i from New Horizons Diagnostics Corp., USA. The detection limit of the proposed assay is 10⁴ CFU/g and duration is ~ 35 min/sample. We analysed ground beef samples (n = 20) whose TBC varied in the range 10⁻²–10⁻¹ CFU/g by both bioluminescent assay and plate count. A good correlation was observed (R = 0.96). The intervals of bacterial ATP concentration (mole/g) were established for ground beef samples: good hygiene quality (<10⁻¹³), contaminated (10⁻¹³–10⁻¹⁰) and low hygiene quality (>10⁻⁸).

Bioluminescent assay of sterility or cleanliness in the hospital environment
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Bioluminescent assay was applied for the rapid control of sterility or cleanliness in hospital environment. Bacterial cells were collected using a swab technique from different surfaces, including surgical tables, furniture, the floor in resuscitation units, medical equipment, linen, hands of medical personnel, etc. Samples with total bacterial contamination (TBC) above 50 CFU/100 cm² were immediate analysed by bioluminescence. Samples with a lower TBC value were incubated in nutritive media for 6–12 h, followed by bioluminescent assay. For removal of non-bacterial ATP and/or concentrating bacterial cell, the samples analysed were filtered through Filtravette™ (a special luminometric microcuvette with the bottom made from bacterial membrane filter). ATP release and measurement of bioluminescent signal were performed in the same Filtravette™. A positive correlation between CFU determined by standard plate count and bacterial ATP concentration measured by bioluminescent assay was observed.

Dioxetane chemiluminescence mechanisms
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The reaction mechanisms of dioxetane chemiluminescence are discussed, based on our new experimental observations: (a) the high pressure effect on chemiluminescence quantum yield and chemiluminescence spectra of photo-induced chemiluminescence of a thermally stable 3-methoxyphenyldioxetane derivative; (b) the temperature effect (77–300 K) on photo-induced chemiluminescence; (c) the magnetic field effect on chemiluminescence quantum yield of a base-triggered dioxetane chemiluminescence at room temperature; (d) the high pressure effect on the quantum yield and emission spectra of a base-triggered dioxetane chemiluminescence at room temperature. Combining these observations and quantum mechanical study, we propose the potential energy surface of the chemical transformation.

Control of microorganisms by singlet oxygen
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Recently, the control of microorganisms has been increasing in importance, because food poisoning and infection disease by microorganisms happens frequently. Additionally, environment-conscious techniques for the control of microorganisms that are less burdensome for natural environment are sought after. Thus, we tried to purify environmental contamination by microorganisms with dyes (Rose Bengal and phthalocyanine). The dyes generate singlet oxygen (¹O₂), a molecular species of active oxygen, by visible light irradiation.

In this paper, we describe the control of microorganisms by singlet oxygen that: (a) inhibits growth of Escherichia coli;
(b) decontaminates water bloom; and (c) kills *Ichthyophthirius multifilis*. In summary: (a) singlet oxygen decreases colony forming units of *E. coli*, indicating that O$_2$ influences *E. coli*; (b) singlet oxygen decontaminates water bloom containing *Microcystis aeruginosa*; (c) singlet oxygen showed high mortality to *Ichthyophthirius multifilis*, which causes white spot disease in goldfish (*Carassius auratus*). These results suggest that the O$_2$-generating system is a simple, safe and effective technique that is applicable to water purification.

**Synthesis and electrochemistry of 5,5′-dimethyl[12,12′]bibenzo[b]acridyldiene**

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A redox-type electrochemically-generated chemiluminescence (ECL) of lucigenin 1 has been known. For the purpose of extending the light wavelength, we planned to synthesize 5,5′-dimethyl[12,12′]bibenzo[b]acridyldiene, 2. The benzo derivative 2 was prepared by application of the conventional method for 1. Acylation of aniline with 3-hydroxy-2-naphthoic acid, 3, to give 3-anilino-2-naphthoic acid 4, followed by intramolecular Friedel–Craft acylation to give 12-chlorobenzo[b]acridine 5, hydrolysis of 5, methylation with CH$_3$I, and finally coupling reaction with Zn-HCl provided the object, benzo derivative 2.

Regarding electrochemical properties, benzo derivative 2 showed typical cyclic voltammetric curves. The waves peaking at −1.30 V, +0.48 V, +0.73 V and +1.14 V correspond to the reduction of 2$^{2-}$, and those peaking at −2.10 V, −0.87 V, −0.69 V and +0.60 V correspond to oxidation of 2. Weak ECL emission was observed in absolute DMF.

**Dual-antibody sandwich format combined with enzyme-linked immunomagnetic chemiluminescence for the detection of *Escherichia coli* O157:H7**

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Contamination of foods with the pathogenic bacterium *Escherichia coli* O157:H7 has been implicated in numerous cases of food-borne illnesses. Many rapid methods have been developed for the detection of this pathogen in foods in order to circumvent time-consuming plate culture techniques. However, many of these methods are solely used for screening and are therefore considered presumptive, since they do not claim to confirm the presence of *E. coli* O157:H7. Using a relatively affordable and portable chemiluminescence detection method coupled with an enzyme-linked sandwich immunoassay and paramagnetic microparticles (enzyme-linked immunomagnetic chemiluminescence: ELIMCL), we have developed a rapid method that shows promise for confirming the presence of *E. coli* O157:H7. The key feature of this assay is that it combines the highly selective synergism of both anti-O157 and anti-H7 antibodies in a dual-antibody sandwich immunoassay format. This work presents the application of a one-step ELIMCL to the detection of live *E. coli* O157:H7, with apparent detection limits of approx. 1 × 10$^5$–1 × 10$^6$ of cells/mL. In addition, the sandwich-based immunoassay portion of this method is performed in a one-step format that only requires a total assay time of approx. 1 h.

**Patterns and chemistry of siphonophore bioluminescence**

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Siphonophores are planktonic hydrozoans that are widely distributed in the oceans. Despite their relative obscurity, they can be dominant members of the carnivorous plankton. The vast majority of species are bioluminescent (with some interesting exceptions), and this phenomenon has been investigated in some detail within the genus *Nanomia*. Here we examine the variety of forms that siphonophore luminescence may take. Like other hydrozoans, they appear to produce light through the use of calcium-activated photoproteins. A species, or even a particular specimen, may produce a range of colours. Although most species previously examined have light-emitting patches of cells on their bracts and nectophores, in deep-sea species we have found novel locations of photocytes, suggesting a variety of functions.

**Bioluminescence intensity modelling and optimization of sampling strategies**

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The objective of this study was the development of methodology for short-term oceanic bioluminescence (BL) predictions and the optimization of spatial and temporal BL sampling strategies. It was shown that short-term changes in some of the salient features in coastal BL can be explained and predicted by the assimilation of BL observations into the advection–diffusion–reaction (tracer) model, with velocities and diffusivities from the circulation model. This approach has been tested with BL observations collected during the Augusts of 2000 and 2003 in the Monterey Bay area. During August 2003, BL surveys were collected during an extended (~1 week) upwelling-favourable event, and in August 2000 during a strong wind
Chemiluminescent analysis of the antioxidant and immunomodulating effects of several psychotropic drugs on peritoneal macrophages

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The ability of peritoneal macrophages to produce superoxide radicals and to induce luminol-dependent chemiluminescence was used to test the antioxidant and immunomodulating effects of several psychotropic drugs: three-cycled antidepressants (imipramine, IMI, amitriptyline, AMI), phenothiazines (chlorpromazine, CPZ) and thioxanthenes (chlorprothixene, CPX). The induction of luminol-dependent chemiluminescence was carried out by activation of protein kinase C- or calmodulin-dependent ‘oxidative burst’ of macrophages, using phorbol-12-myristate-13-acetate (PMA) and calcium ionophore A23187, respectively. The viability of the macrophages was determined by detection of ATP bioluminescence as a result of luciferase-catalysed luciferin + ATP reaction.

It was observed that the neuroleptics (CPZ, CPX), in concentrations higher than 1 µmol/L (corresponding to clinically relevant doses), markedly decreased the chemiluminescent index of PMA- or A23187-activated macrophages in a dose-dependent manner. The inhibitory effect of CPZ on the PMA-/A23187-induced cell chemiluminescence was higher than the ability of the drug to decrease KO2-induced chemiluminescence in a pure chemical system, as a result of its scavenger activity against superoxide radicals only. Presumably, the inhibitory effect of CPZ on the PMA-/A23187-induced macrophage chemiluminescence was also a result of its immunomodulating activity.

In contrast, the antidepressants (IMI, AMI) manifested a weak effect on the luminol-dependent chemiluminescence of the macrophages and did not express any effect on KO2-induced chemiluminescence.

It was also observed that the suppression of the macrophage chemiluminescence by all the investigated drugs was not a result of their cytotoxicity. Moreover, it was established that all the drugs dose-dependently enhanced the macrophage ATP bioluminescence, which is an indirect evidence for an immunomodulation.

Molecular recognition for charge-transfer-induced chemiluminescent decomposition of optically active dioxetanes bearing a bisnaphthyl moiety triggered with an optically active crown ether/potassium t-butoxide

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There exist four optical isomers, viz. the (Ra,Ra)-, (Ra,Sa)-, (Sa,Sa)- and (Sa,Ra)-forms, for bicyclic dioxetanes bearing a 6-hydroxynaphthalen-1-yl moiety substituted with a 5-(naphthalene-1-yl). All these optically pure dioxetanes were synthesized in 14 steps and characterized by means of NMR, IR, mass and X-ray crystallographic analysis. When these dioxetanes were treated with TBAF/DMSO or t-BuOK/(18C6)/THF-PhH, they decomposed rapidly to emit orange light, with few differences to one another in their chemiluminescent properties. On the other hand, on treatment with a (Sa,Sa)-bis(binaphtho)-22-crown-6-t-BuOK complex in THF-benzene, four optically isomeric dioxetanes caused CT-induced decomposition to give light with properties different from each other. When the (Ra,Ra)-bis(binaphtho)-22-crown-6-t-BuOK complex was used as a triggering system in place of its (Sa,Sa)-isomer, these dioxetanes exhibited chemiluminescence with properties quite reverse to the case using a (Sa,Sa)-isomer. The results presented here show that the optically active dioxetanes synthesized here can recognize a host complex which acts as a base.

Quantitative analysis of chemiluminescence intensity and toxicity in silico

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The chemiluminescence detection technique is highly sensitive. Chemiluminescence is produced by a chemical reaction, the sensitivity and intensity of which depends on the reactivity of super oxide. The intensity of chemiluminescence was quantitatively analysed using computational chemical calculations based on a radical reaction mechanism in which a keto-enol rearrangement produces superoxide, and the superoxide reacts...
with luminol or lucigenin to produce the chemiluminescence. The partial charge of the carbon atoms of a carbonyl group, calculated using the MOPAC function of the CAChe™ program, changed significantly and strongly correlated with the relative intensity of the chemiluminescence. This computational chemical analytical method can be used to determine the relative sensitivity of the chemiluminescence reaction using luminol and lucigenin.

Superoxide is toxic in vivo. The chemiluminescence was therefore related to the toxicity. The toxicity was calculated using the TOPKAT™ program from Fujitsu. The correlation coefficient between the intensity of the chemiluminescence of phenacyl alcohol derivatives and rat oral LD$_{50}$ was 0.949 ($n = 5$), and that between the partial charge change and the rat oral LD$_{50}$ was 0.912 ($n = 5$). These results indicate that measurement of the intensity can provide a quantitative measurement of the toxicity of an analyte. Furthermore, the calculation of the partial charge change by the computational chemical method can be used to estimate the rat oral LD$_{50}$. These experimental and computational chemical methods will help to speed up screening of certain drug candidates by chemiluminescence assays.

Many steroid drugs are used for the treatment of skin diseases. Superoxide produced from steroids should also produce chemiluminescence by the same mechanism. Therefore, the above approaches were applied to study the efficacy, i.e. toxicity, of steroid drugs. The intensity of the chemiluminescence strongly correlated with the partial charge change of targeted atoms as well as with the rat oral LD$_{50}$.

**On the mechanism of arylboronic acid 'enhancement' of peroxidase-catalysed reactions**

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The utility of peroxidase enzymes such as horseradish peroxidase (HRP) as labels in enzyme-linked chemiluminescent assays is augmented by including a phenolic compound to enhance the production of light and, in some cases, to reduce non-enzymatic background signal. Chemiluminescence HRP-catalysed reactions subject to enhancement include the oxidation of diverse substrates, including hydrazides such as luminol, acridan esters, thioesters or sulphonamides, and acridan derivatives bearing electron-rich double bonds, such as Lumigen APS, PS-atto and TMA-6. While various hydroxy-substituted aromatic and heterocyclic compounds enhance peroxidase-catalysed chemiluminescence, studies have shown that only those phenolic compounds with oxidation potentials lying within a particular range are effective. Compounds outside of the range are either ineffective or diminish light production. The enhancement effect has been attributed to the phenolic compound mediating electron transfer between enzyme and substrate. Subsequent to the discovery of enhancement by phenols, the use of certain arylboronic acids and arylboronate esters was reported to enhance the same reactions. The boron compounds were considered to represent a new class of enhancers. No mechanistic rationale was put forth to explain the putative enhancement ability of these compounds. We provide experimental evidence to demonstrate that arylboronic acid derivatives are not the actual enhancers. In fact, these compounds are unstable to peroxide under typical assay conditions and are rapidly converted to the corresponding phenols. Moreover, the oxidation of arylboronic acids and esters by hydrogen peroxide to produce phenols was previously reported. Since peroxidase-catalysed reactions are always conducted in the presence of excess peroxide, rapid and complete conversion of arylboronic acid compounds to the phenols is inevitable.

**Imaging fluorescence and bioluminescence in living cells**

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Luminescent and fluorescent probes allow us to non-invasively interrogate living cells under different environmental conditions. Advances in imaging technologies have enabled tracking of multiple targets simultaneously in live samples.

In 2001 LUX Biotechnology Ltd was established to develop filamentous fungal biosensors. LUX technologies harness the power of bioluminescence and fluorescence by engineering fungal cells whose light output gives an indication of the presence of specific molecules. LUX technology is unique in that it utilizes fungi; filamentous fungi are more versatile, grow in more diverse conditions and are easier to store and transport than mammalian cells, bacteria or yeast. It is predicted that LUX biosensors will make important contributions to environmental testing, drug discovery, toxicology and R&D. In addition to the biosensors, LUX has developed a range of novel calibration devices called Glowells™. Glowells™ produce an extremely stable glow, with specific wavelengths and intensities. Glowells™ are used to calibrate plate-reading luminometers, fluorometers and other light-measuring equipment. The products also give a standard with which to compare to biological samples during experiments. The latest product is an array of light sources termed a 'Low Light Imaging Standard™', which is designed for use with CCD imaging systems. For further information, visit www.luxbiotech.com

**Single-molecular imaging of protein in the living cell by pin-fibre video-microscope**

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A novel method for single-molecular imaging was developed using the pin-fibre video-microscope, which utilizes a single optical fibre for sample illumination. This method has several advantages over conventional fluorescence microscopy. In particular, the linearity of the instrument in measuring the fluorescence intensity allows for more accurate calculations of the number of fluorescent molecules on a single cell. Furthermore, the pin-fibre video-microscope is highly versatile, allowing for imaging in diverse environments such as living cells. This technology promises to revolutionize the field of single-molecular imaging, providing new insights into the behavior of proteins and other cellular components.
Development of the chemistry of the imidazopyrazinone bioluminescence system: from the bio- and chemiluminescence mechanism to a design of sensor molecules

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The imidazo[1,2a]pyrazin-3(7H)-one (imidazopyrazinone) ring system is a core structure of the luminescent substrates isolated from marine bioluminescent organisms, such as the jellyfish Aequorea and the crustacean Cypridina. To develop the chemistry of the imidazopyrazinone bioluminescence system, we have systematically investigated the bio- and chemiluminescent properties and the physical properties of imidazopyrazinone derivatives on the basis of the pioneering study of the Goto group. As a result of these studies, the unique π-electronic character of the imidazopyrazinone ring system is clarified. In this paper, we explain the fundamental chemistry of imidazopyrazinone derivatives and discuss the problems of molecular recognition in aequorin bioluminescence and the chemiluminescence reaction process. In addition to these studies, we found that the π-electronic character of imidazopyrazinone derivatives was sensitively affected by interactions with molecular environments, such as a hydrogen-bonding interaction and a Lewis acid–base interaction. These interactions with molecular environments caused the continuous spectral change of imidazopyrazinone derivatives. From these results we show that imidazopyrazinone derivatives are useful as sensor molecules for determining the hydrogen-bond donor strength of a solvent molecule and the Lewis acidity of a metal ion.

Ionic structure of the excited light-emitter in calcium-activated photoprotein bioluminescence

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The calcium-activated photoprotein aequorin was discovered as a bioluminescent substrate of the jellyfish Aequorea by Shimomura and Johnson. After the discovery of aequorin, several calcium-activated photoproteins, including obelin, were found and their chemistry has been developed. In the year 2000, the crystal structure of aequorin was revealed and the supramolecular structure of aequorin was clarified, which contained the coelenterazine peroxide included in the active site of apo-aequorin. We are now in a position to study the molecular process of the bioluminescence of the calcium-activated photoprotein system. We have studied the mechanism of aequorin bioluminescence from the viewpoint of supramolecular photochemistry. We pay special attention to the problem of elucidating the ionic structure of the excited light-emitter coelenteramide in calcium-activated photoprotein bioluminescence. To solve this problem, we have systematically investigated the fluorescent properties of the anion species of coelenteramide analogues. Based on these studies, we confirm that the singlet-excited state of the phenolate anion of coelenteramide has an intramolecular charge-transfer character and its fluorescence emission is sensitively affected by a change of molecular environments. On the other hand, the singlet-excited amide anion of coelenteramide has a fluorescent character that is insensitive to molecular environments. We report here these fluorescent properties of the coelenteramide anion species and our conclusion that the excited phenolate anion of coelenteramide is the light-emitting structure in calcium-activated photoprotein bioluminescence.

In vivo imaging with fluorescent proteins: the new cell biology

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We propose a new cell biology in which the behaviour of cells can be visualized in the living animal. An example of the new cell biology is dual-colour fluorescence imaging, using red fluorescent protein (RFP)-expressing tumours transplanted in green fluorescent protein (GFP)-expressing transgenic mice. These models show with great clarity the details of tumour–stroma interactions, especially tumour-induced angiogenesis, tumour-infiltrating lymphocytes, stromal fibroblasts and macrophages. Another example is the colour coding of cells with RFP or GFP, such that both cell types can be simultaneously visualized in vivo. Stem cells can also be visualized and tracked in vivo. Mice in which the regulatory elements of the
Chemiluminescent microspheres for measuring reactive oxygen species (ROS) in phagocytosis

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Analogues of Cypridina luciferin, viz. CLA, MCLA and FCLA, were bound to polymer microspheres for the purpose of measuring ROS released into phagosomes during phagocytosis. CLA and MCLA were absorbed into or adsorbed onto microspheres (ca. 1 µm) of a methyl methacrylate co-polymer, while FCLA was covalently bound to microspheres (ca. 2 µm) of a glycidyl methacrylate co-polymer derivative. Throughou amide group that was formed by the condensation reaction of an amino group in the polymer with a carboxyl group in FCLA. ROS were generated by O$_2$–hypoxanthine–xanthine oxidase (O$_2$–), KO$_2$(O$_2$–), NDPO$_2$(O$_2$–), H$_2$O$_2$–MPO–Cl or H$_2$O$_2$–Fe$_2$$^+$ (HO$^-$). Desorption of CLA or MCLA from microspheres impregnated with them was less than 5% at pH 7.4 (PBS) and pH 5.2 (citrate buffer). The release of FCLA from FCLA-bound polymer microspheres was negligible. MCLA-impregnated microspheres and FCLA-bound microspheres emitted stronger chemiluminescence than commercially available ABEI-bound polymer microspheres. The HO' radical generated in the H$_2$O$_2$–Fe$_2$$^+$ system elicited stronger luminescence from FCLA-bound microsphere than O$_2$ liberated from KO$_2$. O$_2$ from NDPO$_2$ elicited insufficient chemiluminescence from any kind of these microspheres, whereas it produced strong chemiluminescence with the solution of every kind of these Cypridina luciferin analogues. In conclusion, polymer microsphere impregnated with MCLA and FCLA-bound polymer microspheres are promising probes for measuring O$_2$ and HO' released into phagosomes in phagocytosis.

Chemiluminescence reaction of 4-styrylphthalhydrazides. Remarkable substituent effect on the emitting species and chemiluminescence efficiency

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A series of 4-styrylphthalhydrazides were prepared from the reaction of the corresponding dimethyl 4-styrylphthalates and hydrazine and their chemiluminescence reactions examined in aerated aproic solvents under basic conditions. One of them provided stronger chemiluminescence than luminol. The chemiluminescence quantum yields, as well as the emitters, were found to be variable, depending on the substituents attached on the terminal phenyl groups. Strongly electron-donating substituents increase the chemiluminescence efficiency remarkably in these cases the emitters were unambiguously identified as the corresponding excited phthalate ions, similarly to the chemiluminescence reaction of luminol, while the reactions of 4-styrylphthalhydrazides lacking strongly electron-donating substituents showed only a faint light emission under similar reaction conditions, and the emitters were not the phthalate ions but the weakly fluorescent phthalhydrazide anions, whose decay was spectroscopically monitored. The fluorescence quantum yields of dipotassium 4-styrylphthalates were found to be linearly decreased with increase of the Hammett rho-values of the substituents. Thus, the excited phthalate ions are the emitters when they are strongly fluorescent, while the energy transfer chemiluminescence from the excited phthalate ions to the phthalhydrazide monoanions would take place when the phthalate ions are not (or weakly) fluorescent.
Chemiluminescence assay for lipase activity in human serum by using a proenhancer substrate

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Recently, we developed the chemiluminescence (CL) assay for lipase activity with synthetic substrate, HDI-laurate, which is a lauric acid ester of 2-(4-hydroxyphenyl)-4,5-diphenylimidazole (HDI). The method was based on the CL measurement of cleaved HDI from HDI-laurate by lipase, in which HDI acts as an enhancer of the CL reaction of luminol–H2O2–horseradish peroxidase (HRP). In this study, we tried to develop the assay of lipase activity in human serum for the purpose of clinical diagnosis of pancreatic disorders.

Assay procedure: a serum sample (100 µL) was mixed with 1.0 mL 0.1 mol/L phosphate buffer (pH 6.5) and DMF solution of HDI-laurate (10 µL) for 90 min at 37°C. After incubation, this solution was applied to a solid phage extraction (SPE) cartridge (Sep-pak C18) to remove serum matrices that interfere in the CL reaction. Retained HDI was eluted by acetonitrile (1.5 mL). To 100 µL eluate was successively added Tris–HCl solution (0.3 mol/L, pH 6.4) of luminol and HRP (50 µL each) and 200 µL H2O2 aqueous solution. The CL emission was integrated from 1.0 to 5.0 min. The detection range of lipase activity was obtained in the range 2.6–10.8 U HDI (1 U HDI = enzyme activity that liberates 1 µmol/min of HDI from the substrate at 37°C) with the detection limit (blank signal + 3 SD) of 0.09 U HDI. The reproducibility of replicate assays gave the RSDs of <3.0% and <4.5% for within- and between-day assays, respectively. A comparison study with a conventional method is now in progress to confirm the reliability of the method.

Effect of caloric restriction and ageing on the generation of reactive oxygen species in rat liver mitochondria and peroxisome

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Significant fractions of molecular oxygen utilized in aerobic organisms, including humans, are converted to reactive oxygen species (ROS) in and around the mitochondria, endoplasmic reticulum and peroxisomes. Long-lasting production of ROS results in the accumulation of DNA damage in both nucleus and mitochondria. Caloric restriction has been shown to retard the ageing processes in various organisms, but the underlying mechanism remains unknown.

ROS generation by rat liver mitochondria and peroxisome was analysed using a highly sensitive CHL probe, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione (L012). Mitochondrial generation of ROS was increased by the presence of a respiratory substrate, such as succinate and glutamate, but decreased by the presence of ADP, which activates state III respiration. In the presence of urate, highly purified rat liver peroxisomes also generated ROS, as monitored by L012-CHL. The extent of ROS generation by peroxisome-free mitochondria was fairly low, even in the presence of urate. Biochemical analysis revealed that uricase was responsible for the enhanced generation of ROS in peroxisomes.

To clarify the effects of ageing and caloric restriction on the relationship between energy metabolism and ROS generation, properties of liver mitochondria from young and aged rats were analysed before and after starvation. Although mitochondrial generation of ROS was higher with young (19 week-old) than with aged (2 year-old) rats, it decreased significantly in both animal groups after starvation for 2–3 days. Although ROS generation by peroxisomes was also higher with young rats than with aged animals, effects of age restriction were not apparent in either of the animal groups, even in the presence of urate. These results suggest that ROS generation was greater in and around the mitochondria and peroxisomes in young rats than in aged animals, and caloric restriction suppresses the ROS generation by mitochondria but not peroxisomes in both animal groups. Based on such information, the biomedical significance of caloric restriction in aged subjects is discussed.

Chemiluminescence detection of 3-nitrobenzanthrone and 2-nitrotriphenylene in airborne particles with on-line reduction HPLC system

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Strong direct-acting mutagenic 3-nitrobenzanthrone (3-NBA) and 2-nitrotriphenylene (2-NTP) were successfully determined at sub-p.p.b. level in the soluble organic fraction of airborne particles, by chemiluminescence detection with an HPLC system equipped with a reducer column composed of alumina-supported platinum-rhodium catalysts. The sensitivity of the detection was considerably higher than conventional GC–MS techniques, including a negative ion-detecting one, having a limit of detection of 20 fmol and 40 fmol for 3-NBA and 2-NTP, respectively. The average atmospheric concentrations of 3-NBA and 2-NTP in autumn in central Tokyo were 7.3 fmol/m³ and 1.4 fmol/m³, respectively while 2-nitrofluoranthene (2-NF), 3-nitrofluoranthene (3-NF), 1-nitropyrene (1-NP), 6-nitrochrysene (6-NC), and 1,8-dinitropyrene (1,8-DNP), which are conventionally studied nitrated polycyclic aromatic hydrocarbons, were found in the same samples at the concentration levels of 0.79 pmol/m³, 2.0 fmol/m³, 0.11 pmol/m³, 3.2 fmol/m³ and 1.8 fmol/m³, respectively. Application of the method to the analysis of atmospheric occurrence revealed that daily
A novel orange fluorescent protein from the Cnidarian tube anemone Cerianthus sp. and its multicoloured fluorescent mutants

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The first native orange fluorescent protein (OFP) was discovered from the tentacles of the Cnidarian tube anemone Cerianthus sp. It contains 222 amino acid residues and shows only a modest 26% and 37% sequence identity to Aequorea GFP and Discosoma red fluorescent protein DsRed, respectively. OFP has the excitation maximum peaked at 548 nm and the emission maximum peaked at 565 nm. It also has a quantum yield of 0.64 and a molar absorption coefficient \( \varepsilon_{\text{max}} \) of 60 000 mol/L/cm. OFP is an obligate tetramer, as revealed by its X-ray crystal structure, and shares the same \( \beta \)-can structure and its multicoloured fluorescent properties with only a single amino acid substitution. All these findings indicate that a subtle change in the micro-environment in the vicinity of the chromophore can drastically alter the spectral properties of a fluorescent protein.

Development of tandem bioluminescent enzyme immunoassay for angiotensin I and endothelin-1

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We have developed a simultaneous bioluminescent assay of acetate kinase (AK) and pyruvate phosphate dikinase (PPDK). In this method, the detection limits (at blank + 3SD) of AK and PPDK were 1.03 \( \times 10^{-20} \) and 2.05 \( \times 10^{-20} \) mol/assay, respectively (1). Previously, we succeeded in applying tandem bioluminescent enzyme immunoassay (BL-EIA) for the simultaneous measurement of insulin and c-peptide. In this study, we also applied the method to tandem BL-EIA for angiotensin I and endothelin-1, which are hypertension-related peptides. The tandem BL-EIA was used in competitive immunoreaction for angiotensin I and in sandwich immunoreaction for endothelin-1, respectively. Using the proposed BL-EIA, the measurable range of angiotensin I and endothelin-1 were 7.81–1000 pg/mL and 15.63–1000 pg/mL, respectively. The intra-assay coefficients of variation of angiotensin I and endothelin-1 with each standard point were below 9.7% and 11%, respectively. The BL-EIA was applicable to measure for rat serum samples.

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Studies on the chemiluminescence mechanism of Cypridina luciferin analogues: dissociation constants of singlet-excited Cypridina oxyluciferin analogues

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The chemiluminescence reaction of Cypridina luciferin analogue (CLA), 2-methyl-6-phenylimidazol[1,2-\( \alpha \)]pyrazin-3(7H)-one, with molecular oxygen or superoxide anion in aqueous media gives two light-emitting species, i.e. the singlet-excited state of 2-acetamido-5-phenylpyrazine (\( 1^{\text{OCLA}} * \)) and its conjugate base (\( 1^{\text{OCLA}}^- \)), and the ratio of these two species is affected by the medium’s pH. Also, a substituent at the 6-position of the imidazopyrazinone ring is known to influence the ratio. Although a proton transfer is supposed to be involved among the molecules in singlet excited states, the detailed mechanism is still unclear. To clarify the molecular mechanism for the formation of these two species, five kinds of 2-acetamido-5-arylpypyrazines, possessing various substituents at the \( p \)-position of the 5-phenyl group, were synthesized, and their fluorescent properties, especially the \( \text{pK}_a \) values for the \( \text{N} - \text{H} \) dissociation in the singlet excited states (\( \text{pK}_a^* \)) in aqueous media were investigated. The estimated \( \text{pK}_a^* \) values were found to be larger than 11, and increased with increasing the electron-donating character of the substituents. A proportional relationship between the Hammett’s substituent constants and the \( \text{pK}_a^* \) values was observed. These results indicate the proportional chemiluminescence mechanism of CLA, that the anionic \( 1^{\text{OCLA}}^- \) is produced first and then protonated to give \( 1^{\text{OCLA}}^* \), to be implausible.

Chemiluminescence in the Ru(bpy)_2^2+ -catalysed Belousov–Zhabotinsky reaction

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Much interest has been shown in the study of the light-sensitive oscillatory reaction systems. The Ru(bpy)_2^2+ -catalysed Belousov–Zhabotinsky (BZ) reaction is a BZ variant which interacts with visible light and shows chemiluminescence and
We present here further experiments on the oscillating chemiluminescence in the Ru(bpy)$_3^{2+}$-catalysed BZ reaction in a closed system. Chemiluminescence originates from the emitting excited state, [Ru(bpy)$_3^{2+}$], which is produced by the reduction of Ru(bpy)$_3^{3+}$ in the oscillating reaction. The oscillating chemiluminescence has the same period but is out of phase compared with the photoluminescence oscillation of Ru(bpy)$_3^{2+}$. In the time course of the reaction, the chemiluminescence oscillation damps more rapidly than the absorption oscillation of Ru(bpy)$_3^{2+}$. The oscillating curve of chemiluminescence does not exactly reflect the smooth change in the concentration of Ru(bpy)$_3^{3+}$. The effects of pH and temperature on the chemiluminescence oscillation were investigated. It was found that the apparent chemiluminescence yield in the oscillating system is higher at higher pH. Increasing temperature tends to slightly increase the apparent chemiluminescence yield in early stage of the oscillating reaction. These oscillatory behaviours of chemiluminescence are discussed on the basis of the chemical mechanism of the Ru(bpy)$_3^{2+}$-catalysed BZ reaction.

**Chemiluminescent polymeric probe for the sensitive detection of DNA**

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We have been developing chemiluminescent polymeric compounds for the sensitive detection of a genomic DNA telomere. The telomere has a specific DNA sequence at the ends of chromosomes, consisting of a TTAGGG repeat-unit. The telomere sequence protects the chromosome from fusion and degradation of the cell, and it becomes shortened by each cell division. Thus, the length of the telomere in the chromosome has been of interest in ageing and tumor genesis. Due to the crucial role of the telomere, we addressed the task of detecting a single-stranded telomere sequence by two simple new approaches utilizing chemiluminescent polymeric compounds.

In one approach a unique chemical derivatization reagent, trimethoxyphenylglyoxal (TMPG), was utilized. This reagent reacts specifically with guanine bases in nucleic acids to quickly produce chemiluminescent derivatives under mild reaction conditions (1). TMPG gave an increasing CL intensity; depending on the content of guanine base in the DNA molecule at pmol level (2). Thus, we tried immobilized hybridization assay of the telomere DNA binding to its cDNA on a nylon membrane. TMPG detection with a CCD camera allowed high sensitivity as low as sub-fmol level of the telomere DNA after binding to biotinylated large DNA for signal amplification by the reaction between avidin and biotin. On the other hand, we are synthesizing dextran-based polymeric compounds for use as sensitive chemiluminescent signal probes. The compounds contain numerous low-molecular weight chemiluminescent substances and biotin in the large molecular dextran. These compounds were detectable at sub-fmol level on a nylon membrane. The synthesized polymeric chemiluminescent compounds are expected to be useful for sensitive DNA and other assays.

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**DNA analysis method by luciferase-based bioluminescence detection and a miniaturized luminometer for the bioluminescence assays**

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For spatial and quantitative analysis of protein–protein interactions (PPIs) in living mammalian cells, a method was developed in which PPI-induced complementation of split Renilla luciferase triggers spontaneous emission of luminescence, with a cell membrane-permeable substrate, coelenterazine. Consequently, unlike conventional complement enzymes that lead to stable diffusive fluorescent products, this split Renilla luciferase complementation read-out is capable of locating the PPIs with emission of bioluminescence only at the sites and time of their occurrence in living cells.

To monitor the interaction between two proteins, A and B, the N-terminal half of the split Renilla luciferase is fused to protein A, and protein B to the C-terminal half of the split Renilla luciferase. Interaction between proteins A and B and the consequent juxtapositioning of the split Renilla luciferase simultaneously leads to formation of the complement Renilla luciferase, thereby spontaneously emitting bioluminescence with its cell membrane-permeable substrate, coelenterazine, in situ in living mammalian cells. This split Renilla luciferase complementation read-out was shown to work for locating a PPI between tyrosine-phosphorylated peptide (Y941) of IRS-1 and SH2 domain of PI3K among insulin signalling pathways in living Chinese hamster ovary cells overexpressing human insulin receptors (CHO-HIR). It was thereby found that the insulin-stimulated interaction occurred near to the plasma membrane in the cytosol.

The PPI between Shc and Grb2 was also studied using the same technique.
Bioluminescence detection method is widely used in the biomedical and environmental fields. Especially, the ATP assay based on the firefly luciferase system has become an important tool, not only for the immunoassay but also for hygiene monitoring commercially. The luminometer for the bioluminescence assay has several advantages, e.g. no excitation light source such as a laser required and simply operation; also, the background noise of the bioluminescence detection is lower than that of the fluorescence detection, which is the most popular method in DNA analysis. Since the DNA sequencing method based on the real-time PPI assay, ‘pyrosequencing’, has been reported, many researchers nowadays use it for short-sequencing and SNPs typing. Today’s commercial luminescence detection system is expensive and large because of a cooled CCD camera system. Therefore, miniaturized low-cost luminescence detection systems have been increasingly required in various fields.

In this session, we present a new SNPs typing method by bioluminescence assay coupled with a primer extension method, which does not use gel-electrophoresis. In addition, the miniaturization of a luminometer for this method is presented. The miniaturized luminometer consists of air-driven microdispensers, microreaction cells and a diode-photosensor array. The microdispenser system can supply 0.01–100 µL reagent to each reaction cell and the reproducibility (RSD) of the multiple injections is 3–5% (n = 20). The high photoemission collecting efficiency of about 7% and measurement in a small reaction volume of 5 µL was achieved in this system because the photodiode array was closely positioned under the reaction cells. All the signals from four reaction cells are simultaneously detected by the photosensor array. This method is promising for the achievement of a low-cost DNA analysis system because of its compact size and easy operation.

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Determination of particle-associated nitro-PAH using a HPLC–chemiluminescence detection system

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The concentrations of several kinds of nitricated polycyclic aromatic hydrocarbons (nitro-PAH), such as 1-nitropyrene (1-NP), 2-nitropyrene (2-NP), 2-nitrofluoranthene (2-NF) and 2-nitrotriphenylene (2-NTP), in the soluble organic fraction of airborne particles were determined by the column-switching HPLC–chemiluminescence detection system. The HPLC system consisted of four pumps, a six-port switching valve, two separation ODS columns, a Pt/Rh column for the reduction of nitro-PAH, a concentration column and a chemiluminescence detector. Airborne particulate sample collection was performed every 3 h to clarify their diurnal variation in a non-polluted rural area and in a slightly polluted residential area. In the residential area, the diurnal variation of the concentration of 1-NP was similar to those of NOx (NO + NO2) and CO, which were primarily emitted from combustion processes, such as diesel-powered vehicles, while the concentration of 2-NF, which is produced by atmospheric reactions, showed different patterns of the diurnal variability. On the other hand, in the rural area, the change of nitro-PAH including 2-NF in concentration was accompanied by those of NOx and CO. The results suggest that the concentrations of particle-associated nitro-PAH observed in the rural area are mainly controlled by the transportation of the air mass, including the gaseous air pollutants. In this study, the obtained diurnal variations of the nitro-PAH concentration are classified into patterns, and the individual variabilities are discussed in relation to their controlling factors, such as photochemical reactivity, meteorological conditions, and others.

Chemical studies on bioluminescence of the acorn worm, Ptychodera flava: isolation and characterization of luminous substances

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Cormier and Dure reported the bioluminescent phenomenon of Balanoglossus biminiensis, a kind of acorn worm, which emitted light through a luciferin–luciferase reaction with additional hydrogen peroxide as a co-factor. They found that horseradish peroxidase can promote the light emission, so they suggested the luciferase to be a kind of peroxidase. Higa found Ptychodera flava, a smaller acorn worm, in Kattore bay, Kohama Island, Okinawa. P. flava emitted green light by stimulation with hydrogen peroxide. In the present studies we have been aiming at elucidation of the bioluminescent substances and the luminescence mechanism of P. flava. The bioluminescence was recorded by using a live specimen and the spectrum showed wave length centring at 500 nm. The luminous substances were extracted not in aqueous buffer but in organic solvent. The luminescence assay was established by adding pH 8.5 buffer and 3% hydrogen peroxide aqueous solution into a part of a given fraction. This organic extract was purified by silica gel column chromatography to give three luminous substances. One of them, the major substance, was obtained as a crystal. This crystal was analysed by NMR, MS and X-ray analyses, but it did not show the fluorescence from itself, suggesting the absence of a visible light emitter. A possible light emitter showing green emission was found in the methanol extract from 59 g wet sample. This extract was purified by several silica gel column chromatography runs to give 100 µg, and the structure was determined from UV, fluorescence, ESI-Q–TOF–MS, MS–MS, H/D exchange MS and MS–MS data. All the details are discussed in this symposium.

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Spectral modulation of bioluminescence from Vibrio fischeri strain Y1 cells

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The modulation of bioluminescence (BL) of V. fischeri Y1, producing yellow fluorescent protein (YFP) and Y1-blue fluorescent protein, was characterized in a seawater complete liquid medium at varying concentrations of O2 and hydrogen ions. Moreover, the relationship between the density of a single cell and the BL spectral distribution was studied, based on the sucrose density-gradient centrifugation method. From the measurement of BL during the period of the cycles of aeration on and off, it was found that an increase in the O2 concentration leads to the enhancement of the yellow BL (~535 nm) and that the BL spectral modulation reversibly occurs in accordance with the aeration on-off cycle. In this case, the fluctuation of the blue-green BL intensity was not significant. The enhancement of the yellow BL intensity due to the supply of O2 was somewhat greater in weakly acidified media (~pH 6), where the yellow BL band is not so clear. By contrast, supplying O2 to Photobacterium phosphoreum cells, producing lumazine protein responsible for the blue-shifted BL, took no recognizable effect on the spectral distribution, although the total BL was considerably intensified. These results may suggest that the change in the respiratory activity affects the properties of YFP, possibly being present close to the cell membrane, to cause the reversible spectral modulation of V. fischeri Y1 BL. It was also found that an increase in a single cell density of V. fischeri Y1 causes an increase in the ratio of the yellow BL intensity at 535 nm to the blue-green one at 470 nm. The observed increase in the intensity ratio with an increase in the single cell density might indicate that there is a possibility that V. fischeri Y1 cells also modulate the spectral distribution in the cell cycle.

Biosynthesis of Cypridina luciferin from free amino acids in Cypridina (Vargula) hilgendorfii

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In the bioluminescence of marine organisms, Cypridina luciferin and coelenterazine, known as an imidazopyrazinone-type luciferin, are widely used for the luciferase reaction. But it is not clear what kind of organisms could synthesize these luciferins. The chemical structure of an imidazopyrazinone-type luciferin strongly suggests that the luciferin could be synthesized from several amino acids. To investigate the biosynthetic mechanism of imidazopyrazinone-type luciferin, we tried to identify a synthetic component of Cypridina luciferin in living Cypridina (Vargula) hilgendorfii. After feeding the stable isotope-labelled L-arginine and L-tryptophan to specimens for 5–15 days, Cypridina luciferin was extracted and analysed by LC/ESI–TOF–MS. A significant incorporation of stable isotopes into Cypridina luciferin was detected, suggesting that L-arginine and L-tryptophan are components of Cypridina luciferin synthesis.

Determination of basic compounds with peroxyoxalate chemiluminescence detection

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The chemiluminescence (CL) reaction requires no exciting light source, and thus interference accompanied by light scattering is avoidable. Since CL permits attainment of a large signal:noise ratio, determination of a wide range of analytes may be carried out. Luminol and its analogues are well-known CL reagents, directly able to produce emission of light by themselves due to an oxidation reaction with oxygen species such as hydrogen peroxide (H2O2). The CL reaction involving an energy transfer reaction is also well known. Oxalates react with H2O2 to yield intermediate peroxides, which produce light by energy transfer to a co-existing fluorophore. Therefore, the combination of oxalate with fluorophore is important to yield intense emission. A large number of fluorophores are detected by CL reaction with aryloxalates and H2O2. Among the aryloxalates, bis(2,4,6-trichlorophenyl) oxalate (TCPO) is one of the most popular for the CL reaction. Fluorophores derived from target compounds and H2O2 in samples have been determined in this CL detection system. Since this CL reaction using TCPO and H2O2 proceeds smoothly in the presence of a base catalyst, the determination of basic compounds may also be possible. Based upon this speculation, the determination of basic compounds involving drugs was carried out by flow injection analysis (FIA), using the peroxyoxalate CL reaction. In the present research, TCPO and H2O2 were used as CL reagents, whereas DNS-amino acid was selected as the fluorophore. The concentration of these reagents, which affect the CL intensity, was optimized, and the concentrations of DNS-phenylalanine, TCPO and H2O2 were selected as 2.5 µmol/L, 0.125 mmol/L and 20 mmol/L, respectively. Higher emission was observed in some basic compounds such as histamine under the proposed CL reaction conditions. Detection of basic compounds in microtitre plates by multilabel counter is also discussed.

Non-competitive inhibition of the rare earth element-induced oxidative burst by zinc in tobacco cells: a chemiluminescent analysis

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Phytotoxic rare earth element (REE) ions induce an oxidative burst in tobacco cell culture by stimulating the NADPH oxidase-dependent superoxide-generating mechanism. Previous report has shown that Zn ions inhibit the REE-induced...
oxidative burst. In the present study using tobacco BY-2 cell suspension culture and a superoxide-specific chemiluminescent probe, Cypridina luciferin analogue, the mode of interaction between the ions of Zn and REE (La) in induction of oxidative burst was determined as non-competitive inhibition, suggesting the inhibitory binding of Zn to both the REE-bound and REE-free target molecules.

Chemiluminescence in the reactions of uranium and lanthanides

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We report on new peculiarities of chemiluminescence of 4f (Pr, Nd, Eu, Tb, Dy) and 5f elements (uranium) in aqueous and organic solutions as well as in solid phase:

1. Formation of excited states in the oxidation of trivalent lanthanide complexes by the organic oxidant dimethyldioxirane occurs due to energy released upon oxidation of the ligands.
2. Chemiluminescence of uranyl ion (UO$_2^{2+}$) is observed during the oxidation of U(IV) by the inorganic oxidants XeO$_3$, XeF$_2$, O$_3$, S$_2$O$_8$, etc. Electron transfer is responsible for UO$_2^{2+}$ excitation. A chemiluminescent method for analysis of extremely low concentrations (as low as 10$^{-14}$ mol/L) of uranium is suggested.
3. Lanthanides in ground and excited states catalyse the decomposition of dioxetanes (DO). Formation of complexes of DO–lanthanide chelates precedes DO decomposition. Quantum chain reactions with energetic branching are realized during this catalytic process. Excited europium [Eu(III)] ions are formed in two excited states which take part in branching of the chain. It was shown that Eu(III) ion in the excited state forms a more stable complex with DO than in the ground state. In this connection, the problem of f-shell participation in chemical bonding is discussed.
4. Chemiluminescence occurring upon thermal solid phase decomposition of Eu persulphate and uranyl ions has been revealed.

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On the role of the singlet oxygen dimolecule in chemiluminescence of dioxirane reactions

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Three-membered cyclic peroxides, dioxiranes, remarkably combine two significant properties—the ability to act as strong oxidants and the ability to form products in the electronically excited state upon thermal decomposition and oxidative reactions (1). Recently attention has been focused on a new aspect of these peroxides, viz. infrared chemiluminescence (IR-CL) of singlet oxygen in the chemical transformations of dioxiranes (2). For instance, IR-CL of O$_2$ has been observed in N-oxide-catalysed decomposition of dimethyldioxirane (DMDO) and methyl(trifluoromethyl) dioxirane (TFD), in anion-induced reactions of DMD and TFD, and in the ketone-catalysed decomposition of monoperoxyxysulphate ion via intermediary dioxirane. We report an observation of chemiluminescence in the visible (Vis-CL) spectral region in these catalytic reactions. We also report evidence (spectral analysis of the emission, influence of the solvent on Vis-CL intensity, etc.) that the singlet oxygen dimolecule (O$_2$)$_2$ plays an important role in Vis-CL. Reosibility of energy transfer from the (O$_2$)$_2$ to the luminescent lanthanide complexes is discussed in terms of the Khan and Kasha hypothesis (3) on the O$_2$-sensitized luminescence.

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Design and synthesis of luminescent lanthanide complexes with a luminescence off–on switch

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Luminescent lanthanide sensors are of current interest and much effort has been devoted to designing luminescent sensors. However, there is no coherent strategy available for this purpose. So, we here present a practical strategy. We employed the photoinduced electron transfer (PeT) mechanism as a basis for luminescence switching and synthesized lanthanide complexes \([\text{Ln-1}–\text{Ln-12}]\) with various luminescence off–on switches. We showed that the luminescence intensity can be predicted from the HOMO level of the off–on switch. i.e., the HOMO levels of luminescent complexes were below \(-5.80\) eV and those of non-luminescent complexes were more than \(-5.80\) eV. These results show that we can develop novel luminescent sensors by choosing an off–on switch which steps across the threshold upon reaction with the target analyte. In addition, \([\text{Ln-1}]\) shows a large pH-dependent luminescence enhancement; \([\text{Ln-1}]\) emission is switched on in acidic media, with a pK_a of 4.6 for \([\text{Eu-1}]\) and 3.6 for \([\text{Tb-1}]\). This result shows that \([\text{Ln-1}]\) works as a pH sensor. This strategy should be applicable to develop a range of luminescence probes that can recognize various target molecules.

**Development and biological application of a novel fluorescent probe for ratiometric imaging of protein tyrosine phosphatase activity**

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We have designed and synthesized a novel ratiometric fluorescent probe \(1\) for protein tyrosine phosphatase (PTP) activity, based on spectral overlap integral switching of FRET. The fluorescence spectra of \(1\) exhibited a large shift in their emission wavelength after reaction with PTPs in vitro. Within the \(1\)-labelled cells, an increase in the emission ratio was observed, and it was suppressed by the addition of a PTP inhibitor, orthovanadate, and hydrogen peroxide. So it was shown that \(1\) could detect PTP activity in living cells. Next, we applied this imaging method to biological studies on the regulation of PTP activity during contact growth inhibition of normal cells. In normal cells, PTP activity of dense cells was markedly enhanced compared to that of sparse or medium density cells. The increased PTP activity was also observed in sparse cells treated with a reducing agent, lipoxygenase inhibitor, and PLA2 inhibitor, and resulted in cell growth inhibition. These observations suggest that PTP activity could be regulated by ROS produced in lipoxygenase-mediated arachidonate metabolism and play a key role in density-dependent growth inhibition.

**Luminescence techniques in reinvestigation into photocatalytic reactivity of TiO_2**

J.-I. Kimura

Titanium oxide (TiO_2) has been used as a photocatalyst for more than 30 years. It is an interesting fact that the source of the active species from TiO_2 is still debated. In this study, we tried to reinvestigate a reagent-grade titanium oxide (TiO_2) by luminescence techniques. In the thermally excited luminescence of TiO_2, emission spectrum measurements and temperature dependence of luminous intensity are essential to interpret the observed luminescence. Titanium oxide exhibited luminescence when it was heated in temperatures of 100–160°C under a nitrogen atmosphere. Temperature dependence of the luminescence intensity provided activation energy to be 92 kJ/mol. The thermally excited luminescence spectrum of TiO_2 measured at 160°C in nitrogen exhibited two spectral groups: an emission peak with a maximum at 440 nm and a set of line spectra comprising 480, 580 and 680 nm due to singlet oxygen. In this study photocatalytic luminescence is defined as the light emission from a photocatalyst after cessation of UV-visible light irradiation. Photocatalytic luminescence of TiO_2, irradiated with blacklight (365 nm) and fluorescent light in nitrogen showed almost the same spectrum as the thermally excited spectrum stated above. They are different from those obtained for metal oxides such as stainless steel and quartz, which provide only green emission with a peak near 500 nm but no emission due to singlet oxygen. Active species of TiO_2 have been believed to be due to hydroxyl radical (·OH) and superoxide anion (O_2·−). From the experimental evidence obtained in this study, singlet oxygen will be a good candidate of active species because of their strong oxidizing power. We can propose regeneration process of oxygen for TiO_2, i.e. it can generate active oxygen by daylight and absorb oxygen at night.

**Green luminescence emitted from adsorbed oxygen. Has aurora appeared on the surface of materials?**

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Many investigators have reported anomalous initial peaks in CL intensity–time curves for a variety of materials, but the origin of the CL peaks has long been unsolved. We examined the characteristic luminescence peaks of a filter paper by heating and light irradiation. A filter paper was heated at different temperatures ranging from 90°C to 120°C in nitrogen. The filter papers exhibited luminescence peaks at all the temperatures, followed by exponential decays. The slopes of the luminescence decay curves followed, Arrhenius-fashion. Activation energy for the luminescence decay curves was 29 kJ/mol, which is as low as one-third of reported activation energies for thermal decomposition of organic hydroperoxides (96–116 kJ/mol). A filter paper was irradiated with a black light (365 nm) for 5 s and then luminescence measurement started after waiting for 5 s. The emission spectrum of photo stimulated luminescence showed two peaks at 480 nm and 520 nm. These emission

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bands seem to be of Gaussian profile. The shorter wavelength (480 nm) of the peak correspond to the shortest visible band (480 nm) of electronically excited molecular oxygen. The emission intensity from UV-irradiated filter paper was so high that we could take a colour picture with a highly sensitive colour CCD camera. The colour of UV-irradiated filter paper was green, as we expected from the observed emission spectrum. Green light emission from filter paper resembles the popular green aurora due to atomic oxygen in the upper atmosphere. We have confirmed the presence of significant amounts of adsorbed oxygen on the surface of oxygen-containing organic compounds and metal oxides. The adsorbed oxygen remains on the surface and emits green light on irradiation with light. On the contrary, when they were heated, adsorbed oxygen molecules are desorbed from the surface to form energetic oxygen, which acts as a strong oxidizer to organic materials.

**Relationship between heat of reaction and chemiluminescence efficiency of chemiluminescent reactions**

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Primitive organisms had to excrete oxygen due to its toxic nature. A luminous organ is said to have been provided as a tool for oxygen treatment. Luciferins are key compounds in chemi- and bioluminescence. In 1966, White and McCapra noted that the 1,2-dioxetane structure is important as a chemiluminescence intermediate. A 1,2-dioxetane structure has been recognized as the key intermediate in both chemiluminescent and bioluminescent reactions. The dioxetane structure should be a crucial part of the tool for oxygen treatment, because the dioxetane is an important structure for changing thermal energy, which may damage thermally susceptible organisms, to harmless visible light. We measured the heat of reaction of chemiluminescent systems in a quartz cell capable of shielding light generated during the reaction, and in a quartz cell capable of releasing thermal energy, which may damage thermally susceptible organisms, to harmless visible light. We measured the heat of reaction of chemiluminescent systems in an aluminium capsule, capable of shielding light generated during the reaction, and in a quartz cell capable of releasing light energy by chemiluminescence.

We selected two typical chemiluminescence systems, lophine peroxides and dioxetanes. The dioxetanes gave off a larger amount of light, resulting in generating a smaller amount of heat. It was shown that the sum of light energy and heat energy measured in a quartz cell equals the heat of reaction measured in an aluminium cell. The lophine peroxides generated basically the same amount of heat due to lower CL yields.

**Generation of chemiluminescence upon reaction of phenyltin(IV) compounds irradiated with UV light and tris(2,2'-bipyridine) ruthenium(III)**

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The chemiluminescent system using tris(2,2'-bipyridine) ruthenium(III), Ru(bpy)₃⁺, has recently become a powerful tool for the determination of compounds that have a tertiary amine or diketone group. We have found that phenyltin(IV) compounds were decomposed into some degradation products which react with Ru(bpy)₃⁺ by UV irradiation with a low-pressure mercury lamp. Chemiluminescence intensity depended upon the number of phenyl groups and UV irradiation time. One of the decomposition products was identified as oxalic acid. Although other products are unknown, they ended up by generating oxalic acid with continuous UV irradiation (~60 min). On the basis of these findings, a method for the chemiluminescent determination of phenyltin(IV) compounds has been developed using high-performance liquid chromatography combined with a photochemical process. A calibration graph for triphenyltin(IV), based upon cation exchange separation and subsequent on-line UV irradiation (3.5 min), was linear in the range 0.1–100 µmol/L and the detection limit was 5 pmol (S/N = 3).

**The high energy key intermediates in the peroxyoxalate chemiluminescence of 2,4,6-trichlorophenyl N-aryl-N-tosyloxamates**

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In peroxyoxalate chemiluminescence, dioxetanedione and substituted dioxetanones have been generally proposed as high-energy key intermediates that interact with fluorophores. To elucidate which is the suitable key intermediate, the chemiluminescence reaction of 2,4,6-trichlorophenyl N-aryl-N-tosyloxamates in the presence of DPA (9,10-diphenylanthracene) as a fluorophore was carried out. A Hammett relationship between the maximum intensity and the sigma-value was shown, in which the rho-value under neutral conditions (+2.66) was larger than that under basic conditions (+1.20). This result means that a substituted dioxetanedione still bearing the eliminating group is the suitable high-energy key intermediate, because the proton transfer from the hydroxyl group of the substituted dioxetanone to the nitrogen atom should be involved in order to generate dioxetanedione by liberation of tosylamide from the substituted dioxetanone. When an oxamate having a weakly fluorescent naphthyltosylamide group reacted with hydrogen peroxide, only a faint chemiluminescence was observed, but external addition of the
The chemiluminescence reaction mechanism of imidazopyrazinones: effects of the reaction conditions for the chemiluminescence

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Cypridina luciferin, which is one of the imidazo[1,2-α]pyrazin-3(7H)-one (imidazopyrazinone) derivatives, is the bioluminescence substrate of Cypridina hilgendorfig and constructs a luciferin–luciferase system with Cypridina luciferase and molecular oxygen. One of the remarkable properties of Cypridina bioluminescence is that it shows a high quantum yield (Φ = 0.28). On the other hand, a chemiluminescence reaction in which Cypridina luciferin reacts with molecular oxygen in an organic solvent and emits light gives a lower quantum yield than that of the bioluminescence. The reason for the difference in the quantum yield between bio- and chemiluminescence has not yet been elucidated. It is an important research subject to clarify the problem of the chemiluminescence efficiency for establishing the reaction mechanism. To approach this problem, we investigated the chemiluminescence reactivity of Cypridina luciferin analogue (CLA) by using systematic reaction conditions, which were employed for evaluating the effects of organic solvents, bases and concentrations of molecular oxygen in solutions. We found that the reaction rate and the quantum yield of the chemiluminescence continuously varied, depending on these conditions. From the results of these findings, we discuss the molecular process for the chemiluminescence reaction of CLA in detail.

Excited states of dioxins as studied by ab initio quantum chemical computations: anomalous luminescence characters

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Photochemical studies, such as the luminescence of dioxins, are becoming more important because of diverse applications, such as spectroscopic detection and photodecomposition. The extreme toxicity of dioxins prevents us from studying in safety on the experimental side, although elaborate studies of the excited states of dioxins serve for accurate prediction of their energy levels and luminescence characters. In this context, quantum chemical approaches are of value. Documented calculations do not reproduce experimental observation. The quantum yield of fluorescence (Φf) of unsubstituted dibenzo-p-dioxin (DD) is 0.003 (1). So far, this very small Φf and weak band were presumed to be caused by a rapid intersystem crossing to the nπ* state around the lowest excited singlet states (S1). We have calculated the excited singlet states of dioxins by an ab initio method (SAC-CI) and the calculations reproduced experimental electronic spectra accurately. This calculation shows that S1–S0 transition of DD is forbidden and that any nπ* transition conditions for the chemiluminescence.
states are not located around $S_0$. The observed band can be assigned the vibronic transition from the optically forbidden $S_1$ state or from the lowest allowed state by thermal activation.

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Enzyme-based biosensors based on bacterial bioluminescence for environmental monitoring
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The current situation regarding biosensors for the monitoring of aquatic ecosystems is profoundly analysed. This analysis is to provide a basis for the concept of creating a universal system of biosensors for ecological monitoring. The approaches to the creation of this system using bioluminescent organisms and their enzymes and to devise a laboratory model of a biosensors system are discussed.

To estimate water quality, original bioluminescent biosensors, characterized by rapidity, simplicity, high sensitivity and accuracy, have been devised and successfully used. On the basis of the Collection of Luminous Bacteria (IBSO; http://www.bdt.org.br/bdt/msdn/ibso), the integral bioassay for the monitoring of the environment is being developed, using lyophilized luminous bacteria and the luminescent system isolated from them. Bioluminescent assays have an evident advantage over other biological assays: luminescence is easy to measure, the method is rapid, the measurements can be automated, and the data can be statistically processed.

The preliminary results show a correlation between physicochemical characteristics of inhibitor (activator) molecules and changes in kinetic parameters of the bioluminescent reaction. Such correlations are closely related to the physical mechanism of bioluminescence; they are a biophysical basis for bioluminescent ecological monitoring. These data will provide a basis for comparing sensitivities and choosing test organisms and enzymatic systems to be included among the sensors of the automated system of bioassays.

Acknowledgements
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NO in photophores of bioluminescent fish
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Nitrogen oxide synthase-like immunoreactivity (NOS-IR) was detected by immunohistochemistry in the light organs of three mesopelagic fish species, Argyropelecus hemigymnus, Cyclothone braueri and Electrona rissoi. These species belong to different families (Sternoptychidae, Gonostomatidae and Myctophidae, respectively) with distinct photophore organizations but in all species the photophores are ventrally located and mainly used for counter-shading. Strong NOS-IR was present in nerve fibres as well as in other cells central for the production or modulation of light in these species. In Argyropelecus, NOS-IR fibres surround the photophores and fibres are also present in the filter and lens areas. Lens cells, in particular in the outer layers, showed strong IR throughout the cytoplasm. Pharmacological studies suggested that NO modulates adrenaline-stimulated light emission in isolated photophores from Argyropelecus. Adrenaline is known to produce two different types of light response: a slow, long-lasting, high-intensity response, or a fast and weak response of short duration. Incubation of photophores in the NO donors sodium nitroprusside or S-nitroso-N-acetylpenicillamine prior to adrenaline stimulation reduced both intensity and duration of the strong and long-lasting type of response, but had little or no effect on the weakly responding photophores. The NOS inhibitor L-thiocitrulline potentiated the adrenaline response; the weaker the adrenaline effect, the stronger the potentiation caused by L-thiocitrulline. The results suggest that NO has an important role in the control of light emission from Argyropelecus hemigymnus photophores, and the presence of NOS in other species suggests that this might be a general mechanism.

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Electron-excited states and energy transfer processes in bioluminescent reactions of luminous organisms
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The mechanism of electron-excited states formation in bioluminescent organisms is currently of great interest. Emitters of all bioluminescent organisms are products of oxidative reactions. They are heterocyclic compounds with high fluorescence yield, being specified by upper excited states of ππ* type.

The hypothesis of involvement of the upper electron-excited states in a bioluminescent process has been suggested. The hypothesis was verified in bioluminescent reactions of luminous bacteria, coelenterates and fireflies. A series of exogenous fluorescent compounds were chosen; the energy of their fluorescent states exceeded that of a bioluminescent emitter and their absorption spectra did not overlap with a
bioluminescence spectrum, so trivial light absorption and intermolecular resonance S–S transfer were excluded. Bioluminescent spectra in the presence of these compounds were examined. A weak sensitized fluorescence was registered in the case of bacterial and coelenterate bioluminescent reactions, indicating the population of the upper electron-excited states of the emitters. No sensitized fluorescence was found in the firefly reaction. This result might be an indication of extra-effective intramolecular (intracomplex) energy transfer in the emitter of the firefly bioluminescence.

Interactions of the fluorescent compounds with the enzymes were studied using steady-state and time-resolved fluorescence techniques. Non-specific hydrophobic interactions of the molecules with the enzymes were confirmed, providing a resonance mechanism of intermolecular energy transfer as a means for the excitation of the exogenous compounds in the bioluminescent systems.

Potassium halides and xanthenic dyes were used to study the effect of external heavy atoms in bioluminescent systems. It was found in bioluminescent bacteria but not in the firefly reaction. The effect was shown to be a result of interaction of heavy atoms with enzymes and a change of efficiency of S–T energy conversion in the bioluminescent emitter.

Effects in rats of forced exercise, started from different ages, on the chemiluminescent response and cytokine excretion of alveolar macrophages

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We prepared three groups of rats to evaluate the effects of forced exercise started from different ages on the lucigenin-dependent chemiluminescence (LgCL) and cytokine excretions of alveolar macrophages (AMs). Training by a treadmill was started from 5, 11 and 17 weeks old (groups A, B and C, respectively) and was continued for 12 weeks. After 6- or 12-week training, AMs in the bronchoalveolar lavage fluid (BALF) were counted using a Coulter Counter ZM (Coulter Electronic Inc.). AMs were suspended in Dulbecco’s MEM after adjustment of cell numbers to 6 × 10⁵ cells/mL. AMs were placed in each well of a 96-well black microtitre plate (Greiner Japan Co.) and were cultured for 12 h. Supernatants of AMs were collected and stored for the cytokine measurements. LgCL were measured by a parallel luminometer (Alpha-Basic 47, Kokken Inc.) using opsonized zymosan as stimuli. The chemiluminescence was measured by a parallel luminometer (Alpha-Basic 47, Kokken Inc.). AMs were suspended in Dulbecco’s MEM after adjustment of cell numbers to 6 × 10⁵ cells/mL. AMs were placed in each well of a 96-well black microtitre plate (Greiner Japan Co.) and were cultured for 12 h. Supernatants of AMs were collected and stored for the cytokine measurements. LgCL were measured by a parallel luminometer (Alpha-Basic 47, Kokken Inc.) using opsonized zymosan as stimuli. The chemiluminescence was indicated by peak height (PH) and peak time (PT). Concentrations of tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and interferon-γ (IFNγ) were measured by ELISA kits (BioSource Inc.). According to ageing, PH of LgCL in the control groups was significantly enhanced. Except for Group B, there was no practical difference in PH between the control and the forced training groups. PH of the 12-week training group of Group B was suppressed significantly. Furthermore, significant increase of TNFα, decrease of IL-1β and increase of IFNγ between the control and the 12-week training groups of Group B were observed. The largest number of cells in BALF was observed at 11 weeks old. These results suggest that forced training started from 11 weeks old may be the turning point of lung immunity and affect AM activity. The chemiluminescent technique is useful for evaluating the changes of AM activity.

Determination of artemisinin by HPLC with on-line photoreactor and peroxyoxalate chemiluminescence detection

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Artemisinin is an antimalarial drug with a sesquiterpene lactone structure containing an internal endoperoxide linkage in its structure. We attempted to determine artemisinin based on the fact that organic peroxides can be converted to hydrogen peroxide under UV irradiation. In the proposed method, artemisinin eluted from the HPLC column is UV-irradiated to generate hydrogen peroxide, which is detected by peroxyoxalate chemiluminescence (PO–CL).

The HPLC system was optimized on a mobile phase, post-column CL reagent containing 2,4,6,8-tetramethoxynaphtalene (TMN), fluoro-phore and bis(2,4-dinitrophenyl)oxalate (DNPO), UV source and UV irradiation time. The optimum conditions established were as follows: mobile phase, imidazole-HNO₃ buffer (pH 7.5, 20 mmol/L) containing 60% CH₃CN (flow rate, 0.5 mL/min); CL reagent, CH₃CN containing 0.5 mmol/L DNPO and 1.5 μmol/L TMP (flow rate, 1.0 mL/min); UV irradiation, 45 s by an UV lamp at 254 nm with 10 W. Under optimum conditions, the calibration curve showed good linearity (r = 0.9998) between CL intensity and concentration of artemisinin over the range 0.01–1 mmol/L. The detection limit at signal-to-noise ratio of 3 was 5 μmol/L (100 pmol/injection). The proposed method is highly selective for artemisinin, and thus would be useful for its monitoring in biological samples.

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Studies on the bioluminescent mechanism of the symplectin photoprotein

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Determination of artemisinin by HPLC with on-line photoreactor and peroxyoxalate chemiluminescence detection

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The HPLC system was optimized on a mobile phase, post-column CL reagent containing 2,4,6,8-tetramethoxynaphtalene (TMN), fluoro-phore and bis(2,4-dinitrophenyl)oxalate (DNPO), UV source and UV irradiation time. The optimum conditions established were as follows: mobile phase, imidazole-HNO₃ buffer (pH 7.5, 20 mmol/L) containing 60% CH₃CN (flow rate, 0.5 mL/min); CL reagent, CH₃CN containing 0.5 mmol/L DNPO and 1.5 μmol/L TMP (flow rate, 1.0 mL/min); UV irradiation, 45 s by an UV lamp at 254 nm with 10 W. Under optimum conditions, the calibration curve showed good linearity (r = 0.9998) between CL intensity and concentration of artemisinin over the range 0.01–1 mmol/L. The detection limit at signal-to-noise ratio of 3 was 5 μmol/L (100 pmol/injection). The proposed method is highly selective for artemisinin, and thus would be useful for its monitoring in biological samples.

Reference

1. Wada M, Nakashima K, Kuroda N et al. J. Chromatogr. A 2003; 987: 189–195.
Tobiika (*Symplectoteuthis oualaniensis* L.) is a flying squid that emits blue light (470 nm). We have studied its bioluminescent mechanisms and confirmed that the light comes from its photoprotein, 'symplectin', which is a 60 kDa protein and possesses dehydrocoelenterazine (DCT) as an organic substance. DCT exists as a thiol-bound form with the symplectin active site cysteine to construct the chromophore for bioluminescence. The structural analysis of the symplectin active site is our main research focus now.

From the sequence analysis of symplectin with LC–Q–TOF–MS, MS–MS and cDNA, symplectin has a 501 amino acid sequence. Partial degradation of symplectin with trypsin afforded the 40 kDa protein symplectin A', which is the C-terminal part of symplectin and also has bioluminescent activity. We suppose that the active site of symplectin must exist in the 40 kDa symplectin A', therefore we synthesized fluorinated dehydrocoelenterazine (F-DCT) as a probe to investigate the active site of symplectin. We found that F-DCT strongly binds to sulphhydryl residue of cysteine to afford a stable chromophore, which was conformed with NMR and MS methods. Unfortunately, however, we could not perfectly deduce the active site cysteine residue of symplectin.

We then synthesized a photoprobe to label the active site of symplectin. As a model chromophore of symplectin, azide-fluoro-coelenterazine was photo-irradiated in a solution. Hexafluoropropionol was the best solvent for converting the azide to nitrene from the data MS analysis of photo-irradiated products.

We are now investigating the active site by using photoaffinity labelling and nano-LC–Q–TOF–MS analysis, and also studying the relationships between DCT structures and symplectin bioluminescent activities.

**Bioluminescence on the Web: the Digital Photobiology Compendium**

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The Digital Photobiology Compendium (www.photobiology.info) is an educational resource comprising 13 sections covering the various sub-disciplines of the field. Each section consists of an introductory module, and usually three to nine more advanced modules. The coverage is designed for the mid-to upper undergraduate level, and all the modules can be organized into a format to suit the aims of any particular course. The DPC Bioluminescence section leads the student from a general view of the subject to more advanced modules that deal with 'cutting edge' investigations, which are written in such a way as to not overwhelm the student with the detail found in a literature review.

Interactive tools are used to aid understanding and some structural examples developed by Leo Lin from McGill University are especially impressive. The first module introduces the student to the wide range of occurrence of bioluminescence, the second gives a brief treatment of historical aspects, and the third describes a number of experimental exercises suitable for the undergraduate laboratory. These three are written in a way to be quite suitable for high school or beginning college level students. Four advanced modules deal with the most active subjects of research; the bioluminescence mechanism of the firefly, the bioluminescent bacteria, the calcium-regulated photoproteins, and the application to clinical diagnostics. Another module describes bioluminescent systems occurring on land and adds some recent studies of their biochemistry, and the last covers the great variety of species from the ocean depths. Overall, we try to make clear that the majority of bioluminescent systems have hardly been studied, there are probably many yet to be discovered, and that there is a bountiful supply of subjects for future investigation.

**Acknowledgements**

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**The crystal structure of a Ca**2+-**discharged photoprotein supports the proton relay mechanisms proposed for the calcium trigger and bioluminescence**

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Ca**2+-regulated photoproteins are members of the EF-hand calcium binding protein family. The addition of Ca**2+ produces a blue bioluminescence by triggering a decarboxylation reaction of protein-bound hydroperoxycoelenterazine to form the product, coelenteramide, in its excited state. Based on the spatial structures of aequorin and several obelins, we have postulated mechanisms for the Ca**2+ trigger and for the formation of different excited states that are the origin of the different colours of bioluminescence. Here we report the crystal structure of the Ca**2+-discharged photoprotein obelin at 1.96 Å resolution (PDB code 1S36). The results lend support to the proposed mechanisms and provide new structural insight into details of these processes. Global conformational changes caused by Ca**2+ association are typical of the class of ‘calcium signal modulators’ within the EF-hand protein superfamily. Accommodation of the Ca**2+ ions into the loops of the EF-hands is seen to propagate into the active site of the protein, now occupied by the coelenteramide, where there is a significant repositioning and flipping of the His175 imidazole ring as crucially required in the trigger hypothesis. Also, the H-bonding between His22...
and the coelenterazine found in the active photoprotein is preserved at the equivalent position of coelenteramide, confirming the proposed rapid excited-state proton transfer that would lead to the excited state of the phenolate ion-pair, responsible for the blue emission of bioluminescence.

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Microchip electrophoresis with chemiluminescent detection and its possible application to environmental analytical chemistry

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In this present work, the principle of microchip technology was briefly described. A glass microchip was designed on the basis of flow injection–chemiluminescent devices and fabricated by standard photolithography technology, wet chemical etching and heat bonding technology. The preparation of microchip electrophoretic systems utilizing polyester with an in situ polymerization method was also introduced (1, 2). Four different detection methods—chemiluminescence (2, 3), LED-induced fluorescence (4), absorption and amperometric detection (5)—were compared in our laboratory. As the examples, trace amount of Cu^{2+}, Co^{2+} and Ni^{2+} were separated and detected with chemiluminescent method on the chip. The detection limits of Cu^{2+}, Co^{2+} and Ni^{2+} can be obtained to 5.0 × 10^{-9}, 5.0 × 10^{-11} and 1.0 × 10^{-7} mol/L, respectively. Dopamine and catechol were separated and detected using a permanganate chemiluminescent system on the prepared microchip. The samples were electrokinetically injected into the double-T cross-section, separated in the separation channel, and then oxidized by chemiluminescent reagent delivered by a home-made micropump to produce light in the detection cell. The detection limits for dopamine and catechol were 20.0 μmol/L and 10.0 μmol/L, respectively. Successful separations and detections of metal ions and organic compounds demonstrated the distinct advantages of integration of chemiluminescent detection on a microchip for rapid and sensitive analysis.

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Lophine peroxides as an efficient organic source of singlet oxygen

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Singlet oxygen is widely recognized as a very important and reactive species in both organic chemistry and biological systems. As singlet oxygen sources are limited, any new efficient source is desirable. Dufrasie et al. successfully isolated what they termed ‘photo-oxide’ after photolysis of 2,4,5-triphenylimidazole(lophine) in the presence of air, from which solid state pyrolysis at 170°C led to the formation of 50% molecular oxygen. There has been no report, however, that the molecular oxygen stems from formation of singlet oxygen. Photo-oxide was assigned the endoperoxide structure. Later, the structure was correctly assigned to C4-hydroperoxide, which is also the key intermediate in the long-recognized phenomenon of chemiluminescence from reactions between lophine and hydrogen peroxide in base. The structure of lophine peroxide was confirmed to be 2,4,5-triphenyl-4H-imidazol-4-yl hydroperoxide (1a), and not the 2,5-endoperoxide, by X-ray analysis. We have confirmed the formation of singlet oxygen from lophine peroxide in 50% yield. Further, we investigated 2-(4-Xphenyl)-4-hydroperoxy-4,5-bis(4-phenyl)imidazole [1b: X = NO_2; 1c: X = N(CH_3)_2]. Peroxide 1b provides singlet oxygen in >50% yield, whereas 1c provides only chemiluminescence, but no singlet oxygen.

We discuss the chemistry of lophine peroxides as a singlet oxygen generator more on the basis of its substituent effect.

Overview of new analytical tools for bioluminescent biomass estimation

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The luciferase assay of ATP has been used for estimation of biomass for over 50 years. Industrial applications include: (a) hygiene monitoring in the food industry; (b) estimation of microbes in process water, drinks (e.g. UHT milk, fruit juices, beer), foods and personal care products; and (c) bioassay of assimilable organic carbon (AOC) in water. Several improvements have been introduced during recent years. The availability of recombinant luciferase has allowed development of reagents with a high luciferase activity. Such reagents degrade their own ATP background and emit high levels of light,
The function of Trp residues in obelin bioluminescence

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The Ca2+-regulated photoprotein obelin has four Trp residues, which lie in the hydroperoxycoelenterazine binding cavity and are strictly conserved among all known photoproteins. Trp92 and 179 surround the 6-(p-hydroxy)-phenyl substituent of coelenterazine, whereas Trp114 and 135 are found at the other end of the chromophore near the 2-(hydroxy)-benzyl group. To determine how these residues influence bioluminescence, we produced single and double obelin mutants in which Phe was substituted for Trp, and studied a number of their physicochemical properties (emission spectrum, specific activity, sensitivity to Ca2+, stopped-flow kinetics, Ca2+-independent luminescence, temperature stability, and efficiency of charging the apo-protein). The substitutions of Trp114 and 135 had very little effect on the emission spectrum, but changed all the other properties studied. In contrast, the mutations of Trp92 and 179 resulted in significant changes in the spectral distribution of the bioluminescence and in the kinetics, along with only minor changes of the other physicochemical properties. The W92F and double W92F+179F mutants display bimodal bioluminescence spectra with violet (\( \lambda_{\text{max}} = 405 \) nm) and blue (\( \lambda_{\text{max}} = 475 \) nm) maxima. On the basis of fluorescence studies of coelenteramide it has been suggested that the violet and blue emissions originate from the neutral and ion-pair states of excited coelenteramide.

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Ca2+-regulated photoproteins of the bioluminescent ctenophores: cloning, expression and some properties

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The bright blue-green luminescence of the ctenophores studied is reversibly inhibited in sunlight and conditioned by light-sensitive Ca2+-regulated photoproteins. We have isolated the cDNAs for ctenophore photoproteins from the expression cDNA libraries of several bioluminescent ctenophores, using a functional screening method. The open reading frame of
each cDNA encodes 206–208 amino acid proteins with calculated molecular weights of 24 500–24 900. The sequence analysis has shown that novel ctenophore photoproteins belong to the EF-hand superfamily of Ca\(^{2+}\)-binding proteins and have three Ca\(^{2+}\)-binding loops like those of the early cloned photoproteins from jellyfishes. However, the novel ctenophore photoproteins display very weak homology with them, sharing maximum identity of 29% with *Obelia longissima* obelin. The ctenophore photoproteins were overexpressed in *E. coli* cells and accumulated inside cells in inclusion bodies. The recombinant apo-photoproteins were purified, refolded and reactivated with coelenterazine into active photoprotein. The recombinant photoproteins reveal light sensitivity similar to that of native photoproteins. Some biochemical and biophysical properties were studied. The successful expression of the cloned ctenophore photoproteins in mammalian cells suggests that these new photoproteins function well as bioluminescent reporters for monitoring of gene expression and for measurement of intracellular calcium.

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**Synthesis and bioluminescent activities of the *Latia* luciferin analogues**

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The limpet-like snail *Latia neritoides* inhabits stony-bottomed, clear and shallow streams on the North Island in New Zealand. Of the luminescent animal species, *Latia* is the only bioluminescent animal that lives its whole life cycle in fresh water. On stimulation, physically or chemically, the snails globules of mucus, from which greenish light (\(\lambda_{\text{max}} = 536 \text{ nm}\)) is emitted. While the predator is paying attention to the twinkling green balls that move downstream, the snails are thought to escape from the predator. Shimomura reported that the bioluminescence system of *Latia* exhibits a typical luciferin–luciferase reaction in the presence of oxygen. Our recent study indicated that the bioluminescence system requires no cofactors but is able to produce green light with only the luciferin and the purified luciferase component, clearly indicating that the light emitter is present in the luciferase, as suggested by Shimomura. The luciferin has a characteristic enol formate structure and the 2,6,6-trimethyl cyclohexene ring system, the same as the vision pigment, retinal. Previous works suggested that the enol formate moiety is an essential functionality for the bioluminescent reaction. However, the chemical structure of light emitter and precise mechanism of the bioluminescent reaction are still unclear. In order to clarify the relationship between the structure and bioluminescence activity, we synthesized various *Latia* luciferin analogues, modified at enol formate moiety and 2,6,6-trimethyl cyclohexene ring system, and their bioluminescent activities were studied. The results from these studies are presented.

**Photina™: an improved Ca\(^{2+}\)-sensitive photoprotein**

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Calcium-sensitive photoproteins are important tools for analysing all aspects of calcium-mediated signal transduction processes in mammalian cells. One of their characteristics is the immediate photon release (flash luminescence) upon calcium binding to the coelenterazine–photoprotein complex, which makes this system extremely useful for studying receptor–ligand interactions or ion channels if the mobilization of calcium is involved.

In order to create a very sensitive photoprotein, the sequence and structural similarities and differences of seven well known photoproteins were carefully analysed. Based on this analysis, a photoprotein was created with an optimized codon usage for mammalian cells, with a reduced number of cysteine residues and, most importantly, with a modified calcium binding site.

This new photoprotein, called Photina™, has been used as a reporter system in cell-based assays, and data showing these results are presented. Transfected cells exhibit a robust flash luminescence signal, making the system well suited for HTS and uHTS applications.

**Chromatography and mass spectrometric analysis of isoforms of recombinant apo-aequorin**

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Anion-exchange (MonoQ) column chromatography of recombinant apo-aequorin carried out in the presence of Ca\(^{2+}\) revealed the presence of two isoforms of apo-aequorin, designated A and B. The N-terminal amino acid sequences of the two isoforms were found to be identical. On native PAGE, the two isoforms moved as a doublet, with B moving slightly ahead of A. When treated with 2-mercaptoethanol and then subjected to native PAGE, isoform B moved at the same rate as A, indicating that isoform A is the reduced, and isoform B the oxidized, form of apo-aequorin. When isoform A was analysed
by electrospray ionization (ESI) mass spectrometry, two peaks were obtained, corresponding to one or three Ca\(^{2+}\) per molecule, whereas isoform B gave three peaks corresponding to one, two or eight Ca\(^{2+}\) per molecule. When isoform B was treated with EDTA and 2-mercaptoethanol- and then subjected to ESI mass spectrometry, Ca\(^{2+}\) was found to be absent. From these results, we conclude that both EDTA and 2-mercaptoethanol are necessary for the complete removal of Ca\(^{2+}\), as required in the regeneration of apo-aequorin into aequorin.

**Effects of various antifungal agents on reactive oxygen species generation by Candida albicans**

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It is known that Candida albicans can generate reactive oxygen species (ROS), and that some antifungal agents affect the ROS generation by C. albicans. The effects of seven antifungal agents [terbinafine (allylamine), amorolfine (morfoline), ciclopirox olamine (hydroxyypyridone) and four imidazoles antimycotics, including miconazole, bifonazole, sulconazole and clotrimazole] on the ROS generation by C. albicans were examined on a plate, using a fluorescence analyser. C. albicans strain K, the antifungal agents and 2,7′-dichlorofluorescein diacetate (DCFH–DA) as a fluorescent probe were put into a 96-well plate and incubated for 4 h at 37°C. To examine the participation of glucose metabolism on the ROS generation, glucose (10 mmol/L) was added to the separately-prepared 96-well plate.

The results of the ROS generation showed that: (a) terbinafine affected suppression; (b) amorolfine had little effect; (c) miconazole and other imidazoles affected enhancement; and (d) ciclopirox olamine affected extreme enhancement. Although in six antimycotics little effect was observed on glucose additive ROS generation when compared to the basic ROS generation mentioned above, only ciclopirox olamine affected suppression. These results suggested at least that the mechanism of ROS generation is affected differently by hydroxypyridone and the other antimycotics examined.

**Visualization of superoxide generated from colonies of Candida albicans**

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Chemiluminescent (CL) visualization of superoxide generated by Candida albicans was conducted. The wild-type strain K of C. albicans cultured overnight in liquid PYG medium at 37°C with shaking was diluted and 0.1 ml of dilutions containing 50 cells was spread on PYG agar plates. After incubation at 37°C for 1–5 days, reactive oxygen species (ROS) generation by colonies of C. albicans on the PYG agar plate was examined with a ultralow light image analyser equipped with a photon-counting CCD camera.

To visualize ROS generated by C. albicans, methyl-Cypridina-luciferin analogue (MCLA) as a CL probe and the herbicide paraquat (PO) as a respiration-dependent ROS generator were used. After taking photographs of colonies under light, a mixture of 0.1 mol/L PO and 0.05 mmol/L MCLA (1:1) was gently dropped onto the colonies. To examine the effects of antioxidants, SOD or l-cysteine was added to the PO–MCLA mixture. The MCLA-dependent CL due to ROS generated by the colonies was recorded for 5 min in a light-tight box.

CL from the colonies was observed at 1 day after incubation and whole of the colonies were weakly luminous. In parallel with the increase in the colony size after incubation for 3 and 5 days, the marginal regions of the colony were strongly luminous. These results indicate that Candida colonies expand by division of metabolically active cells in the marginal regions, leaving aged cells in the central regions. CL from the colonies was almost completely extinguished by the addition of SOD and/or l-cysteine. In addition, we also achieved CL visualization of ROS generated by hyphal-form cells of C. albicans in a 96-well plate. To our knowledge, the present report is the first CL visualization of ROS including superoxide generated by C. albicans.

**Change of colour and decay rate by microenvironments for base-induced chemiluminescence of dioxetanes bearing a biaryl moiety**

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One marvellous phenomenon in luminous organisms is the colour change observed in the bioluminescence of beetles, in which luciferases use the same luciferin as a precursor of the emitter to display light ranging in colour from green to red. Many efforts, including an excellent study using luciferase mutants, reported very recently, have been made to elucidate how the colour is modulated by microenvironments in relation to the luciferase structures. On the other hand, there has been little known of the colour change of chemiluminescence derived from CT-induced decomposition of dioxetane, although this type of reaction is responsible for the bioluminescence of beetles, in which the participation of a high-energy dioxetanone intermediate, a family of dioxetane, is believed to occur. We report here a first example of the dioxetane-based chemiluminescence changing its colour of emission, depending on the base used for triggering; a dioxetane bearing an aryl-substituted phenolic moiety displays light ranging from orange to crimson near the infrared region, while another dioxetane emits light with two peaks in its spectrum. In addition to the colour modulation, we report also chemiluminescence of an optically active dioxetane substituted with a biaryl in a triggering system with an optically active base.
Can hydrogen peroxide be generated from natural antimicrobial materials?

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Recently, it was reported that tea catechin generated hydrogen peroxide continuously in solution and the antimicrobial activities of tea catechin were higher than that of hydrogen peroxide in the same molecule concentration (1). There are many kinds of natural antibacterial materials beside tea catechin, and we assumed that these materials might also generate hydrogen peroxide as well as tea catechin. To confirm this hypothesis, we investigated the generation of hydrogen peroxide from a natural plant extract that showed bacteriocidal activities, using the peroxoxylate chemiluminescent reaction. As a result, a number of materials showed hydrogen peroxide generation and several plants exhibited higher generation of hydrogen peroxide than tea catechin (e.g. Origanum vulgare, Mosla chinesis, Melaleuca alternifolia, Pimenta officinalis, Magnolia officinalis). Since these plants do not include very much catechin, the mechanism of hydrogen peroxide generation in these plants may differ from that in tea catechin. In addition, we investigated the correlation between the antibacterial spectrum and hydrogen peroxide generation in these plant extracts. Consequently, the materials, which generate much hydrogen peroxide, showed antimicrobial effects against both Gram-positive and Gram-negative bacteria. It was concluded that several antimicrobial materials were able to generate hydrogen peroxide, and this generation was considered one of the important mechanisms to exert the antibacterial effect of natural materials.

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‘BIOLUMBASE’—the electronic collection of bioluminescent organisms

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Wide application of bioluminescence in medicine and ecology demands the ordering of information about structure and functioning of bioluminescent systems in native and transgenic microorganisms. The electronic collection (EC) of bioluminescent organisms, ‘BIOLUMBASE’, has been created to collect all information on these problems. To develop it, we worked out respective database and client software. Two main sections of ‘BIOLUMBASE’, ‘natural luminous bacteria’ and ‘transgenic luminous bacteria’, contain textual, formalized and graphic information about molecular-genetic, morphological, physiological–biochemical and other characteristics of native and genetically modified luminous microorganisms, their bioluminescent systems, and regulation of the lux operon expression (near 280 characteristics). Users can receive not only catalogue information about strains, but also information about properties, functions, application, bibliography references, to make search of strains on any set of attributes. The database will collect all information that can be useful in the decision of different tasks of microbial ecology and biotechnology where the luminous bacteria, luminescent systems selected from them and lux genes cloned in others organisms are used.

The website of the electronic collection (http://www.ibp.ru/collection/default.htm; http://lux.ibp.ru), which allows publishing the collection’s data on the Internet, has been worked out. Additionally, information about collection strains of CCIBSO WDCM836 has been published in the consolidated Catalogue of microorganisms of Russian collections (http://www.vkm.ru/).

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Luminescent behaviour in the New Zealand glowworm, Arachnocampa luminosa (Insecta; Diptera; Mycetophilidae)

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The larvae of the mycetophilid Arachnocampa luminosa, commonly known as glowworms, emit a nearly continuous blue light. The larvae occupy a nest made of thin silk and produce numerous vertical silk threads that are covered with droplets of sticky mucus and hang from the ceiling of the limestone cave. With their light, the glowworms lure small insects from subterranean waters into their mucus-coated ‘fishing lines’. Until now there has been no study of any possible rhythmicity in the glowworms’ luminescence, under either field or laboratory settings. We examined the glowworms’ luminescence behaviour in the field to determine whether the emission of luminescence followed distinct on and off patterns. The glowworms’ luminescence behaviour was recorded with a highly sensitive VTR camera in Waitomo Cave, New Zealand. We found that in daytime in the cave almost all the individuals monitored continued to emit light for at least 7282 s. The percentage of individuals luminescing, without ceasing light emission even once, was greater than 80%. On the other hand, individuals that we observed at night in the cave continued to emit light continuously for about 5960 s. The percentage of such individuals was 35%. There are more individuals at nighttime than during the day that stop emitting light or emit light only weakly. Pauses in luminescence lasted for about 1500 s (25 min) both at nighttime and during the day. It was predictable that if an individual ceased to produce light, that individual would usually emit light again within 1500 s. We suggest that the glowworm has adapted its luminescence activity to the activity maximum of its prey, because emitting light is indispensable to attract prey. Why individual glowworms in the cave more often stop emitting light at night than during the day is, however, not entirely clear at the moment, but if it can
be shown that aquatic insects emerge from subterranean waters in greater numbers during the day, it would confirm our suggestion.

Novel method for visualization of protein–protein interactions in living mammalian cells using red fluorescent protein, DsRed

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Fluorescent proteins show bright fluorescence in the absence of exogenous substrates and co-factors. They are therefore very useful for monitoring gene expression, localization of proteins and so on, even in living cells, and are used for many purposes in the various fields of bioscience.

The red fluorescent protein DsRed from Discosoma coral undergoes oligomerization as an essential and highly rate-limiting step of its fluorescence maturation. While trying to overcome this apparent drawback, we found that DsRed is rapidly degraded through the ubiquitin–proteasome pathway in mammalian cells, but its tandem-linked dimer can escape this proteolysis to show markedly enhanced fluorescence. Taking advantage of this dimerization-dependent stabilization of DsRed, we have developed a new versatile two-hybrid vector system that can directly track protein–protein interactions in living cells by using conventional types of flow cytometers and fluorescent microscopes. This RFP-using bimolecular fluorescence yielding (RUBY) system has been successfully utilized to visualize the intracellular assembling and localization of two known heteromeric protein pairs, Runx2–PEBP2β and p65RelA–IxBα.

Furthermore, we have developed new variants of DsRed1 and established a multicolour system to detect alternative protein–protein interactions among three proteins in living cells.

Chemiluminescence involving phosphorus chemistry. Phospha-1,2-dioxetanes as the most likely high-energy intermediates in autoxidation of phosphonate carbanions

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The phospha-1,2-dioxetanes are the most likely intermediates in autoxidation of the phosphonate carbanions, in which chemiluminescence (CL) can be detected if suitable substrates are employed, because such intermediates are the structural analogues of well-known chemiluminescent 1,2-dioxetanes. Autoxidation of phosphonate carbanions derived from several 9-phosphorylacridanides in aerated aprotic solvents provided CL along with the formation of fluorescent acridones. Detection of the large difference in the CL efficiencies between N-methylacridan and N-hydroacridan allows us to apply a CIEEL process, as established in the firefly luciferin system. The CL quantum yields, as well as the time course of the light emission, depended highly on the phosphoryl substituents, which were connected with those found in the Wittig-type olefination reactions. Since the structure of the phosphoryl group is known to affect the rate of oxaphosphatane ring formation in the Wittig-type reaction, a similar effect observed in the present CL reactions can be linked together, which suggests that the most likely intermediates are the phospha-1,2-dioxetanes. On the other hand, energy-transfer CL to the externally added fluorescers was also observed in autoxidation of the phosphonate carbanions, giving no fluorescent carbonyl products. Consequently, CL involving phosphorus chemistry is not a phenomenon peculiar to the 9-phosphorylacridanides but is a general event for the phosphonate carbanions if the conditions are adjusted.

New methods for development of FRET-based biosensors with expanded dynamic range

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Green fluorescent protein (GFP) from the bioluminescent jellyfish Aequorea victoria and its colour variants have revolutionized our ability to uncover the complicated details of protein dynamics and gene activation. In addition, combination of GFPs with a fluorescence resonance energy transfer (FRET) technique allows us to develop genetically-encoded fluorescent biosensors that enable visualization of localized molecular events in their natural environment, within a living cell in real time and space. To date, increasing number of sensor that report concentrations of second messenger molecules and activation of signalling components have been developed and successfully used in various cell types. While most indicators have cyan- and yellow-emitting fluorescent proteins (CFP and YFP) as FRET donor and acceptor, respectively, their poor dynamic range (100% or less) often prevents detection of subtle but significant signals.

Here, by using new construction methods that allow rigorous alteration in relative position between donor and acceptor GFPs in the sensor molecules, we developed FRET-based Ca2+ and active caspase-3 sensors that show much bigger change in the ratio of YFP:CFP, in accordance with Ca2+ concentration (600% dynamic range) and caspase-3 activation (900% dynamic range), respectively. The new indicators enable visualization of the subcellular dynamics of Ca2+ and caspase-3 activation, with better spatial and temporal resolutions than before. Our study provides an important guide for the development and improvement of indicators using GFP-based FRET.

Development of fluorescence probes for biological applications, based on photoinduced electron transfer

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Fluorescence probes are essential tools for biological imaging, affording high sensitivity, real-time detection and simple measurement. Fluorescein is known to have a high quantum yield of fluorescence in aqueous solution and to be excitable at long wavelength light, and is a fluorophore widely used for fluorescence probes. Recently, we have found that the fluorescence properties of fluorescein derivatives can be controlled by intramolecular photoinduced electron transfer (PeT). We considered it appropriate to divide the fluorescein structure into two parts, i.e. the benzene moiety as the PeT donor and the xanthene moiety as the fluorophore. We have synthesized various kinds of fluorescein derivatives and examined the relationship between their fluorescence properties and the oxidation potentials of their benzene moieties. It was concluded that the fluorescence properties of fluorescein derivatives are controlled by PeT process from the benzene moiety to the xanthene moiety. The results obtained provided the basis for a practical strategy for rational design of functional fluorescence probes to detect certain biomolecules, such as NO, Zn$^{2+}$ and highly reactive oxygen species (hROS). All these probes were almost non-fluorescent before reaction or binding with target molecules, and highly fluorescent after the reaction or binding.

The PeT-dependent fluorescence off–on switching mechanism has also been applied to other fluorophores, such as BODIPY, and novel fluorescence probes for nitric oxide (NO) have been developed based on BODIPY structure.

Our recent findings on the PeT mechanism and its application in the development of useful fluorescence probes as biosensors are reported.

**A novel tricolour reporter assay system for simultaneous monitoring of multiple gene expressions**

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Reporter assay systems using firefly and *Renilla* luciferases are routinely employed as conventional and powerful tools for monitoring gene expression in many organisms, because they are highly sensitive and strictly quantitative. Typically, firefly luciferase (yellow-green emission), is used to monitor gene expression, while *Renilla* luciferase (blue), is used as an internal control (dual reporter assay system). Even with this current system, however, more than one gene expression cannot be monitored simultaneously, because one of two luciferases must be used as an internal control to minimize experimental variability. Additional luciferases and a novel measuring technique are therefore required for simultaneous monitoring. A reporter assay system to monitor multiple gene expressions and/or interactions simultaneously is greatly needed to investigate cascades of transcriptional regulations, which form the molecular basis of various biological functions. We have therefore developed a tricolour reporter assay system, in which two gene expressions can be monitored simultaneously from different luciferase emitters. The system consists of green- and red-emitting *Phrixothrix* luciferases as dual reporters, and blue-emitting *Renilla* luciferase as an internal control. The activity of the green and red luciferases can be measured simultaneously using an optical filter. We successfully applied this technique to the analysis of the molecular mechanism of the mammalian circadian clock, and directly compared the effects of the clock gene products on the enhancer elements of *Bmal1* and *Per1* promoters. Here we present a technique allowing direct analyses of two synergistic gene expressions in the same cell population.

**HPLC with fluorescence detection of morphine in rat plasma using 4-(4,5-diphenyl-1H-imidazol-2-yl) benzoyl chloride as a label**

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A highly sensitive method has been developed for the determination of morphine (MOR) in rat plasma by using derivatization with 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl), a solid-phase extraction, a reversed phase column and a fluorescence detection. Cyclazocine was used as an internal standard. Derivatization could be achieved under mild conditions (e.g. 10 min at room temperature). To remove excess DIB-Cl, a solid-phase extraction with an ODS cartridge was used and the obtained recovery for MOR spiked to plasma sample was 92% (n = 4). DIB-MOR was separated within 40 min by an ODS column and a step-wise elution programme with acetonitrile and 0.1 mol/L acetate buffer (pH 5.4). The fluorescence of DIB-MOR at 486 nm with excitation at 355 nm was monitored. A calibration curve of MOR spiked to a rat plasma sample showed a good linearity (r = 0.998). The detection limit of MOR in rat plasma at signal/noise ratio of 3 was very low (0.09 ng/mL). Both intra- and inter-day precision of the proposed method was also satisfactory (less than 9.4% RSD, n = 4). The applicability of the proposed method was confirmed to determine MOR in plasma after a single administration of MOR (2.5 mg/kg, intraperitoneally) to a male Wistar rat. The MOR in plasma could be monitored at 240 min after administration. Pharmacokinetic parameters of MOR obtained from the concentration–time profile were 610 ± 212 ng/mL for t$_{1/2}$, 62 ± 8 min for T$_{1/2}$ and 52.9 ± 15.5 µg/mL/min for AUC$_{inf}$.

**Photosensitive luminol-releasing compounds**

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The derivatives of luminol are used for the simple and highly sensitive chemiluminescence (CL) assays of DNA and enzyme activity. Luminol produces 3-aminophthalic acid and emits light in the presence of peroxide in an alkaline medium.
Photosensitive compounds, which have a 2-nitrobenzyl group in their structure, have been utilized for developing novel caged compounds, such as a caged ATP. E. H. White reported that the luminal derivative, which has a methyl group at the carbonyl oxygen of luminal, was non-CL. Thus, we synthesized luminal-O-2-nitrobenzylate (I), which has a 2-nitrobenzyl group at the carbonyl oxygen of luminal. We found that I released luminal upon light irradiation at 366 nm, and used I for light power measurements. The released luminal emitted light in the presence of NaOH and H2O2. In the concentration range 0.01–1 µmol/L, the CL intensities of the released luminal increased with the increasing concentration of I with a light irradiation time of 5 min. I (1 µmol/L in DMF) was photoirradiated for 10 s with 366 nm light, using an ultrahigh-pressure mercury lamp. The CL intensity of I after the light irradiation linearly increased with the light power in the range of 450–650 mW/cm². These results indicated that I was useful as a photosensitive CL compound. Photosensitive CL compounds of the acridinium ester, lucigenin and lophine have not been reported.

More recently, we synthesized luminal-O-4,5-dimethoxy-2-nitrobenzylate (II), and confirmed that II released luminal upon light irradiation. The light power measurement with II is also discussed.

Fluorescence and chemiluminescence characteristics of bisindoles

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Indole derivatives have been used for fluorescence (FL), chemiluminescence (CL) and bioluminescence assays. The FL and CL characteristics of indole derivatives were determined. However, indole derivatives have not been used frequently compared to typical FL and CL reagents, such as dansyl chloride, fluorescein, luminal and acridinium esters, from the standpoint of emission wavelength and intensity. For the selective and highly sensitive FL and CL assays, indole derivatives, which have a long emission wavelength and lasting CL, are needed.

We observed changes in the FL and CL of the indole derivatives by changing the number of indole molecules. In this study, we designed bisindoles which have two indole molecules in the structure, such as 1,2-bis(1H-indole-3-yl)oxaoethyl)ethylenediamine (I) and 3,4-bis(3-indolyl)-1H-pyrrole-2,5-dione (II) and then synthesized them. The FL of indole, I and II were measured at the concentration of 10 µmol/L, in dimethylformamide (DMF). Their excitation (ex) and emission (em) wavelengths were as follows: indole (ex: 272 nm, em: 326 nm), I (ex: 325 nm, em: 487 nm) and II (ex: 360 nm, em: 572 nm). The emission wavelengths of I and II were apparently long compared to that of indole, and the stork shifts of I and II were 162 and 212 nm, respectively. The FL intensity of II linearly increased with the increasing concentration of II in the range 0.1–10 µmol/L. II should be a useful reagent for selective FL assays. The CL characteristics of I and II in DMF are also needed.

Synthesis and chemiluminescence of novel π-conjugated bisimidazopyrazinone derivatives

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The imidazo[1,2-a]pyrazin-3(7H)-one ring (imidazopyrazinone) makes a core structure of the luminescent substrates of the jellyfish Aequorea and the crustacean Cypridina. Imidazopyrazinone derivatives have bio- and chemiluminescent abilities by a reaction with molecular oxygen. In addition, we found that imidazopyrazinone derivatives had a remarkable spectroscopic property in which their electronic absorptions and fluorescence emissions were observed in the visible region on account of the unique π-electronic structure of the imidazopyrazinone ring system. In this study, we design π-conjugated bisimidazopyrazinone derivatives, which are expected to have a two-step chemiluminescence ability, because their two imidazopyrazinone rings work as the luminescent sites. Each step of the two-step chemiluminescence process would show their individual characteristics, including a luminescence wavelength and an efficiency. On the basis of this molecular design, we succeeded in syntheses of novel π-conjugated bisimidazopyrazinone derivatives, in which two imidazopyrazinone rings were connected at the 2-, 6- and 8-positions. We report here the chemiluminescent properties of these novel bisimidazopyrazinone derivatives and discuss the characteristics and the molecular process of their chemiluminescence.

Isoluminol chemiluminescence detection of reducing sample using the ligand exchanging effect

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A detection method for reductant (sample) using Co(III) complex using the isoluminol-H2O2 system was developed. The methods based on the rate of ligand exchanging of the Co(III) complex. Many papers reported that the rate was very low compared with the rate of the Co(II) complex. It was confirmed that the Werner’s type Co(III) complex, such as [Co(III)EDTA]−, [Co(III)(NH3)]3++, etc., could be used for the system.

The resulting solution prepared with the reaction each of these complexes and reductants, such as SO32−, hydroxylamine, etc., enhanced the CL. The CL enhancement was ascribed to the Co(II) released from the Co(II) complex generated from the reaction of Co(III) complex and reductant, since the rate of ligand exchange of Co(II) complex is high. The difference in the rate of ligand exchange of Co(III) complexes and that of Co(II) complexes has been explained based on the ligand-field theory. A d-orbital of Co ion configurated by ligand is split to eg-level and t2g-level. The split scale is decided by the
Rapid and simultaneous bioluminescent assay using of aequorin and firefly luciferase

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We have developed a highly sensitive simultaneous bioluminescent assay of firefly luciferase and aequorin, and applied this assay to a tandem bioluminescent enzyme immunoassay (BL-EIA). The luciferin–luciferase reaction is specific and sensitive for the determination of ATP; therefore, this reaction has been used, e.g. for hygiene monitoring. Aequorin binds specifically to Ca2+ and then emits blue light; thus, aequorin is a valuable technique for studying intercellular Ca2+. We developed simultaneous assay of aequorin and luciferase by these different bioluminescence reactions. In the proposed assay, each of 10 µL aequorin and luciferase solution were added to a microtitre plate, and 100 µL Ca2+ solution was added, and then the bioluminescent intensity was integrated for 1 s immediately. Then, 100 µL reagent solution containing ATP, luciferin and Mg2+ was added to the same wells. The bioluminescent intensity obtained in the luciferin–luciferase reaction was integrated for 1 s after a delay of 2 s. The detection limits (at blank + 3SD) of aequorin and luciferase were 7.6 × 10−20 and 3.16 × 10−18 mol/assay, respectively. Therefore, this proposed assay provides high-throughput and sensitive detection. Furthermore, we have applied this assay to tandem immunoassay for prostate-specific antigen and prostatic acid phosphatase.

New aspects of the molecular mechanism of Latia bioluminescence

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The limpet-like snail Latia neritoides is only found in clear and shallow streams of North Island in New Zealand, and is the only luminous animal that lives its whole life cycle in fresh water. On mechanical stimulation, the snails secrete mucus with greenish light (536 nm) is emitted. The bioluminescence system exhibits a typical luciferin–luciferase reaction. Shimomura established the structure of the luciferin having a characteristic, enol formate functionality, and proposed that the bioluminescence requires the luciferin, the luciferase (178 000 Da), a co-factor so-called ‘purple protein’ (38 000 Da) (red fluorescent protein), and molecular oxygen. However, the precise bio-oxidation mechanism of the luciferin and the detailed molecular bases for the light-emitting process is still unclear. For understanding the Latia bioluminescence reaction, we studied the relationship between the structure and bioluminescence activity of the luciferin analogues. Luciferin possesses the characteristic structural profiles: the 2,6,6-trimethyl cyclohexene ring, similar to the vision pigment retinal, and the enol formate functionality. On saturation of the cyclohexene ring into the corresponding cyclohexane one, or on replacement of the ring system to the phenyl group, the bioluminescence activity disappeared, implying that the 2,6,6-trimethylcyclohexene ring moiety is important for substrate recognition with the luciferase. While the corresponding enol ether analogue had no bioluminescence activity, the corresponding enol acetate and enol benzoate analogues exhibited substantial light production. In addition, the rate of light production was delayed when the enol acetate and enol benzoate analogues were used. These results indicate that the formate group in the luciferin is not the essential one for bioluminescence reaction, and that the hydrolysis step of the enol ester at the active site of the luciferase may be the rate-determining step of the light production reaction. Further studies on the relationship between structure and luciferin activity are currently under investigation.

Repetitive assay for enhanced detection of immobilized horseradish peroxidase by imidazole chemiluminescence coupled to the technique of on-line regeneration of inactivated peroxidase

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A method for enhanced detection of horseradish peroxidase (HRP) immobilized in a chip by microflow injection–imidazole chemiluminescence is presented in this study. The imidazole chemiluminescence consisted of imidazole, HRP and hydrogen peroxide (H2O2) in Tricine buffer (pH 9.4), and was employed for assaying HRP activity. The principle of the method was as follows. HRP immobilized in a chip set in a cooled CCD-chemiluminescence monitor reacts with H2O2, resulting in light emission and HRP inactivation. The inactivated HRP is then reactivated on-line with alkaline imidazole solution to react with H2O2 again. This allowed the same HRP in a chip to keep on reacting with H2O2 injected repetitively at intervals. The final goal of the study was to accumulate the lights from the same HRP with the chemiluminometer for enhanced detection of HRP.

Firefly luciferase and the Drosophila CG6178 gene product are fatty acyl-CoA synthetases

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Recently we found that firefly luciferase is a bifunctional enzyme, showing both luminescence activity and synthetic activity of fatty acyl-CoA from various long-chain fatty acids in the presence of ATP, Mg2+ and CoA (1). In the Drosophila melanogaster genome, the most homologous gene to firefly luciferase gene is CG6178 and was not characterized. To understand the function of the CG6178 gene product, the cDNA was cloned and expressed in E. coli cells. The purified CG6178 gene product showed fatty acyl-CoA synthetase activity similar to that of firefly luciferase. However, the luminescence activity of the CG6178 gene product was not detected in the presence of firefly luciferin, ATP, Mg2+ and CoA. Luciferyl adenylate was not formed during this reaction. These results and phylogenetic analyses strongly suggest that firefly luciferase may be evolved from the fatty acyl-CoA synthetase in an insect lineage.

Reference

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A novel method to enhance the subcutaneous detection of bioluminescence in the facultative anaerobe Streptococcus pyogenes by DMSO-assisted transdermal oxygen delivery

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Streptococcus pyogenes is a common Gram-positive organism that can cause severe systemic and invasive disease. Xenogen developed a bioluminescent strain of S. pyogenes harbouring the LuxABCDE construct; however, under anaerobic conditions, e.g. subcutaneous abscesses, the oxygen-dependent bioluminescence is reduced. To enhance subcutaneous detection of bacterial growth, we developed a novel method to deliver oxygen to subcutaneous sites. 100 µL BHI broth or blood containing 2.6 × 10⁶ S. pyogenes CFUs was injected subcutaneously into mice, following depilation, and allowed to grow for up to 24 h. At various time points after inoculation, the subcutaneous sites were examined for bioluminescence using the Hamamatsu Low Light Imaging System and Metamorph imaging software. Various concentrations of DMSO and H₂O₂, separately and in combination, were applied over the inoculation sites for bioluminescence detection.

The administration of 30% DMSO combined with 0.6% H₂O₂ enhanced bioluminescence nearly 10-fold. The delivery of oxygen was still evident 3 h after DMSO and H₂O₂ administration. H₂O₂ alone (0.6%) transiently increased bioluminescence four-fold but appeared to be toxic, as evidenced by a subsequent decrease in bioluminescence and a poor response to DMSO + H₂O₂ application after further incubation (3 h). DMSO alone (30%) had no effect on bioluminescence detection.

These findings demonstrate that DMSO + H₂O₂ is a novel and useful in vivo approach to enhance the oxygen-dependent bioluminescence of the LuxABCDE luciferase product in an anaerobic environment. This technique will increase sensitivity in animal models, allowing more precise monitoring of infection by this bioluminescent strain of S. pyogenes.

Comparative study of ROS scavengers in terms of quenching MCLA-dependent chemiluminescence

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Scavengers of reactive oxygen species (ROS) were compared in terms of their quenching effect on MCLA-dependent chemiluminescence. The substances examined were ascorbic acid, glutathione, superoxide dismutase, α-tocopherol, β-carotene and electrochemically reduced water. As ROS, O₂·− was generated by O₂/hypoxanthine/xanthine oxidase or released from KO₂. ¹O₂ was generated from NDPO₂ and ‘OH was produced by H₂O₂/Fe²⁺. H₂O₂/myeroperoxidase/Cl⁻ also was examined. As a scavenger of O₂·−, ascorbic acid was more effective than glutathione, while the situation was reversed in the case of ¹O₂. α-Tocopherol was effective against ‘OH but not against O₂·− or ¹O₂. β-Carotene was a good scavenger of O₂·− and ¹O₂. Electrochemically-reduced water effectively suppressed MCLA-dependent chemiluminescence caused by O₂·−, ¹O₂ and ‘OH, but rather enhanced the chemiluminescence by H₂O₂/myeroperoxidase/Cl⁻. Further, it quenched luminol-dependent chemiluminescence by H₂O₂/myeroperoxidase/Cl⁻. Distilled water saturated with H₂ did not affect the intensity of chemiluminescence caused by any kind of ROS. In the analysis of electrochemically-reduced water, 2.8 p.p.b. Pt was detected. Hence, the effect of addition of colloidal Pt to the chemiluminescence generation reaction solution was examined. Colloidal Pt did not affect chemiluminescence intensity by itself, but clearly reduced chemiluminescence intensity when used in combination with H₂ saturation. Therefore, it was concluded that scavenging ROS by electrochemically-reduced water was due to the Pt-catalysed reduction of ROS by dissolved H₂. It should be noted that electrochemically-reduced water enhanced chemiluminescence intensity when H₂O₂ was present in the reaction system.

Solvent effect on the NMR and absorption spectra of firefly luciferin in tetrahydrofuran

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To clarify the light emission mechanism of firefly luciferin (Ln) by the experimental and the theoretical method, we have studied the elementary reaction path for firefly Ln chemiluminescence without the enzyme luciferase. It had been shown previously that chemiluminescence can be observed on adding molecular oxygen and potassium t-butoxide (t-BuOK) to Ln in deoxidized dimethyl sulphoxide (DMSO) through intermediates M440 and M420.

To study the solvent effect of tetrahydrofuran (THF) on Ln chemiluminescence, we reproduced the $^1$H-NMR and absorption spectra of Ln in THF to compare with its NMR and absorption peaks calculated by the \textit{ab initio} method at the B3LYP/6-311+G(2d,p) level.

In this report, we discuss the structural and the physicochemical characteristics of Ln interacting with THF.

### Specific detection of singlet oxygen using vinylpyrene derivatives as chemiluminescent probe

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In this study, we developed a novel chemiluminescent probe, trans-1-(2-methoxvinyl)pyren (t-MSVP), for singlet oxygen ($^1$O$_2$) based on trans-1-(2-methoxvinyl)pyren (t-MVP), previously synthesized and characterized. It is considered that by reacting with electrophilic $^1$O$_2$, both probes transform into the corresponding dioxetane intermediates, and then their cleavages induce an excited state of 1-pyrene carboxyaldehyde, emitting light by the intramolecular CIEEL mechanism. To compare with t-MVP, t-MSVP was characterized with regard to its analytical and chemiluminescent properties. As a result, t-MSVP provided a limit of detection 100 times lower than t-MVP. This comes from fact that since a dioxetane with higher electron density is more thermostable, the dioxetane intermediate from t-MSVP with a -SMe group would more readily cleave than that from t-MVP with a -OMe group. Furthermore, the specificity for $^1$O$_2$ was as good as t-MVP, the signals for H$_2$O$_2$, O$_2$ and OH being 0.0004%, 0.1% and 10%, respectively, of that for $^1$O$_2$.

### Preparation of a crown-ether-modified isoluminol derivative and its chemiluminescence properties in an organic medium

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The isoluminol derivative of the title, having an aza-15-crown-5-ether function 1, has been prepared through two-step synthesis. 4-Amino-N-methylphthalimide was treated with penta(ethylene glycol) ditosylate in the presence of sodium hydride to construct an aza-crown ether on its amino group. The resulting caged phthalimide then reacted with hydrazine to afford the desired isoluminol 1, which is the first luminol analogue possessing an aza-crown ionophore.

The crowned isoluminol 1 displayed very weak chemiluminescence (CL) in acetonitrile in the presence of hydrogen peroxide and tetrabutyl ammonium hydroxide (TBAOH). However, addition of alkaline metal salts (as iodide) to this mixture triggered off intense blue CL emission. The CL emission was short-lived and ceased within a few seconds at room temperature. In aqueous solution, however, such an intense CL emission was not observed upon addition of alkaline metal iodide in the presence of TBAOH and hydrogen peroxide. The maximum of the CL spectrum was affected little by the alkaline metal cation used (Li+, Na+, K+, 450–470 nm), whereas, the intensity...
of the CL emission changed depending on the cation; the relative intensity of the CL emission increased for Li⁺ < Na⁺ < K⁺.

High-pressure effects on green fluorescent protein (GFP) chromophore models
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Two types of green fluorescent protein (GFP) chromophore models (GFP models) were synthesized and characterized. It is well known that a solution of GFP model in a fluid solvent such as ethanol does not emit fluorescent light; however, noticeable fluorescence emission is available in a viscous solvent such as glycerol. We studied the high-pressure effect (1–4500 bar) on fluorescence quantum yield and geometrical isomerization of the ground state and S1 state of the models in glycerol. Increase of pressure dramatically increased the fluorescence quantum yield. Increase of pressure retarded rates of geometrical isomerizations of the GFP chromophore models of the ground state and the S1 state, from which activation volumes for the isomerization were determined. The activation volume for the S1 state geometrical isomerization was found to be smaller than that for the ground state GFP chromophore models. We discuss behaviour of S1 state GFP chromophore models in solutions.

In vivo observation the super-weak luminescence of single living cotton cells
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In first super-weak luminescence of separate living cotton hair cells was investigated for the cotton plant varieties Tashkent-I, C-4727, Gossypium hirsutum, Turfan guza G. herbaceum, C-6063 G. barbadense L. and the wild form G. raimondii. This phenomenon was observed under optical microscope Neophot-2, using a so-called replica–reprint technique for preparation living cotton cells. For this purpose, a polymer solution (gelatin) was used for visualization of track luminescence in the bulk of deposited polymer film on cotton cell hairs. It was shown that luminescence was not generated for lifeless and mature plant cells but maximal luminescence was detected from the apical parts of cotton hairs in the early growth stage (4–6 days after flowering). We have shown that luminescence has a cone-like form a different varieties of cotton plant. This fact indicates a focusing of radiation connected with the morphological and structural features of the apical parts of cotton cell hairs at early stages of their evolution. The electromagnetic nature of cotton cell luminescence has been shown by experiments using photoelectric multiplexer (PEM) sensing of ultraviolet radiation. At insertion in darkness of a cotton seed-bud on a PEM window, the dark current increased 6–8%. Actually, radiation flux from a developing cotton hair is more high-powered, as through a PEM window only a small part of the radiation was fixed. Therefore, on the basis of the large experimental information obtained by many investigators on miscellaneous biological objects, it is possible to say that the luminescence of cotton hairs is high-energy ultra-violet irradiation.

The bacterial bioluminescence as a protection from oxidative stress
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Defence mechanism of bacterial luminescence against oxidative stress is proposed. Some species of luminescent bacteria were examined for superoxide anion (O₂⁻) production and superoxide dismutase and catalase activities. The extracellular generation of O₂⁻ were discovered through logarithmic phase of bacterial growth. Luciferase activity increases with increasing light intensity. The specific activities of superoxide dismutase and catalase were increased as an adaptive response of bacteria under oxidative stress. The Fe³⁺-ions induced the bioluminescence. The dark mutants lost their resistance to oxidative stress. Although the mechanism of O₂⁻ production is unknown, this phenomenon may be important for hosts colonization.

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Bioluminescent bioreporter integrated circuit sensing of the chemical and biological spacecraft environment
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Advance in lux-based bioluminescent bioreporter design and application have produced novel monitoring technologies for rapid (<30 min), sensitive (parts per billion) and specific detection of chemical and biological agents within complex environmental matrices. These bioreporter systems rely on lux gene fusions with characterized promoter elements responsive
to the agent(s) of interest for autonomous, real-time generation of bioluminescent signals. Typical application emphasis has been towards chemical contaminant detection and monitoring, but novel engineering schemes have produced bioluminescent bioreporters sensitive to microorganisms (i.e., bacterial pathogens) as well. In addition, bioreporters can be microelectronically interfaced with miniaturized integrated circuit luminometers, for wireless sensor deployment for pinpointing locations or time events of potential biohazardous concern. The low-power, low-mass, and low-maintenance characteristics of these bioluminescent bioreporter integrated circuit (BBIC) detectors provides extensive compatibility with current spacecraft monitoring needs. We present an overview of our current research efforts towards the establishment of multiarray BBICs for tailored sensing of the spacecraft environment.

The mating system and alternative mating strategies of a marine bioluminescent Caribbean ostracod

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In shallow reef areas in the Caribbean there is a wide array of complex and formalized light displays at dusk. Each consists of patterns of bioluminescence secreted into the water by small (<2 mm) myodocopid ostracods (Crustacea). These displays are species-specific, produced only by the males, and are used for courtship. Observations of a grassbed species of a new genus (Photeros sp.) at Southwater Caye, Belize, indicate that they are the first crustacean species in which all criteria for a classic lek mating system are satisfied. The development of new observational techniques has allowed us to observe the swimming and display behaviour of individual ostracods, both in the laboratory and in the field. We find that males are able to switch readily between producing luminescent displays and 'sneaking' on other males' displays as an alternative mating strategy, and do so multiple times during a single night of courtship. Our study will increase our understanding of the rapidity and plasticity of alternative mating strategies. This is one of the first in-depth behavioural studies of the complex signals of any bioluminescent marine invertebrate, and could yield invaluable insights into the evolution of behaviour of bioluminescent organisms in general, and of crustaceans in particular.

Luminescent probes for sensitive and specific optical microscope imaging

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The detection and localization at microscopic level of analytes present at very low concentrations in single cell and tissue samples is one of the most exciting challenges for bioanalytical chemistry. Fluorescence microscopy is commonly used for the sensitive localization of analytes in biological samples through immunohistochemical and in situ hybridization reactions with fluorescent-labelled probes. However, this technique suffers from two main drawbacks that reduce the sensitivity, i.e. the autofluorescence of the sample and the light scattering in the apparatus.

We previously demonstrated that chemiluminescent enzyme-labelled probes allow the sensitive localization of DNA sequences and antigens, due the high specificity of the chemiluminescent reactions and low background noise. Chemiluminescence also provides high spatial resolution and the possibility of quantifying the amount of analyte present on a given surface area.

Time-resolved fluorescence (TRF) microscopy is a promising alternative to conventional fluorescence microscopy that allows for elimination of the sample autofluorescence. Due to their peculiar photophysical properties, luminescent lanthanide chelates are the most suitable labels for this technique. Unfortunately, only a few, expensive, commercially available labels have been developed up to now. We have designed and synthesized new stable Eu³⁺ and Tb³⁺ lanthanide chelate labels suitable for binding to primary amino groups. These Eu³⁺ and Tb³⁺ complexes are characterized by long luminescence lifetimes (0.6 and 1.5 ms) and high emission quantum yields (0.08 and 0.30), thus being particularly suitable for TRF applications. Preliminary experiments on model samples proved the suitability of these labels for TRF imaging microscopy. Immunohistochemistry experiments are in progress, using either biospecific probes labelled with the chelate or biotinylated probes revealed by a complex between streptavidin and a labelled biotinylated polymer, in order to assess the potential of this new family of lanthanide labels for TRF imaging purposes.

Oxidative processes and catalytic properties of cytoplasmatic NAD-malate dehydrogenase in rat heart at norm and under ischaemia

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The increase of free-radical processes is a universal non-specific link relating the development of many ischaemia diseases. According to this hypothesis, some protective mechanisms begin functioning in the cell at earlier stages of ischaemia under hypoxic conditions. Malate metabolism, realized by malate dehydrogenase (NAD-MDH; EC 1.1.1.37), is considered to play an important role, determined by its participation in tricarboxylic acid, malate–oxaloacetate shunts or biochemical adaptation to hypoxia. The present work aims to understand the role of MDH in antioxidant mechanisms protecting the tissue during stress. The intensity of free-radical oxidation has been investigated by a biochemiluminescence method. It has shown that parameters such as light sum and maximum flare amplitude increase by 2.5 and 2.9 times in the myocardium cytoplasm of rat under ischaemia, when compared with the norm. These changes testify to the increase of free-radical oxidation in the cytoplasm.

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processes in the tissue under ischaemic damage. At the same time, the tangent of curve fall angle, $\tan \alpha_2$, and coefficient $K$ equal to ratio $I_{\text{max}}/S$, characterizing the antioxidative potential of the sample, increase by 52% and 16%, respectively, under ischaemia. The data suggest an activation of antioxidative systems controlling tissue free-radical processes. NAD-MDH has been purified from the cytoplasm of the rat myocardium under normal conditions and under ischaemia and the same catalytic properties analysed. The specific activity of NAD-MDH in the rat heart under ischaemia fell by 2.7 times. $K_m$ for oxaloacetate (0.040 ± 0.003 and 0.050 ± 0.004 mmol/L) and for NADH (0.050 ± 0.003 and 0.075 ± 0.004 mmol/L) has been determined for the enzyme from rat heart cytoplasm in normal conditions and under ischaemia, respectively. Thus, some peculiarities of MDH functioning under conditions of intense free-radical processes have been detected.

### Oxidative status and glucose-6-phosphate dehydrogenase (G6PDH) activities in rat liver and blood serum at norm, toxic hepatitis and melatonin treatment

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The intensity of oxidative and antioxidative processes plays a key role during the stress response. Tissue capacity to recover from the stress depends on its antioxidative potential and NADPH production by metabolic pathways. The intensity of free radical processes, measured through a biochemiluminescence method, and the activity of G6PDH (EC 1.1.1.49) in liver and blood serum from normal rats and animals with toxic hepatitis or under melatonin treatment, has been compared. This study aims to investigate the participation of antioxidative processes in the tissue response to stress. Chemiluminescence parameters, the light sum of slow flash ($S$) and the slow flash intensity ($I_{\text{max}}$), characterizing the intensity of free radical process increases in toxic hepatitis, were higher by 5.4- and 9.6-fold in blood serum and 1.8- and 1.7-fold in liver, respectively. Antioxidative activity, reflected by the tangent of curve fall angle, $\tan \alpha_2$, increases ~10 times in blood serum and ~1.5 times in liver, when compared with normal rat tissues. The data suggest a mobilization of the antioxidant system in response to oxidative processes during toxic hepatitis. Melatonin treatment of rats with toxic hepatitis seems to decrease free radical production and antioxidative processes in rat liver and blood. The $S$ value decreases ~2.4-fold in blood serum and ~1.6-fold in liver homogenate, when compared with rat tissues under toxic hepatitis. Similar variations in $I_{\text{max}}$ and $\tan \alpha_2$ are also observed. The increase of antioxidative activity appears to be accomplished by an increase of G6PDH activity, which takes place in both rat tissues, but is higher in liver than in blood and decreases during melatonin treatment of toxic hepatitis.

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6,8-Diarylimidazo[1,2-a]pyrazin-3(7H)-ones as potential chemiluminescent pH/superoxide double sensors

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Imidazo[1,2-a]pyrazin-3(7H)-ones possessing different para-substituted phenyl groups at the 6- and 8-positions were synthesized, and their superoxide-triggered chemiluminescence was investigated, using the hypoxanthine–xanthine oxidase system as the source of superoxide in the phosphate buffer, under various pH conditions. 2-Methyl-6-(4-methoxyphenyl)-8-(4-trifluoromethylphenyl)imidazo[1,2-a]pyrazin-3(7H)-one (1a) showed chemiluminescence at around 460 nm under neutral conditions. This luminescence was ascribed to the singlet-excited state of 2-acetamido-5-(4-methoxyphenyl)-3-(4-trifluoromethylphenyl)pyrazine (2a), the product of the chemiluminescent reaction of 1a with superoxide. On raising the pH of the buffer solution, the emission at 540 nm was increased, with decreasing emission at 460 nm. This largely bathochromic-shifted luminescence was produced from the singlet-excited amine anion of 2a, in which large energy stabilization with extended conjugation at the 8-position is supposed to cause the large shift in the emission maximum. Also, the electron-withdrawing trifluoromethyl group, which can stabilize the anion, would be responsible for the red-shifted luminescence. The other imidazopyrazinones, 6-(4-hydroxyphenyl)-8-(4-methoxyphenyl)-1b and 6-(4-dimethylaminophenyl)-derivatives (1c), showed no luminescence under the same conditions, probably because the specific interaction between the solvating water and the hydroxyl or dimethylamino group quenches their emission. The observed pH-dependent bimodal chemiluminescence of 1a may allow us to apply 1a for spontaneous detection of superoxide and the local pH in a cell or in a droplet.

### Rapid and on-site BOD sensing by luminous cells-immobilized chip

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A BOD monitoring system, based on a bio-chip containing immobilized luminous bacterium in µm-order holes that were arrayed and fabricated by micro-machine techniques, was developed. The acrylic chip (3 cm × 3 cm) comprises nine micro-holes (diameter, 700 µm or 1 mm; depth, 100 µm) arranged in a 3 × 3 array. Cells of the marine luminous bacterium, Photobacterium phosphoreum IFO 13896, which was grown at...
15°C for 15 h, were immobilized with 3% or 15% sodium alginate gel. BOD standard solutions or actual sample solution (approximately 50 µL) was dropped onto the cell-arrayed chip, and then the chip was incubated at 25°C for 30 min. After incubation, bioluminescence from each hole was grey-scaled and measured by a chemi-imager or newly developed on site-type monitoring system using a digital camera. BOD values less than 50 p.p.m. could be detected by the chip, in particular, linear relationship at the concentrations 0–50 p.p.m. could be observed when the luminous cells were immobilized with 3% sodium alginate gel. Furthermore, simultaneous detection of BOD values in various samples could be employed in a single chip. These results show that a monitoring system with biochips can achieve high-throughput and on-site BOD detection.

Development of a range of bioluminescent food-borne pathogens for assessing in situ heat inactivation and recovery of bacteria during heat treatment of foods

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Bacteria expressing lux genes were used as real-time, in situ reporters of the effects of food surface pasteurization processes, within the ‘Bugdeath’ programme for predicting microbial death during heat treatments on food. Food-borne pathogens were transformed with plasmids carrying lux genes, in order to cause minimum alteration of the bacterial phenotype. The plasmids all carry the luxCDABE genes from Photobacterium phosphorum, controlled by a constitutive promoter; however, different vectors were used to give stable lux gene expression in Escherichia coli O157, Salmonella typhimurium DT104 and Listeria monocytogenes Scott A. The bioluminescent reporter constructs were monitored in nutrient media over 24 h and showed good correlation between bioluminescence and growth. For assessing heat inactivation and recovery, S. typhimurium DT104 pGLITE was inoculated onto food surfaces at 10^5 cfu/cm² and exposed to dry heat treatments, raising the temperature of the food surface from 4°C to 90°C over 30–40 s and holding for 3 min. For wet heat treatments, steam was passed over the food surface for 1 min. Bioluminescence was monitored throughout heating, using a photon-counting camera. The bacterial regrowth, after heating, was monitored, positionally and in real time, by light output at room temperature for up to 24 h. Bioluminescence declined at a constant rate as the temperature increased above 32°C for all foods tested. It then remained below detection level throughout the period of heating. The 90°C dry heat treatment had only a limited cidal effect on S. typhimurium adhering to different food surfaces, causing 6–7 h of metabolic inhibition on vegetables and chicken. This period increased to 9–18 h for all other meat samples. Steam treatment prevented bacterial recovery for at least 24 h on all food surfaces tested. Bioluminescence monitoring indicates that steam is significantly more effective at preventing bacterial recovery than dry heating at a similar temperature.

Improved polymer-supported photosensitizers

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The photosensitized oxidation of compounds containing carbon–carbon double bonds is widely used for the preparation of 1,2-dioxetanes. Polymer-supported photosensitizers are particularly useful, due to the ease of their removal from the reaction mixture by filtration. Lumigen has developed novel polymer-supported photosensitizers, which consist of a cross-linked polymer backbone containing covalently bound ammonium or phosphonium groups and immobilized photosensitizers. The photosensitizers are bound either covalently or ionically to the polymer. These new polymer-supported photosensitizers are superior to existing polymer-supported and soluble photosensitizers in their ability to catalyse the photo-oxygenations of carbon–carbon double bonds. The ability of the polymers to swell in protic solvents allows for the efficient generation of singlet oxygen and accelerates the photooxygénation of ionic substrates. The reduced reaction times result in significant decreases in by-product formation. The particle size and amount of cross-linkage in the initial polymers affect the rate of photo-oxygenation.

Simultaneous measurement of fluorescence and chemiluminescence using neutrophil-like culture cells

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We have developed a novel method to measure fluorescence and chemiluminescence simultaneously in real time to investigate function of the cells. It is well known that the superoxide ion from neutrophils kills bacteria and viruses and plays an important role in biological defence. It is also well known that the calcium ion is a kind of intracellular mediator. However, details about the relationship between the superoxide ion and calcium ion are not clear. To reveal the relationship between them, the time-courses of calcium ion concentration and superoxide ion concentration from neutrophil-like cells were measured in real time. Calcium ion concentration in the cells was measured by a fluorescent calcium indicator, fluo-3(1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N',N'-tetraacetic acid), and superoxide ion concentration was measured by the chemiluminescent reagent CLA (2-methyl-6-phenyl-3,7-dihydroimidazo(1,2-)pyrazin-3-one).

When the sample containing neutrophil-like cells was irradiated with excitation light chopped, both fluorescence and chemiluminescence were detected. When the sample was not irradiated, only chemiluminescence was detected. Fluorescence is a difference in both fluorescence and chemiluminescence and only chemiluminescence. The chopping frequency is faster.
than cell response. Thus, the measurement of fluorescence and chemiluminescence are practically simultaneous.

We found out that the calcium ion concentration increased and then the superoxide ion concentration increased on addition of a stimulating agent such as f-MLP. We also found out that the neutrophil-like cell response was affected by additional reagents, such as metal ions.

Acknowledgements
Our study has been carried out through the Research and Development Programme for New Bio-industry Initiatives.

Modelling of the signal intensity in the various reaction chambers of the NO–O₃ chemiluminescence nitrogen oxides monitor to obtain higher sensitivity
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A NO–O₃ chemiluminescence nitrogen oxides monitor is used frequently for measuring nitrogen oxides. However, it is not possible to measure the nitrogen oxides (NOₓ) concentrations sufficiently using commercial NOₓ monitors, because the concentration of NOₓ is low at the clean air region. In this study, we discuss the change in signal intensity and thus the sensitivity of NOₓ monitors, by changing the mixing condition of sample gas and ozone using various types of reaction chambers, among which the formerly reported one is also included. We tried the following chambers: the sample and ozone gas were collided near a photomultiplier tube; the gases were flowed into the reaction chamber from wall of chamber radially; and so on. We constructed a model of the chemiluminescence of the NO–O₃ reaction in the reaction chamber used in the experiments. In the model, the space of the reaction chamber was divided into several parts, and the concentration of excited NOₓ and the intensity of illumination to detector were calculated in each of the divided parts. The conformity of this model was verified experimentally. Findings from this study, suggest that a stronger intensity is expected to obtain also in the best reaction chamber used in the experiment by changing the mixing conditions, e.g. the manner of gas flow in the reaction chamber.

Chagas assay using recombinant antigens on a fully automated chemiluminescence immunoassay analyser
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The Abbott PRISM® is a fully automated, chemiluminescence-based immunoassay analyser (ChLIA). It has been used to screen blood specimens for five different viral markers. A prototype assay is being developed on the PRISM analyser for the detection of Chagas’ disease (American trypanosomiasis). The disease, caused by the protozoan T. cruzi, is endemic to most regions of Latin America. Of the estimated 18 million infected people, approximately 50 000 die from this disease yearly. The estimated seroprevalence of the disease in US blood donor populations was as high as 0.48% and the trend is increasing with the increase in the Hispanic population.

The assay uses: (a) microparticles coated with rAgs of T. cruzi to capture antibodies to T. cruzi; (b) a 9-N-sulphopropyl acridinium derivative labelled mouse anti-human IgG to tag the captured human Ab on the microparticles; and (c) alkaline peroxide to trigger the chemiluminescence. Samples from patients with anti-T. cruzi antibodies gave a significantly higher signal compared to the normal control.

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 99.0% detection of confirmed T. cruzi-positive specimens (n = 203). Specificity was estimated to be 99.8% (n = 2000; RR = 4). Further optimization of the assay is in progress.

A prototype Abbott PRISM Chagas assay based on rAg and Chemiluminescence detection was demonstrated. The assay has the potential to be developed into a product for blood screening for the prevention of transmitting Chagas’ disease through blood transfusion.

Aequorin and GFP: an historical account
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Aequorin and the green fluorescent protein (GFP) were discovered at the same time from the luminous jellyfish Aequorea, which displays a bright ring of green light upon stimulation. The luminescence is caused by the calcium-sensitive photoprotein aequorin and the green fluorescent protein (GFP). These two proteins were isolated, purified and cloned. Currently, both the proteins are widely used as important research tools, aequorin as a calcium indicator and GFP as a marker protein. I started the study of Aequorea bioluminescence in 1961 with Dr Frank H. Johnson. The extraction of aequorin from the light organs was difficult because we did not know that calcium triggers the luminescence of aequorin. The extraction was accomplished only after abandoning the concept of the luciferin–luciferase (substrate–enzyme) reaction. Once aequorin was extracted in a solution, it was a simple work to discover the role of calcium in the luminescence of aequorin. Determining the structure of the luminophore of aequorin was a formidable task, because every treatment of aequorin caused an intramolecular reaction that triggers the self-destruction of the luminophore. With many years of effort, the structure of the luminophore was finally determined from a fragment of the luminophore, helped by a similarity to the Cypridina luminescence system that I had previously studied. Determination of the structure of the GFP chromophore was also accomplished with the aid of model compounds of Cypridina luciferin. The process of solving these problems and the advance in the understanding of aequorin and GFP are discussed.
ABSTRACTS

Modules in emerging fields: an overview of bioluminescence

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Connecticut College has received grants from the Howard Hughes Medical Institute and the W. M. Keck Foundation to help incorporate a wide variety of emerging fields of study into undergraduate curricula. This programme brings visiting scholars to campus to educate college professors and undergraduate students and encourage incorporation of these new areas of research into the classroom. We present here an overview of our laboratory’s module on bioluminescence, the emission of light by living organisms. Bioluminescence is widely distributed in nature, with 17 phyla and around 700 genera represented. Perhaps the most well-known example is the firefly, but there are examples of bioluminescent bacteria, fungi and marine organisms. The marine environment hosts the majority of bioluminescent organisms, including bioluminescent jellyfish, crustaceans, molluscs and dinoflagellates. The biological and biochemical diversity of this phenomenon, as well as some of the important bioanalytical applications using bioluminescence and fluorescence of related systems, is presented by a series of invited speakers: Professor J. Woodland Hastings, Harvard University; Dr Keith Ward, United States Department of Homeland Security; Dr Robert Hoffman, Anti-Cancer Inc.; Dr Keith Wood, Promega Corporation; and Dr Aldo Roda, University of Bologna. In conjunction with an instructional designer, we have created a teaching module formatted onto a CD to be distributed, free of charge, for educational purposes.

A new instrument for automated luminescent assays*

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A prototype instrument designed to perform automated fluid handling has been developed for use with light-emitting assays. The instrument is based on an integrated fluidics principle and can perform various bioluminescent assays. The instrument comprises of valves, stepper motors and pumps, which are integrated into an acrylic block containing fluid channels and reservoirs. A photomultiplier tube (PMT) is built into the device to measure light output. The valves, motors and PMT are controlled from a computer via a graphical user interface. Linear worm-drives, stirrers and magnets are incorporated into the instrument to allow the device to perform assays that use magnetic separation steps. The instrument has been compared with an automated flow injection luminometer, and preliminary results have shown the two devices to have equivalent sensitivity. Further work is continuing to develop the device to perform automated specific luminescent assays for a variety of analytes.

Hospital testing of a rapid bioluminescent assay for MRSA*

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Methicillin-resistant Staphylococcus aureus (MRSA) is a major cause of hospital-acquired infections and is directly responsible for about 1000 deaths/year in the UK alone. Standard methods take 2–4 days to determine the presence of MRSA in clinical samples. This limits the value of testing to the monitoring of infection trends, rather than providing information for immediate use in the treatment of patients. Having previously shown the feasibility of using a bioluminescence-based assay to detect MRSA from swabs in 3–4 h, we conducted blind trials on over 300 patient samples to determine the correlation of the rapid test with a standard method. The standard method involved suspension of material from swabs in a salt-containing broth and overnight incubation at 30°C to allow selective amplification of S. aureus. The broth is then plated onto agar containing oxacillin (methyllicin is no longer available) to select for antibiotic-resistant cells. The semi-automated rapid method involved: pre-incubation in salt broth with oxacillin to allow amplification of target cell numbers and to lyse antibiotic sensitive cells; selective extraction and concentration of S. aureus using immunomagnetic separation; and estimation of cell numbers through ATP bioluminescence, following an adenylate kinase-based amplification step. The rapid method was modified during the course of the trials and in final form used fibrinogen as the capture agent on the magnetic beads and lysostaphin as a selective release agent for the endpoint assay. Although some false positive and false negative results (relative to the standard test) were obtained, the overall outcome has justified taking the rapid method forward for larger-scale trials.

A spectral difference between obelin and aequorin is determined by the residue in position 88

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Bioluminescence of Ca2+-regulated photoproteins arises from chemical breakdown of ‘coelenterazine’, an imidazolopyrazine derivative substituted by a hydroperoxy group at the C2-position and tightly but non-covalently bound within the protein. Ca2+ binding initiates decarboxylation, resulting in the excited state of the product, coelenteramide. The well-studied representatives are aequorin and obelin. The aequorin

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bioluminescence maximum is at 465 nm, whereas that of obelin is at longer wavelength, $\lambda_{\text{max}} = 485$ nm. Unreacted photoproteins are hardly fluorescent, but Ca$^{2+}$-discharged aequorin has a strong fluorescence ($\lambda_{\text{max}} = 465$ nm) coinciding with the bioluminescence spectrum, Ca$^{2+}$-discharged obelin having green fluorescence with $\lambda_{\text{max}} = 510$ nm. According to the spatial structures, there is only one remarkable difference between the two photoproteins in the nature of residues making up the substrate-binding site. In obelin Phe is found at position 88, whereas in aequorin the corresponding 82-position is occupied by Tyr, i.e. hydrogen-bonded with the oxygen atom of the 6-($p$-hydroxy)-phenyl group of coelenterazine. To elucidate the influence of the residue in this position on spectral properties, two mutants were constructed: F88Y–obelin and the corresponding Y82F–aequorin. Both mutants show no change in specific activity compared with wild-type photoproteins. They mainly differ in light emission spectra. The obelin mutant shifts both bioluminescence ($\lambda_{\text{max}} = 455$ nm) and fluorescence ($\lambda_{\text{max}} = 488$ nm) to the blue, while the aequorin mutant emits green bioluminescence ($\lambda_{\text{max}} = 501$ nm) and fluorescence ($\lambda_{\text{max}} = 505$ nm). These results clearly indicate that the residue in this position controls the excited electronic energy level of coelenteramide.

Acknowledgements

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A MNDO-PM5 study of the enzyme-free NADH generation from NAD$^+$ in the presence of electron-transfer mediator and an evaluation of the process based on bacterial bioluminescence

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We have recently reported the enzyme-free generation of nicotinamide adenine dinucleotide (NADH) from the oxidized form (NAD$^+$) in a KCl solution, with a cathodically generated violen mononcation radical ($V^{+}$) as an electron-transfer mediator. The following two reactions have been postulated to be responsible for the generation of NADH:

$$\text{NAD}^+ + 2V^{+} + H^+ \rightarrow \text{NADH} + 2V^{2+}$$  \hspace{0.5cm} (1)

$$\text{NAD}^+ + V^{+} + H^+ \rightarrow \text{NADH} + V^{2+}$$  \hspace{0.5cm} (2)

where NAD$^+$ is the 1–e$^-$ reduced NADH and V$^{2+}$ the parent species of V$^{+}$.

In this study, we first verified the postulated reactions (1) and (2) by the MNDO-PM5 molecular orbital method, under the hypothetical condition that all the components, including Cl$^-$ from KCl, are in vacuo. From the calculation, it was expected that the reaction (1) consists of three elementary processes (3–5) and that the processes (4 and 5) are present in (2):

$$\text{NAD}^+ + X^{+} \rightarrow \text{NAD}^+ + X^{2+}$$  \hspace{0.5cm} (3)

$$\text{NAD}^+ + YH^+ \rightarrow (\text{NAD})H^+ + Y$$  \hspace{0.5cm} (4)

$$(\text{NAD})H^+ + X^{+} \rightarrow \text{NADH} + X^{2+}$$  \hspace{0.5cm} (5)

where X$^{+}$ is an electron-transfer mediator, YH$^+$ a proton donor and (NAD)$H^+$ a protonated NAD$^+$. The electron to reduce NAD$^+$ seems to originate from Cl$^-$.

Effect of organic solvents on bioluminescence emission spectra of bacterial luciferase from Photobacterium leiognathi

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The bioluminescence emission spectra of luciferase from luminous bacteria Photobacterium leiognathi in various water-miscible organic solvents were studied. The reaction was initiated by the injection of the chemical recovered FMNH$_2$. The effects of acetone, ethanol, methanol, formamide, glycerol and dimethyl sulphoxide on the positions of the bacterial bioluminescent emission maxima and spectra shape were investigated. It is shown that a small alteration of the solvent composition may modulate luciferase activity and bioluminescence spectra, displaying various degrees of emission spectral shifts. The emission maximum can shift to longer (4–19 nm) or shorter wavelengths (2–6 nm). The effects of organic solvents on the emission spectra of bacterial bioluminescence in vitro depend on the nature of the organic solvent and its concentration in the media. The effects of solvent physicochemical characteristics [dielectric constant, hydrophobicity (logP), polarity index, ionic strength, viscosity, pH, etc.] on the observed charge of emission spectra has been discussed.

Kinetic investigation of bacterial luciferase

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The bacterial bioluminescent reaction has principal non-steady-state kinetics because it is an enzymatic system containing one unstable substrate, FMNH$_2$, in the presence of oxygen. We present a kinetic analysis of the enzymatic system for the case in which the substrate FMNH$_2$ is unstable. A kinetic model of bacterial bioluminescence was proposed.
From the analytical approaches, we can determine a set of elementary constants for the kinetic model. Kinetic analysis of the influence of competitive inhibitors on bioluminescence was carried out. It was shown that competitive inhibitors for aldehyde decreased the maximum light intensity of bioluminescence but had no effect upon the total quantity of emitted light. The competitive inhibitors for FMNH₂ decreased the maximum light intensity of bioluminescence and quantum yield but had no effect upon the rate constant of bioluminescent decay. Such studies are designed to answer questions concerning the quantitative aspects of the biotransformation process and the applied aspects of bioluminescence.

An evolutionary history of Japanese aquatic fireflies inferred from mitochondrial DNA sequences

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The luminescent signal of fireflies is an important method of sexual communication, as the flash pattern in mating is species-specific. Two flash types (fast and slow) have been reported in each of the Japanese aquatic species, *Luciola cruciata* and *Luciola lateralis*. Because the flash pattern is strictly related to mating approach, flashing-time differentiation must function as an important factor in reproductive isolation and speciation. To elucidate the origin of the flash types and the phylogenetic relationships of these species, the mitochondrial cytochrome oxidase II (mt CO II) gene was surveyed by restriction fragment length polymorphism (RFLP) and sequence analyses. The geographical differentiation pattern of CO II haplotypes was congruent with that of flash types in *L. cruciata*, and the slow-flash type (4 s) was considered to be a derived form of the fast-flash form (2 s). In *L. lateralis*, on the other hand, a geographical differentiation pattern of the CO II haplotypes could not be observed, and therefore it is considered that flashing-time differentiation might have evolved through physiological adaptation to the colder climate at the locality. Furthermore, an evolutionary history of Japanese aquatic fireflies is discussed, concerning the related species *L. owadai* and geographical events of the formation of the Japanese Islands.

Purification of the environment by singlet oxygen

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Singlet oxygen has a very short half-life (10⁻⁸ s in water and 10⁻³ s in air). It runs for only a few centimeters in water, then changes to the ordinary molecular oxygen and leaves almost no residual toxicity in the environment. This is a demerit in an ordinary sense but a great merit at the same time as a disinfectant or a microbicide to kill microorganisms attaching to important cells, such as human cells or cultured plant/animal cells. Singlet oxygen would be one of useful tools for purifying the environment without polluting anywhere or injuring the important cells. For example, penaeid white spot syndrome virus (WSSV) attaching to the eggs of the Kuruma shrimp (*Penaeus japonicus*) can be eliminated by singlet oxygen without injuring the eggs.

Many people worldwide lack good drinking water. Over a million children die every year through drinking insanitary water. We would like to offer them a costless sanitary device to make good drinking water. We also describe here some experimental studies on eliminating *Escherichia coli* in drinking water and on plant seeds and eliminating ‘Aoko’, a water-polluting weed (*Microcystis aeruginosa*) in lake Biwa, which supplies tap water for the Cities of Osaka and Kyoto, by singlet oxygen.

Spectral control of 2-arylimidazopyrazinone derivatives: substituent effect and an interaction with biological molecules

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Imidazo[1,2-a]pyrazin-3(7H)-one (imidazopyrazinone) derivatives are important bio- and chemiluminescent compounds as sensor molecules. In addition, we have found that imidazopyrazinone derivatives show solvatochromic shift of the absorption bands in the visible region. This solvatochromic property originates from the characteristic π-electronic structure of the imidazopyrazinone ring system, which has a twitterionic character. In particular, 2-phenyl derivatives have electronic absorption bands in the red region on account of an expansion of the π-conjugated system, and it is easy to recognize the solvatochromism with the naked eye. Since the solution colours of imidazopyrazinone derivatives change depending on solution conditions, imidazopyrazinone derivatives are useful in designing a novel photofunctional dye for biological imaging. On the basis of this idea, we have systematically investigated the spectroscopic properties of 2-arylimidazopyrazinone derivatives. In this study, we focus on the substituent effect of 2-arylimidazopyrazinone derivatives in order to control π-electronic character. From the results we were able to expand the colour range of the solvatochromism. We also report the spectral changes of 2-arylimidazopyrazinone derivatives induced by a supermolecular interaction with biomolecules.

Chemiluminescent study on oxidation of mono-, di- and polysaccharides

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Advanced optical biosensors based on nano-/microtechnology

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Biosensors consist of biomolecular recognition elements and signal transducers. Biomolecules such as enzymes, antibodies and DNA have excellent specificity and sensitivity for target molecules. An optical detection system has been used as a signal transducer for biosensor development. This paper focuses on our examples of advanced optical biosensors, based on nanotechnology and microchip technology.

Micro-fluidic antibody sensor for PCB based on sensitive fluorometric detection

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants that pose a threat to both humans and the biosphere, due to their toxic and endocrine-disrupting effects. There have been many reports in the literature PCB estimating sensors by several other groups. However, the presently available PCB sensing methods are laborious and time consuming and need high-cost investment. So far to the best of our knowledge, there is no report available on a Co-PCB sensing microfluidic device. In this study, we report a novel, rapid and simple Co-PCB microfluidic device with high sensitivity.

Multianalyte immunoassay based on microfabricated encoded particle array

A sandwich format immunoassay was performed using antibody-immobilized particles, peroxidase-labelled antibodies and a luminol-based chemiluminescence detection system. Three analytes, IgA, IgG and IgM, were tested in the assay. The procedure is as follows. Separate sets of particles with an identifiable code were each tagged with a specific antibody. After combining the sets of particles in a plastic tube, immuno-reactants for a sandwich assay format were added sequentially. The immuno-treated particles were then applied to the array in a random fashion and finally detected using a luminol-based chemiluminescence detection system with a CCD detector. The code signature identified the individual particles and, subsequently, the immobilized biocomponents at each specific location in the array.

Silicon microchamber array for sequence-specific DNA amplification and detection

This topic describes on-chip DNA amplification in a highly integrated microchamber array. 40 nL PCR mixture was introduced into each chamber of the microarray precisely by using a nL dispensing system through the oil layer which served as a coverlid. The amplified DNA was then detected with a CCD camera built-in fluorescence microscope, using SYBR Green and TaqMan chemistry.

The details were shown and discussed at the Conference.

Intramolecular CIEEL mechanism on chemiluminescence of phenoxide substituted 1,2-dioxetanes

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The mechanism of chemiluminescence in intramolecular CIEEL were studied for dioxetanes, such as (a) the oxanion of syn-3-(m-hydroxyphenyl)-1,2-dioxetane, (b) the oxanion of anti-3-(m-hydroxyphenyl)-1,2-dioxetane, and (c) the oxanion of 3-(p-hydroxyphenyl)-1,2-dioxetane. The uB3LYP method of Gaussian 94 and 98 programmes were used with the basis function 6 − 31 + G(d). The intrinsic reaction coordinates (IRC) were calculated to find the reaction path, potential energies and geometries of molecules along the IRC. The energies of the excited states were calculated by the MCSCF method. The calculated activation energies were much smaller than those of unsubstituted dioxetanes. These results show that intramolecular electron transfer is decisive for the initiation of the reaction. The ground state (S0) and the lowest singlet excited state (S1), i.e. nπ* excitations of the dioxetane ring, merge after crossing the transition state. The chemiluminescence occurs from the state comprising intramolecular electron
Simultaneous determination of 21 mutagenic nitropolycyclic aromatic hydrocarbons by high-performance liquid chromatography with chemiluminescence detection

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A method using high-performance liquid chromatography with chemiluminescence detection was developed for analysing 21 mutagenic nitropolycyclic aromatic hydrocarbons (NPAHs) in airborne particulates. The system consisted of three pumps, a chemiluminescence detector, a system controller, an integrator, a degasser, an auto sample injector, a column oven, a guard column and two analytical columns connected in series. The mobile phase was 10 mmol/L imidazole buffer (pH 7.6)–acetonitrile (1:1, v/v), and the chemiluminescence reagent solution was an acetonitrile solution containing 0.02 mmol/L bis(2,4,6-trichlorophenyl)oxalate and 15 mmol/L hydrogen peroxide. The sensitivity was improved by increasing the loading time of the sample solution from 38 to 43 min. In order to separate the all NPAHs completely, the length of the separation column was increased from 25 to 40 cm. Graduation elution mode was used to reduce the retention times and to increase the sensitivities of NPAHs, whose retentions were stronger. Twenty-one NPAHs, 1,3-, 1,6- and 1,8-dinitropyrenes (DNPs), 1-, 2- and 4-nitropyrenes (NPs), 3- and 10-nitrobenzanthenes (NBAs), 2-nitrofluorene (2-NF), 4- and 9-nitroanthracenes (NAs), 5-nitroacenaphthene (5-NAc), 4- and 9-nitrophenanthrenes (NPs), 3-nitrofluoranthenes (3-NFR), 7-nitrobenz[a]anthracene (7-NBaA), 2-nitrotriphenylene (2-NTP), 6-nitrochrysene (6-NC), 6-nitrobenzo[a]pyrene (6-NBAP), 1- and 3-nitropyrenes (NPs), were determined chemilumigenically with linear calibration graphs from 3 fmol to 20 pmol. The relative standard deviations (n = 3) were less than 5%. The detection limits (S/N = 3) were 1 fmol for the DNPs, 10 fmol for 1-NP, 7-NBaA and 2-NA, 2 fmol for 3-NPer and 6-NBAP, 4 fmol for 9-NA and 1-NPer, 21 fmol for 3-NFR, 30 fmol for 4-NP, 100 fmol for 5-NAc and 4-NPh, 120 fmol for 9-NPh, 150 fmol for 2-NP and 6-NC, 400 fmol for 3-NBA, 450 fmol for 2-NTP, and 1 pmol for 2-NF, 5.5 pmol for 1-NBA, when the sample injection volume was 100 µL. As an application of the proposed method, NPAHs in airborne particulates collected at a heavy traffic site in Kanazawa were analysed. The pretreatment conditions for NPAHs in airborne particulates were the same as our previous method. All NPAHs were detected from the extracts, except for 10-NBA. The atmospheric concentrations of these NPAHs ranged from 2 fmol/m³ (1,3-DNP) to 0.7 pmol/m³ (2-NF).

A bioluminescent assay for real-time nucleic acid amplification

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Existing real-time PCR (RT-PCR) assays used for quantitative RT-PCR rely upon the detection and quantification of a fluorescent reporter, the signal of which changes in direct proportion to the amount of PCR product in a reaction. Here, however, we describe a means to follow nucleic acid amplification technologies (NAATs) in real time using a bioluminescent assay. This bioluminescent assay for Real-Time (BART) NAATs requires exceptionally simple hardware to perform: just a digital camera and a suitable temperature control unit for the NAAT in question.

The approach combines the use, in one tube, of a NAAT system and a bioluminescent assay using firefly luciferase. The basis of the approach is the detection of inorganic pyrophosphate, a by-product of nucleic acid amplification reactions. The pyrophosphate can be detected either by its direct effect on the firefly luciferase bioluminescence reaction, or via pyrophosphate-dependent generation of ATP (via the ELIDA reaction). We demonstrated this principle by using the isothermal NAAT known as loop-mediated amplification (LAMP) in combination with a modification of the ELIDA method. We demonstrate for BART–LAMP that light emission can indeed be used to follow nucleic acid amplification. The method is quantitative, sensitive, rapid, requires small sample volumes.
A single-step bioluminescent endpoint assay for nucleic acid amplification

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The generation of DNA in nucleic acid amplification technologies (NAATs) is necessarily accompanied by the production of pyrophosphate. Since a sensitive bioluminescent assay for pyrophosphate is available (the ELIDA method), in principle, the outcome of any particular NAAT can be assayed using an ELIDA. However a complication in performing such an endpoint ELIDA is the presence of dATP in the NAAT that can act as a substrate for the firefly luciferase used in the bioluminescent assay. This has two deleterious effects; first, it produces a high background level of light from samples, regardless of whether or not pyrophosphate is present; second, the rate of decay of light emission from the ELIDA becomes increasingly high as the amount of pyrophosphate in the sample increases. These factors seriously affect the sensitivity and reproducibility of such end-point assays. Previously, attempts to use an end-point ELIDA to follow, in particular, PCR, have had to be in the form of (at least) two-step assays to obtain reliable results, essentially because of the high levels of dATP in PCR. Clearly, a one-step mix-and-measure assay would be preferable.

We demonstrate here a single-step, end-point ELIDA for PCR that is sensitive and reliable. This was possible by substituting d-α-S-ATP for dATP in the PCR reaction with appropriate optimization of PCR for use with d-α-S-ATP. The result of this substitution is twofold; first, it greatly reduces the background bioluminescence, since d-α-S-ATP is not a substrate for firefly luciferase; second, it stabilizes the light emission from assays, even at high pyrophosphate concentrations. These improvements greatly increase the utility of end-point ELIDA-based assays for PCR and other NAATs.

Universal chemiluminescent assay for oxidative and antioxidative processes in chemical and biological media: fundamentals and application aspects

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Presented are the fundamentals of a versatile chemiluminescent assay to monitor oxidation processes in a variety of chemical and biological media, with the emphasis on oxidation in the presence of antioxidants. The reason for this emphasis resides in a paramount role of antioxidants in numerous areas of biology, materials science, chemical technologies and analytical techniques. The method utilizes chemiluminescence generated in the oxidation of hydrocarbons, a well-known phenomenon. The mere fact that antioxidants suppress oxidation and thereby quench the light emission opens a direct opportunity for the use of chemiluminescence in the antioxidant analysis. The developed method allows monitoring of both the antioxidant concentration and reactivity. Considered examples illustrate the potential of the presented approach to examine analytes dissolved in different phases, i.e. in liquids (hydrocarbons and aqueous solutions), in the solid state (polymers) and in gas (atmospheric air). The chemiluminescent detection of antioxidants in the ambient air constitutes an unprecedented observation. The biological role of such antioxidative air-pollution species is found to be harmful. Other biologically relevant examples refer to the distinction between antioxidants of different strength that are present in the same probe (in blood plasma and plant extracts), and studies on anti- and pro-oxidative activities of tocopherols.

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Biololuminescence reaction in the firefly squid, Watasenia scintillans

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Each spring, in a far-off region of northern Japan, where the Noto Peninsula projects into the Sea of Japan forming Toyama Bay (maximum depth, ca. 1200 m), the deep-sea squid Watasenia scintillans (mantle length, ca. 6 cm; wet weight, ca. 9 g), comes inshore to lay fertilized eggs. The migration takes place on a vast scale, involving hundreds of millions of squids, and is most notable in Toyama Bay. For nearly 100 years the squid has served as a food source and a local fishery has developed around the squid in the small maritime city of Namerikawa. The squids are caught by setting nets in shallow water, and on one day this March the recorded catch was 3000 tons. The squid has been studied ever since the luminescence was first described by Watašé in 1905, for whom the squid is named. An early visitor was E. Newton Harvey, who made the long journey from Princeton and reported his observations in a paper in 1917. The squid has more than 800 minute luminous organs distributed over its ventral mantle, a row of five prominent organs lining the lower margin of its eye and a cluster of three tiny pigmented organs (less than 1 mm diameter) on the tips of the fourth pair of arms. The ventral organs produce a steady glow of light, whereas the arm organs emit brilliant flashes of light (470 nm). The rhythmic flashing sometimes observed in the arm organs resembles that of a firefly flashing at night, and so the squid is known in Japan as the ‘firefly squid’. The Watasenia reaction is due to a luciferin–luciferase reaction, involving a ‘soluble component’, ‘insoluble component’, ATP, Mg ions and molecular oxygen. The optimum pH is 8.8. If the arm organs are homogenized in Tris–HCl buffer, pH 8.3, and injected with ATP, a bright luminescence is observed. On centrifuging the homogenate, the supernatant is found to contain luciferin as a ‘soluble component’, whereas the pellet yields an unstable membrane-bound luciferase as the ‘insoluble component’. Neither the ‘soluble component’ nor the ‘insoluble component’ give light with ATP, except when reconstituted. There is an absolute requirement for molecular oxygen. Earlier work on structure determination, total chemical synthesis and measurement of light-emitting activity has shown that the luciferin is coelenterazine disulphate. Based on results with Watasenia and other luminescent systems, a hypothetical scheme for the Watasenia reaction is proposed involving: (a) a base/luciferase-catalysed enolization of the C-3 keto oxygen of coelenterazine disulphate; (b) an adenyl formation of the intermediate adenyI coelenterazine disulphate; (c) removal of AMP and the addition of molecular oxygen to the C-2 carbon, forming a dioxetane intermediate; and (d) spontaneous cleavage of the dioxetane ring, yielding carbon dioxide, coelenteramide disulphate and 60 kcal/mol of energy required for the blue light emission. The probable light emitter in the reaction is the excited state amide anion of coelenteramide bound to luciferase.

Capillary electrophoresis with chemiluminescence detector—direct analysis of biomolecules

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Mostly, a labelling procedure is required for chemiluminescence (CL) analysis and such a labelling procedure is sometimes time-consuming and labourious. Direct detection without labelling procedure has attracted much attention in CL analysis. We proposed to develop a direct analysis of biomolecules in capillary electrophoresis (CE) with a CL detector using the luminol–hydrogen peroxide–Cu(II) catalyst CL reaction. Biomolecules such as α-amino acids, peptides and proteins were directly detected by CE with a CL detector without any labelling procedures. The CL peak from biomolecules must appear due to the enhancement of the Cu(II) catalytic activity for luminol–hydrogen peroxide CL. The Cu(II) is more catalytically active when it interacts with biomolecules through the complex formation. Twenty amino acids, four peptides and 11 proteins were examined. Most of them were detected with satisfactory CL intensity. For example, glutamic acid, an amino acid, was determined over the range 2.0 × 10⁻⁷–1.2 × 10⁻⁵ mol/L with a detection limit (S:N = 3) of 0.6 fmol. Glycylglycine, a peptide, was determined over the range 1.7 × 10⁻⁷–1.2 × 10⁻⁵ mol/L with a detection limit (S:N = 3) of 0.9 fmol. Haemoglobin, a haem protein, was determined over the range 1.2 × 10⁻⁷–1.0 × 10⁻⁵ mol/L with a detection limit (S:N = 3) of 0.6 fmol. Representative mixtures of amino acids and peptides were well separated and detected with the present CE–CL detector.

Determination of plasma catecholamine analysis with HPLC-peroxylxalate chemiluminescence reaction detection system in mouse

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Sympathetic nervous system plays an important role in the blood pressure regulation. Plasma catecholamines (CAs) are widely used as an index of sympathetic nervous system activity. Further, CAs are known to be metabolized by catechol-O-methyltransferase (COMT) to produce their 3-O-methyl metabolites. However, until now, no sensitive determination method of plasma CAs (and their 3-O-methyl metabolites) in mouse was developed. Therefore, in this work, we established the sensitive determination method of CAs and their 3-O-methyl metabolites in mouse plasma, using a high-performance liquid chromatography (HPLC)-peroxylxalate chemiluminescence-scence reaction detection system: automated precolumn ion-exchange extraction of diluted plasma was coupled with the separation on ODS column, post-column coulometric oxidation, fluorescence derivatization with ethylenediamine, and finally peroxylxalate chemiluminescence reaction detection. The detection limits of the method were
Bioenergetic confirmation of viable pathogens in foods by ATP-bioluminescence

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The cellular ATP content of 14 freshly harvested bacteria, including Bacillus, Campylobacter, Citrobacter, Escherichia, Lactobacillus, Listeria, Pediococcus, Pseudomonas, Salmonella, Streptococcus and Yersinia, was determined using a luciferin–luciferase bioluminescence approach. Incubation of bacteria with carbonyl cyanide meta-chlorophenyl hydrzone (CCCP), a membrane protonophore, prior to cell breakage, substantially lowered the bioluminescence signals, indicating a decrease of cellular ATP content. The addition of CCCP after cell breakage had no detectable effect on the ATP levels. This differential effect of CCCP was not observed using heat-killed bacteria, i.e. the ATP content was not affected by CCCP incubation. The CCCP effects on cellular ATP level were differential effect of CCCP was not observed using heat-killed bacteria, i.e. the ATP content was not affected by CCCP incubation. The CCCP effects on cellular ATP level were detectable in bacterial suspensions with 10^3–10^6 CFU/mL. This procedure was applied to detect E. coli O157:H7 spiked in ground beef. After a brief enrichment for about 6 h at 37°C, the E. coli cells were captured and concentrated by specific immunomagnetic beads. The captured E. coli cells were then subject to CCCP treatment. The results showed that presence of less than 1 CFU E. coli ground beef could be detected.

Bacterial bioluminescence with flavinmononucleotide activated by N-methylimidazole

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The substrate of bacterial luciferase, flavin mononucleotide, was activated by N-methylimidazole on the phosphate group. Such nucleotide derivatives are highly reactive to nucleophiles. The interaction of activated substrate with the bacterial luciferase from P. leiognathi was investigated. Prolonged luminescence of low intensity is observed using the chemically reduced activated flavin derivative. The activated FMNH₂ derivative competes for the active site of enzyme with native FMNH₂. However, in the absence of exogenous aldehyde in the reaction with activated substrate, the accumulation oxygen peroxide is not observed. It is confirmed that the active centre of luciferase has strongly nucleophilic functional groups, which are able to form a covalent bond with the activated flavin. This luciferase–flavin complex does not interrupt the formation of excited emitter subsequent to light emission.

Luminous bacteria: applied aspects

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The Culture Collection IBSO deposits luminous bacteria of different species (Photobacterium phosphoreum, P. leiognathi, Vibrio fischeri and V. harveyi) and genetically modified Escherichia coli strains with marker lux gene. Luminous bacteria produce target enzymes and biologically active substances. The main ones are luciferase, oxidoreductase and different dehydrogenases. The luminous bacteria can contain considerable amounts of lactate and malate dehydrogenases. The producers of the restriction endonucleases BanI, HaeIII, MboI, AfiIII, AsuI, PstI and Pph 14I are found among the strains of the CC IBSO. The majority of cultures produce such enzymes as L-ornithine, L-arginine, L-lysine decarboxylases, chitinase and cellulase. There are superoxide dismutases (SOD) in luminous bacteria. Strains with a high activity of NAD(P)H:FMN-oxidoreductase were found among those belonging to the species V. fischeri of the CCIBSO. The original technology was developed to obtain highly purified bacterial luciferases from four species (P. phosphoreum, P. leiognathi, V. fischeri and V. harveyi). Transfer of the luminescent system genes into E. coli made it possible to obtain recombinant strains with a high yield of the luminescent system basic enzyme, luciferase (enzyme synthesis 5–10 times higher than the most productive strains of luminous natural bacteria). Strains that produce luciferase and oxidoreductase are widely used for the development and implementation of bioluminescent bioassays to detect the integral toxicity of waters, to test heavy metals, phenol compounds, pesticides, etc.

Interaction of oxyluciferin’s analogues, dimethyl oxyluciferin and monomethyl oxyluciferin, with firefly luciferase

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The fluorescent properties and stability of the oxyluciferin analogues dimethyl oxyluciferin (DMOL) and monomethyl oxyluciferin (MMOL) in large pH range were studied. DMOL and MMOL were used as effectors to study the dynamic properties of the luciferase active site. The binding constants of DMOL and MMOL with native and mutant luciferases were determined at pH 6.0–9.0. DMOL and MMOL were shown to bind to the enzyme more effectively when its phenolic group was protonated. The short-wave shift of the fluorescence was observed.
maximum and an increase in fluorescence intensity were shown for DMOL and MMOL bound to luciferase. This is explained by lower polarizability, $\Delta f$ (in comparison with water solution) of the luciferase active site.

**Bioluminescent spectra of native and mutant firefly luciferase as a function of pH**

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A firefly (Luciola mingrelica) luciferase with point mutation His433Tyr was obtained, that has a bioluminescence maximum at 606 nm, whereas the native enzyme has one at 560 nm. Bioluminescence spectra in the pH interval 5.6–10.2 demonstrated a large difference in the relative content of keto and enol forms of oxyluciferin for native and mutant enzymes. At pH $\geq 7.0$, only enol’s bioluminescence was observed for native luciferase, whereas both forms were observed for the mutant one, and the keto form predominated over the enol form. The relationship between the keto and enol forms is 50% at pH 6.5 for the native luciferase and at pH 8.9 for the mutant. Therefore, the $pK_v$ value for the equilibrium ketone $\leftrightarrow$ enol in the active site of luciferase was shifted to the higher pH after mutation His433Tyr. The mechanism that could be responsible for the $pK_v$ changes observed is discussed.

**Genetically encoded fluorescent and bioluminescent indicators for cellular signalling pathways in live cells**

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For non-destructive analysis of chemical processes in living cells, we have developed genetically encoded fluorescent probes for detecting: (a) second messengers, cGMP, phosphatidylinositol-3,4,5-triphosphate (PIP$_3$) and diacylglycerol (DAG); (b) protein phosphorylation; (c) protein localization in organelles; and (d) protein–protein interactions in living cells. Key molecules and steps in cellular signalling pathways were visualized in relevant living cells using the developed fluorescent and bioluminescent probes. These probes were found of general importance not only for fundamental biological studies, but also for assay and screening of chemicals that inhibit or facilitate these cellular signalling pathways.

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Viable organisms are of particular concern, but methods for their detection and enumeration are fraught with difficulties, notably the fact that different species require different media for growth, so in practice all can never be detected as colony forming units. A firefly luciferase bioluminescence assay that differentiates free extracellular (dead cells, etc.) from intracellular (viable microbes) ATP was used to determine the microbial cleanliness of NASA spacecraft assembly rooms. The viable microbial population as evaluated by ATP was 1–3 logs higher than that indicated by aerobic plate counts. Six major clusters were identified, based on ATP content and purified isolates representing five of these clusters, except that viable but non-cultivable microbes were identified by 16S rDNA sequence analysis and their intracellular ATP concentrations were measured. The 16S rDNA sequences retrieved from these samples that had no cultivable microbes but measurable ATP showed high similarities with the non-cultivable species, such as members of oligotrophic, thermophile, anaerobic and *Ultramicrobacterium* spp. (cell volume 0.3 µm³), etc. In addition, since the AMP level is much higher than the ATP level in spores, AMP may be a better biomarker for spore detection. In a bioluminescence assay developed by Kikkoman Corp., AMP is converted to ATP using pyruvate orthophosphate dikinase, and ATP is subsequently detected by luciferase. We measured the AMP content of several spores of *Bacillus* species using AMP bioluminescence assay and optimized conditions suitable for rapid spore detection. This AMP-based spore assay is rapid and sensitive. It may have wide applications in homeland security, defence against biowarfare agents, environmental monitoring, and in the food and pharmaceutical industries.

Luciferase-like enzymes in non-luminescent beetles: a model of beetle protoluciferase?

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The origin of insect bioluminescence remains a mystery. In beetles, the luciferases arose from AMP/CoA-ligases, a family of enzymes which adenylate carboxylic substrates with their subsequent transfer to CoA. How AMP-ligases acquired the new bioluminescent function during evolution is a challenge. In 1996 we first reported the occurrence of luciferase-like enzymes in the fat body of the mealworm *Tenebrio molitor*, a non-luminescent beetle lacking luciferin, distantly related to fireflies. These enzymes produce a low-level chemiluminescence in the presence of d-luciferin and ATP. As expected, these luciferase-like enzymes are 60 kDa proteins displaying antigenicity against firefly luciferase antibodies. Their chemiluminescence is also stimulated by CoA. These ligases adenylate luciferin, which undergoes a spontaneous oxidation, producing weak chemiluminescence. Similar bioluminogenic AMP-ligases were also found in other non-luminescent larvae of closely and distantly related beetle families. These luciferase-like enzymes could be paralogous enzymes which retained resemblances with the protoluciferase from which luciferases evolved.

Luminol-dependent chemiluminescence of peripheral neutrophils from workers exposed to low-frequency electromagnetic fields

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The production of reactive oxygen species is considered a possible route for cellular damage in the presence of environmental electromagnetic fields. Phagocytes are a main source of free radicals and reactive oxygen species and their activity could be modified by electromagnetic fields. In this study we evaluated the effects of chronic exposure to extremely low frequency electromagnetic fields (ELF) on neutrophils (PMNs) by measuring their resting and stimulated chemiluminescence (CL) activity. Intra- and extracellular CL was also analysed to obtain information on the compartment release of reactive oxygen species. The subjects studied were a group of workers chronically exposed to 50 Hz ELF and a group of age-matched healthy subjects. Resting intracellular CL of PMNs from exposed workers was found to be higher than that of the controls, while the extracellular CL was not significantly different from that of controls. In contrast, extracellular CL emission of activated cells of ELF-exposed subjects was lower than that of controls and no difference was found in intracellular CL. In conclusion, subjects professionally exposed to ELF have a basal production of reactive oxygen species higher than unexposed controls and a modification of the ratio of the intracellular/extracellular CL. The decrease of stimulated CL observed in ELF-exposed subjects is probably dependent on the chronic activation of peripheral blood PMNs induced by ELF exposure.

Bacterial bioluminescence metabolic structure

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The luminous bacteria *lux* operon contains luciferase and its substrate (aldehyde) synthesis genes. However, the presence of the FMN-containing protein and its reduction dehydrogenases is necessary for light emission. Luminous cell-free extracts of marine luminous bacteria and recombinant *E. coli* cell-free luminous extract were obtained. FMN-containing protein and long-chain aldehyde dehydrogenases were detected in the extracts. The functional properties of recombinant luminous *E. coli* and marine luminous bacteria aldehyde dehydrogenases were the same. Two dark mutant *Photobacterium leiognathi* strains were obtained after its incubation with nitrosoguanidine. One of them had no FMN-containing protein; the other had defective aldehyde dehydrogenase. These results therefore led us to propose the important
role of long-chain aldehyde dehydrogenases in the function of the bacterial luminous system.

Possible mechanisms of luminous bacteria adaptations to environment

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Investigations of biopolymer transformation by luminous bacteria revealed not only species (generic) variations in enzyme activity but also variations in ecological adaptation of enzymatic systems of luminous bacteria belonging to different ecological groups. This may serve to prove the existence of true free-living luminous bacteria, contrary to the opinion that sea water is but a temporary habitat for bacteria excreted from the gastrointestinal tract of marine animals.

Three types of bacteria–host interactions (symbiotic, commensal and free-living) are generally recognized. Nothing is known about luminous bacterial lectins. To determine the presence of luminous bacterial lectins and their carbohydrate specificity, we used haemagglutination assay with human Type A blood. The results were estimated semiquantitatively. Strains of the luminous bacteria Vibrio fischeri, Vibrio harveyi, Photobacterium phosphoreum and Photobacterium leiognathi from different ecological groups (symbiotic, commensal and free-living) were investigated. Inhibition of haemagglutination by glucose, maltose, fructose, mannose and N-acetyl-d-glucosamine was observed. This is an argument for the existence of luminous bacterial lectins with proper specificity. At the same time, fermentation of glucose, maltose and fructose is an important test for luminous bacteria species identification. The present study therefore supports the hypothesis about the role of lectins in bacterial nutrition. Mannose and N-acetyl-d-glucosamine inhibition of haemagglutination may be determined by the presence of pili and by the role of the cell wall in adherence process.

Substituent effect on the regioselectivity of ene-reaction of lophine derivatives with singlet oxygen

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Two preliminary studies are known of the substituent effect of the chemiluminescence of 2-(p-X-phenyl)4,5-diphenyl imidazoles as a basic methodology to maximize chemiluminescence efficiencies (Φ/Φ0). Philbrook et al. found a linear Hamnett’s relationship between the relative chemiluminescence efficiencies and σ values of substituents at the para position on 2-phenyl of the triarylimidazole system (ρ = −1.96). Kimura et al. found a non-linear bell-shaped relationship between the relative efficiencies and σ values, using a series of 2-(p-dimethylaninophenyl)-4,5-di(para-Y-phenyl)-4-hydroperoxy-4H-isimidazoles 1, for which a maximum was found for the σ value of fluoride. The substituent effect of a mixture of 2-phenyl-4-(p-Y-phenyl)-5-(p-Z-phenyl)-4-hydroperoxy-4H-isimidazoles 2 and 2-phenyl-4-(p-Z-phenyl)-5-(p-Y-phenyl)-4-hydroperoxy-4H-isimidazoles 2’ (2,2’) = a:b has not been investigated, however. If a dioxetane were an obligatory intermediate, the chemiluminescence efficiencies of any mixture composed of 2 and 2’ would be the same.

In order to confirm this hypothesis of general belief, we have synthesized a series of imidazoles with unsymmetrically para-substituted 4- and 5-phenyl groups and the corresponding mixed peroxides (2 + 2’). The relative chemiluminescence efficiencies [Φ(2 + 2’)/Φ0, Φ(2 + 2’) = chemiluminescence efficiencies for the mixture of (2 + 2’); Φ0 = chemiluminescence efficiency of lophineperoxide as a standard] were measured and corresponding Hamnett’s plots were deduced. A linear relationship was obtained when log Φ(2 + 2’)/Φ0 for the chemiluminescence efficiencies of a series of mixtures of 2 and 2’ was plotted against a/(a + b) σ’ for Z = b/(a + b) σ’ for Y. We present findings of a study of (2 + 2’) that provide additional insight into the mechanism of chemi- and bioluminescence.
Expulsion of symbiotic luminous bacteria from the pony fish, *Leiognathus nuchalis*

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Expulsion of the symbiotic bacterium, *Photobacterium leiognathi*, from the pony fish, *Leiognathus nuchalis*, was investigated. Seawater in the aquarium in which replicates of adult fish were kept under cycles of 12 light:12 dark, began to emit detectable luminescence after the onset of the light period, with its intensity increasing to $3 \times 10^4$ photons/min/mL. The luminescence from seawater then decreased to a level below detection limit ($<3 \times 10^2$ photons/min/mL) towards the dark period. Defecation of luminescent pellets (with light intensities of $5 \times 10^8$–$2 \times 10^{10}$ photons/min/pellet), from which symbiotically competent *P. leiognathi* cells were isolated, coincided with the light period. Similar diurnal patterns of seawater luminescence were also found for juvenile fish that had been infected by the symbiotic luminous bacteria. Changes in the number of viable luminous bacterial cells in seawater corresponded to those in the light intensity from seawater. Up to 85% of the bacterial population in the juvenile light organ was estimated to be released daily. These findings suggest that expulsion of symbiotic bacteria from the light organ of pony fish is controlled by the diurnal rhythm of host physiology and that, at least in adult fish, a significant portion of the symbiotic bacteria is defecated as luminous pellets. Daily expulsion of the symbiotic bacteria via defecation should help to maintain the *P. leiognathi* population in marine environments, thereby increasing the chances of lateral infection to the next generations of the host.

RFLP analysis of the luxA genes of *Photobacterium leiognathi* isolates derived from the symbiotic light organ of the leiognathid fish, *Leiognathus rivulatus*

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The luxA genes were analysed for a total of 85 isolates of *Photobacterium leiognathi* that were obtained from the symbiotic light organs of *Leiognathus rivulatus* for the 3-month period June–August 2002. In June, the luxA gene sequences of the isolates derived from male hosts were found to be clearly distinct from the luxA genes of female host isolates. Comparison of the two luxA gene sequences allowed us to design a pair of restriction enzymes, *Hae*III and *Msp*I, which can be used for restriction fragment length polymorphism (RFLP) analysis of the luxA genes. Except for June, sex-related separation in the luxA–RFLP patterns was not obvious for the symbiotic *P. leiognathi* isolates. Instead, the majority of the isolates showed RFLP patterns that were similar to those found in isolates from females in June. These results indicate that symbiotic *P. leiognathi* populations in the light organs of *L. rivulatus* consist of at least two subpopulations with distinctive luxA gene sequences, and that the dominance of one subpopulation over another is likely to be related to either sex or seasonality, or both.

Development of FIA-chemiluminescence methods to evaluate quenching effects against reactive oxygen species

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Recently, special attention has been focused on quenching effects for reactive oxygen species (ROS) in foods. A simple and rapid evaluation method for a quenching effect against ROS is required to control food quality and human health. In this study, FIA-chemiluminescence (CL) methods to evaluate the quenching effects of functional foods against ROS, such as singlet oxygen ($1O_2$) and hydroxyl radicals (·OH), are developed. The proposed methods are based on the CL reaction between luminol and ROS. A Fenton reaction and a H$_2$O$_2$–NaBr–lactoperoxidase (LPO) system were utilized to prepare hydroxyl radical and singlet oxygen, respectively.

FIA conditions: carrier solution, 0.1 mol/L acetate buffer (pH 7.4) for ·OH, and 0.1 mol/L Hepes buffer (pH 7.4) for $1O_2$; CL reagent, 10 µg/mL LPO in acetate buffer for $1O_2$ and 8 mmol/L diethylenetriaminepentaacetic acid and 200 µmol/L FeCl$_3$ for ·OH. The quenching effect was calculated by the following equation: quenching effect (%) = 100 $\times$ (CL intensity of sample/CL intensity of blank) $\times$ 100. An increased value means an increased quenching effect.

Optimization of FIA conditions (concentration of luminol, flow rate of carrier solution and CL reagent) improved the precision of measurement for ·OH and gave a relative standard deviation of less than 2.5% ($n=5$). Applicability of the proposed methods was confirmed by measuring the quenching effects of grape seed extracts, chalcone, pelargonidine, cyanidine, delphinidine and resveratrol. Quenching effects against ·OH were in the range 3.1–100%. A rapid measurement for both ROS could be achieved by the proposed method (two or three injections/min).

Bioluminescent detection of RNA hydrolysis probes in DNA testing*

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Bioluminescent detection of RNA hydrolysis probes in DNA testing*

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A novel method for the detection of amplicon in nucleic acid amplification technologies (NAATs) is being investigated. The assay is based on the binding of an RNA probe to a DNA amplicon, hydrolysis of the bound RNA to nucleotide mono- phosphates, and consequent production of ATP for detection via firefly luciferase. The combination of an RNA probe-based detection element and a bioluminescent output provides an excellent measurement technique with specificity, great sensitivity, a large dynamic range and improved quantitation.

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**ABSTRACTS**

Dynamics of colonization and clearance of the murine pathogen *Citrobacter rodentium*

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*Citrobacter rodentium* belongs to a family of human and animal enteric pathogens that includes the clinically significant enterohaemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC). EPEC is a frequent cause of infantile diarrhoea in the developing world, while EHEC causes a wide spectrum of illnesses, ranging from mild diarrhoea to severe diseases such as haemorrhagic colitis and haemolytic uraemic syndrome. These pathogens use attaching and effacing (A/E) lesions to colonize the host gastrointestinal tract. However, both EHEC and EPEC are poorly pathogenic in mice. In contrast, the murine pathogen *Citrobacter rodentium* relies on A/E lesion formation as an essential step in both colonization and infection of the mucosa. *C. rodentium* is genetically highly related to *E. coli*, providing an excellent in vivo model. In this study we have used bioluminescence imaging (BLI) to investigate the organ specificity, dynamics of colonization and clearance of mice by *C. rodentium in situ* and in real time. The bioluminescent *C. rodentium* derivative CL2 expresses the luxCDABE operon from the entomopathogenic nematode symbiont *Photorrhhabdus luminescens* and light levels accurately reflect bacterial numbers both in vitro and in vivo. Furthermore, the use of a disarmed transposon to locate the luxCDABE operon and antibiotic resistance gene on the chromosome of the bacteria provides genetic and phenotypic stability in the absence of antibiotic selection.

Development of novel bioluminescent reporter constructs for utilization in mycobacterial species

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Worldwide, tuberculosis causes nearly 2 million deaths/year. Approximately one-third of the world’s population is thought to be infected with *Mycobacterium tuberculosis*, a facultative intracellular pathogen which resides within the macrophages of its host. Work on mycobacteria is compromised by the very slow growth rate of these organisms, with doubling times in excess of 18 h, making it difficult to assess viability by conventional means. Due to these restrictions, many researchers have used bioluminescence (either luxAB from the marine bacterium *Vibrio harveyi* or luc from the firefly *Photinus pyralis*) as an indicator of mycobacterial viability. A further application of bioluminescence is as a reporter of gene expression. While these systems have proved extremely versatile for such a task, there are caveats about their use, mainly surrounding the nature of the luciferases, which are sensitive to high temperatures and conditions such as low pH. The most commonly occurring bioluminescence system is that of deep-sea imidazolopyrazine bioluminescence, reported in approximately 90 genera, including copepods. Coelenterazine is an imidazolopyrazine derivative which, when oxidized by the appropriate luciferase, produces carbon dioxide, coelenteramide and light. *Gaussia princeps* is a bioluminescent marine copepod with a body length of 10 mm, living at depths of 350–1000 m. Bioluminescence originates as a secretion from 30 glands located in the antennae, cephalothorax, thorax and abdomen in response to mechanical, electrical or light stimuli. Recently the luciferase from *Gaussia princeps* has been cloned and sequenced. Expression of this luciferase in *M. smegmatis* results in detectable light expression that is unaffected by many of the conditions that result in denaturation of other commonly used luciferases, such as low pH, H2O2 and high temperatures. This luciferase would be suitable as a reporter in mycobacteria for assessing such systems as those resulting in oxidative damage and the acid tolerance response, which is triggered in response to mild acid and enhances survival at normally lethal pH.

Robust chemiluminescent peroxidase substrates

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Chemiluminescent detection reagents for the widely used enzyme label horseradish peroxidase (HRP) in current use include those based on the oxidation of acridan substrates, such as Lumigen® PS-1, and hydrazides, such as luminol and isoluminol. These reagents must be supplied as two components and working solutions prepared daily because of limited storage stability. We report the synthesis of new, highly stable chemiluminescent peroxidase substrates and the development of enhanced detection reagents. Two compounds selected for commercial development, Lumigen PS-atto for solution assays and Lumigen TMA-6 for solid-phase assays, are structurally quite similar but possess subtle differences in performance properties. The substrates are readily water-soluble and stable to hydrolysis and peroxide. The robustness of the formulations allows storage as ready-to-use, single container working solutions for at least 3 weeks at room temperature and up to 4 months at 4°C without degradation of performance. The reagents, based on an entirely new type of substrate, exhibit a
Development of highly sensitive analysis of glycated protein in human hair by luminol chemiluminescence

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Glycated protein and glucose as indicators of glycaemic control are accepted in the use of blood sample in the medical field. Hair as clinical specimen might be useful for diagnosis with non-invasive sampling and may be stored for long time. In the present study, a highly sensitive, easy and simple method was developed for diabetes screening in hair by luminol chemiluminescence using a microtit replate. It has been reported that the HPLC method for measurement of furosine in hair was developed and required a large amount of hair (10–50 mg). In the present study, we investigated the chemiluminescence method for measurement of glycated protein by small amount (1 cm) of hair. In order to analyse the glycated protein precisely, a new glycation index, which is based on the ratio of glycated protein and arginine in hair protein, was studied. This index of diabetes patients gave significantly higher values than those of healthy subjects (p < 0.003). These indices remarkably correlated with the levels of glycohaemoglobin (HbA1C) in diabetes patients and healthy subjects (r = 0.98). The analysis of glycated protein in hair makes it possible to evaluate past advanced stage of diabetes mellitus. Based on this, we propose that the method could be useful in the monitoring of diagnosis of diabetes mellitus.

Development of the enzyme immunoassay using new chemiluminescence substrate

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Various 1,2-dioxetane derivatives, represented by one bearing a spiroadamantyl group joined at the 3-position and a phenol phosphate at the 4-position, have been synthesized and applied for clinical usage and chemiluminescence enzyme immunoassay (CLEIA). These 1,2-dioxetanes do not, however, necessarily satisfy the demands, namely high thermal stability, ease of handling, and high light yield, to use in the field of clinical applications.

After extensive study, we have reached a new 1,2-dioxetane derivative having a fused furan ring and a bulky substituent, e.g. t-BuO, group and phenol phosphate with superiority to the conventional 1,2-dioxetane derivative. Using such a 1,2-dioxetane derivative, we have realized a new CLEIA system in combination with an enhancer, a tetra-alkyl phosphonium derivative together with fluorescein, and found that this substrate is quite stable and has a very high light-emitting efficiency. Thus, this substrate was stable and intact for more than 1 month at 40°C and for more than 1 year at 10°C. Furthermore, this substrate was found to have a light yield more than six times higher than that for Lumi Phos 530 (AMPPD) at the 5 min end-point in alkaline phosphatase assay.

In conclusion, our substrate comprising a new 1,2-dioxetane and an enhancer was found to have excellent characteristics, with a high thermal stability and a very high light yield, which can be well applied in clinical usage. CLEIA using the new chemiluminescence substrate proved to be highly sensitive, with TSH even less than 0.001 µIU/mL. We discuss in further detail our substrate and the CLEIA results.

Colour modulation for chemiluminescence of a dioxetane bearing a 3-aryl-5-hydroxyphenyl moiety triggered by the potassium tert-butoxide/crown ether complex

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A bicyclic dioxetane bearing a 3-hydroxyphenyl moiety substituted with a 5-(anthracen-9-yl) group (1) and related dioxetanes were synthesized. The dioxetane (1) decomposed rapidly to emit crimson light on treatment with TBAF/DMSO or TBA·t-BuO·PhH, for which a solvent effect on the maximum wavelength of emission was little observed. On the other hand, when the crown ether/t-BuOK complex was used as a base in benzene for triggering of the dioxetane, the colour of chemiluminescence changed significantly, depending on the crown ether used, e.g. \( \Delta_{\text{max}}^{\text{CL}} \) for TBA·t-BuO·PhH was 103 nm. The colour modulation by the crown ether complex was observed also for the base-induced decomposition of dioxetanes bearing a 3-(10-cyanoanthracen-9-yl)-5-hydroxyphenyl (2), 3-hydroxy-5-(naphthalen-1-yl)phenol moiety (3).

An examination of the relationship of \( \Delta_{\text{max}}^{\text{CL}} \) with the stability constant \( (K_s) \) and with the formal surface area of crown ether toward an oxyphenyl anion coordinated with K+ for [K/crown ether] revealed that the colour change of chemiluminescence for dioxetane (1/t-BuOK/crown ether)/PhH is most likely attributable to the steric factor of a crown ether, rather than the magnitude of ionic interaction between an oxyphenyl anion and K+.
Development of a new chemiluminescence substrate for the enzyme immunoassay

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Various 1,2-dioxetane derivatives have been synthesized and reported. These 1,2-dioxetanes do not, however, necessarily satisfy the demands, viz high thermal stability, ease of handling and high light yield in an aqueous solution, to use in the field of clinical applications. For this reason, we have challenged combinations of several different hydrophobic materials and fluorescent dyes for enhancing light yield in an aqueous solution.

After extensive study, we have reached a new chemiluminescence substrate for the enzyme immunoasssay using a new 1,2-dioxetane derivative, 5,5'-butyl-4,4-dimethyl-1-(3'-phosphoryloxy)phenyl-2,6,7-trioxabicyclo[3.2.0]heptane disodium salt, in combination with tetra-alkyl phosphonium derivative and fluorescein as an enhancer. Upon optimization of each component’s concentration, the new substrate has a high light yield with low background by removing impurities using chelating ion exchange resin. Furthermore, this substrate was found to be stable for more than 1 month at 40°C and for more than 1 year at 10°C.

In conclusion, our substrate comprising a new 1,2-dioxetane and an enhancer was found to have excellent characteristics, with a high thermal stability and a very high light yield, which can be well applied in clinical usage. Chemiluminescence enzyme immunoassay using the new chemiluminescence substrate proved to be highly sensitive, with TSH even less than 0.001 µIU/mL.

Syntheses and properties of cell-membrane permeable lucigenin derivatives for the assay of intracellular superoxide

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Since superoxide is first generated and derived into other reactive oxygen species in living cells, its detection within the cells is very important. Although lucigenin (10,10'-dimethyl-9,9'-biacridinum dinitrate) is a useful chemiluminescent probe for detecting and measuring superoxide, it cannot be used for the assay of intracellular superoxide due to its cell-membrane impermeability. The main reasons for the impermeability have been supposed to be its strongly ionic and hydrophilic nature. Therefore, in order to increase the membrane permeability we designed and synthesized less ionic and amphiphilic lucigenin derivatives, consisting of various kinds of carboxylate anions. Their membrane permeabilities were observed by a fluorescence microscope using mouse neutrophils. Lucigenin derivatives with aliphatic and aromatic carboxylate anions, such as monoethyl moronate and monomethyl terephthalate, were found to be membrane-permeable. It is noted that log P values (1.5–5.0) as well as pKₐ values (2.8–5.0) of the conjugate acids of these anions are larger than that (HNO₃; log P = -0.12, pKₐ = -1.8) in lucigenin itself. CT absorptions at 500–700 nm in UV-vis spectra, lower fluorescence-quantum yields and lower redox potentials of these compounds were observed. We conclude that these properties would be usable to evaluate their membrane permeabilities.

Theoretical studies on the charge transfer mechanism for decomposition of dioxetanes and dioxetanones

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The chemiluminescence decomposition of four-membered ring peroxides, known as 1,2-dioxetanes and 1,2-dioxetanones, has been an active area of investigation. When these high-energy peroxides bear a substituent of low oxidation potential, they easily decompose by intramolecular electron or charge transfer and generate singlet excited products with high yields, which lead to the emission of bright light. Although a large number of experimental studies have been performed to elucidate the highly efficient chemical transformation of chemical bond energy of the peroxides into the excitation energy of decomposition products, there is still controversy with regard to the reaction mechanism. This is due to the reactions with complex electron transfer processes, which cannot be directly observed by available experimental techniques. We have performed hybrid density functional calculations to locate endothermic charge transfer transition structures of the decomposition reactions of dioxetanes and dioxetanones, with easily oxidized groups such as phenoxide anion and luciferin anion. It was found that the calculated activation energies for the O-O cleavage of the species are dramatically reduced by the deprotonation of phenol to its anion, showing an important role of charge transfer from the anion site to the O–O σ* orbital. The activation energies for dioxetanone analogues are smaller than those of dioxetanes because of large electron affinities of the former rings. The odd/even selection rule for the chemiluminescence yield is explained by the orbital interaction between the radical orbital of the carbonyl anion and HOMO or LUMO of generated light emitters. Implications of the computational results are discussed in relation to the charge transfer mechanism for chemiluminescence reactions.

Chemiluminescent immunometric detection of SARS-CoV in sera as a early diagnosis of SARS

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SARS is a new epidemic with high lethality and infectivity; it is known that the SARS-CoV is its pathogen. Until now,
the only way of controlling its spread is finding and isolating SARS cases in their early stages. Unfortunately, none of the current assays fit this demand, hence a new sensitive technique for early diagnosis is extremely necessary. Recently a chemiluminescent immunoassay to SARS-CoV has been developed, in which an antibody to SARS-CoV has been labelled by HRP, another was coated in the microwell. In this assay, 50 µL sera and the conjugator were added, respectively followed by 2 h incubation and washing, then chemiluminescence substrate was added and the signal was detected. Higher than 2.1 in S:N ratio was judged as positive. The experiments indicated high sensitivity to six SARS-CoV strains from WHO, CDC, Beijing, Guangzhou and Hong Kong; the detectability was around 60 PFU/mL, while without cross-reaction to other Coronavirus strains from human and animals. The clinical trial taken by the samples collected at the period of spread also represented an outstanding result in early diagnosis: For the 19 cases identified by both clinical and antibody detection, the relevant ratios were 75%, 100%, 71% and 50% during disease days 1–5, 6–10, 11–15, 16–20, while the relevance ratio for 351 cases identified only clinically were 56%, 72%, 42% and 24%, respectively. The data above strongly suggest it perhaps could play an important role for early diagnosis of SARS. This assay can also be applied in other samples; probably it will still act as one of tools in discovering the natural infection of SARS.

Fluorescence sensor peptide for protein phosphorylation
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We are currently focusing on a peptide as a novel biosensor material and have investigated several sensor peptides that can be applied to analysing intracellular signal transduction. We have designed a fluorescence resonance energy transfer-based sensor peptide for analysing protein kinase activity. For instance, we have synthesized a sensor peptide modified with EDANS and dabcyl for monitoring MAPKK activity.

In this work, we synthesized 10-mer and 15-mer peptides which include the amino acid sequence of the phosphorylated site, Arg–Arg–Ala–Ser–Leu, of c-AMP-dependent protein kinase (PKA). Ser is phosphorylated by PKA in the cell. EDANS-modified 10-mer and 15-mer peptides, GLRRASLGE(EDANS)G and GDENLRRASLGEDE(EDANS)G, were synthesized on a bead. After cleavage and deprotection of the peptide side-chain, a dabcyl group was attached at the N-terminal α-amino group. Ser-phosphorylated sensor peptides were also synthesized. The fluorescence property of the peptides modified with EDANS and dabcyl was investigated. Energy transfer from EDANS to dabcyl, quenching by dabcyl, was reduced by phosphorylation of Ser. The peptide main chain was stretched by an increase in hydrophilicity and the distance between the fluorescence dye and quencher increased.

Application of imaging detection using XYZ emission system to food analysis
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Low-level chemiluminescence arose in the presence of reactive oxygen species (ROS; X), hydrogen donor (Y) and mediator (Z) at room temperature and neutral conditions (XYZ emission system). The photon intensity (P) in this system demonstrated the high concentration dependence, which were indicated by [P] = k[X][Y][Z] (k = photon constant). This equation suggested the simple detection system for X, Y and Z species by combination of XYZ reagents (YZ reagent for X detection, XZ reagent for Y and XY reagent for Z). Based on this theory, we developed an imaging quantification for ROS, hydrogen donor and mediator using a charge-coupled device (CCD) camera. We selected the H₂O₂–gallic acid–KHCO₃ in MeCHO system for the standard imaging detection system. The advantages of our chemiluminescence system as a food evaluation system are: (a) short measuring time (10 min); (b) simultaneous measurement of 10–20 samples; (c) applicability to liquid and solid samples; and (d) simplified measurement technique. We also studied the relationship between Y photon intensity and the ROS-scavenging potential of food.
Supplements for Photobacterium phosphoreum RL-1 culture medium to enhance luminescence activity

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In an attempt to exploit bacterial luminescence in commercial use, we have isolated Photobacterium phosphoreum RL-1 from coastal marine sediment. We examined a series of extracts that were prepared from various dried marine foods to maximize the luminescence activity of RL-1 in half-strength SWC medium (Hastings and Nealson, 1981). While extracts from either squid or shrimp increased the luminescence (RLU), those from seaweed had little effects. As dried squid and shrimp are known to be rich in amino acids and chitinous compounds, we further tested whether amino acids and/or chitosan would enhance RLU of the strain RL-1. Among the amino acids tested, both cysteine and asparatic acid strongly enhanced the bacterial RLU, whereas arginine produced an adverse effect on the luminescence activity by these compounds seems to be unique to P. phosphoreum, because none of the other isolates of luminous bacteria so far examined, including the genera Vibrio and Shewanella, showed enhanced luminescence in the presence of the amino acids and/or chitosan.

Enhancement by gold nanoparticles of luminol chemiluminescence

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Luminol, a popular chemiluminescent reagent, can react with a variety of oxidants, giving rise to light emission. In some cases, metal ions can catalyse luminol chemiluminescence (CL). However, to the best of our knowledge, no research has been carried out about the effect of metal nanoparticles on luminol CL. In this work, gold nanoparticles of different sizes were synthesized by the reduction of hydrochloroauric acid and the effects of gold nanoparticles and Au (III) ions on the luminol–hydrogen peroxide system. Gold nanoparticles exhibited strong enhancement. CL spectrum studies showed that the luminophor for the two chemiluminescent systems did not change after the addition of gold nanoparticles or Au (III) ions. UV-visible absorption spectra and fluorescence spectra showed that Au (III) ions were reduced to be Au (0), whereas gold nanoparticles did not change after the reactions. Therefore, the enhancement was assigned to the catalytic activity of the gold nanoparticles. The relationship between the particle size of the gold nanoparticles and the chemiluminescence intensity has been found for the two chemiluminescent systems, which may be helpful for investigating the effects of particle size on their catalytic activity of
Development and validation of a avidin–biotin chemiluminescence enzyme immunoassay (CL-EIA) for the quantitative detection of albumin in urinary

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The objective of this study was to develop and validate a simple and sensitive chemiluminescence enzyme immunoassay (CL-EIA) for albumin determination in urine on microtitre plates, using the avidin coating-well as a solid phase. Biotin was coupled to anti-albumin antibody and through selective biotin-SA immobilization to achieve potential high antibody densities (1). The albumin standards and alkaline phosphatase (ALP)-labelled albumin were prepared in Tris–HCl buffer (pH 7.4) in the competitive immunoassay. The most optimal chemiluminescence substrate for ALP 4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2′-adamantane) (AMPPD) (2) was used in this study. The working range of the chemiluminescence EIA for albumin was 0.15–15 µg/mL. The assay sensitivity was 0.089 µg/mL. Both intra-assay and inter-assay had relative standard deviation of less 15%. The effect of several physicochemical parameters, such as incubation time, detergent concentration and the coating buffer, were also studied. In addition, the studies revealed that using this method the antibody would be reduced to 1/10 of the CL-ELISA method. Therefore, it would have very high potential applications in the market in the future. This method has been successfully applied to albumin detection in urine. Compared with the radioimmunoassay (RIA), the correlation was good.

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Potential mechanism of the enhancement of photoluminescence of CdSe quantum dots under UV irradiation: role of free Cd and Se ions

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Development of a sensitive field test for detecting nerve gases in water

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A chemiluminescence-based assay is presented which is capable of detecting low (sub-p.p.t.) concentrations of nerve gases in water. The system consists of enzyme-containing microtitre strips. Upon incubation of these strips with substrate and sample or standard for 15–20 min, an enhancer is formed which may be detected using subsequent reaction with horseradish peroxidase, hydrogen peroxide and a chemiluminogenic compound (e.g. acridan ester GZ-11), using a camera luminometer. The response time of the test, i.e. the time between sampling and result, is about 25 min. The performance of this system in terms of stability, sensitivity and robustness will be discussed.

Development of a sensitive field test for detecting nerve gases in water

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A chemiluminescence-based assay is presented which is capable of detecting low (sub-p.p.t.) concentrations of nerve gases in water. The system consists of enzyme-containing microtitre strips. Upon incubation of these strips with substrate and sample or standard for 15–20 min, an enhancer is formed which may be detected using subsequent reaction with horseradish peroxidase, hydrogen peroxide and a chemiluminogenic compound (e.g. acridan ester GZ-11), using a camera luminometer. The response time of the test, i.e. the time between sampling and result, is about 25 min. The performance of this system in terms of stability, sensitivity and robustness will be discussed.

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