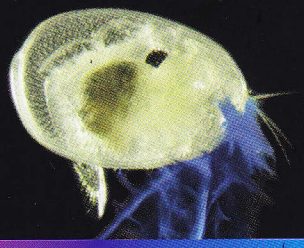
Why Use Luminescence?



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Bioscience & Biotechnology

Why use luminescence?

Photo on the cover: Luminescence emitted by Umihotaru
Umihotaru (Cypridina hilgendorfi, an ostracod sometimes called sea firefly) produces and accumulates luciferin, a luminescent substance, and luciferase, the corresponding enzyme in the body. When stimulated, it releases the luminescent substrate and the enzyme from openings (luminescent glands) around the mouth to the outside of the body, resulting in a reaction between these substances and oxygen in the seawater to generate pale-blue light.

Revival of luminescence system

The 2008 Nobel Prize in Chemistry went to three scientists, including Dr. Osamu Shimomura (a former professor at the Marine Biological Laboratory, Massachusetts, USA) for the discovery and development of the green fluorescent protein, GFP. First isolated from the crystal jelly, Aequorea victoria, GFP has become an indispensable experimental tool to intracellularly investigate various functions of genes and proteins. No researcher would raise any objection to their award.

An alternative experimental tool to GFP is luciferase. However, luciferase luminescence is darker compared to GFP fluorescence, which has lead to a misunderstanding that luciferase is not suitable for biological experiments.

Indeed, this is a "misunderstanding". Let's think of it more simply. It would be hard to say that the light from a firefly is significantly dimmer than that from a jellyfish. Few scientists can surely answer this question. If you had the experience of catching a firefly in your hand in childhood, you would remember that the light of the firefly was bright enough to penetrate your small hands.

In fact, the light emitted by luciferase is "sufficiently" bright. Luminescence-based assay systems—luciferases and their detecting systems—have been greatly improved, capable of covering a wide range of research fields for which GFP-fluorescence systems are not well suited. If used instead of GFP in an appropriate situation, luciferase has the potential to provide a breakthrough for studies in the fields of life science and medicine.

In this booklet, we describe the features of the luciferase-based luminescence systems, how they differ from the GFP-based fluorescence systems, and how these systems could be used for the future development of new research methods based on bioluminescence.

01 Why use luminescence?

"Luminescence" versus "Fluorescence"

*1 Yoshihiro Ohmiya (1960-)
Senior researcher of Research Institute of Genome-based Biofactory at the National Institute of Advanced Industrial Science and Technology
Visiting professor of Hokkaido University Graduate School of Medicine
His specialty is "any living thing related to emitting luminescence" from bioluminescence to molecular imaging in advanced medical practice.

Quest for light-producing organisms

After Dr. Shimomura's Nobel Prize was announced in October 2008, the media found themselves frantically trying to get in contact with him. However, Shimomura was half a world away in Boston. In addition, there were few of his "followers" in Japan. Luckily, the media located a person living in Hokkaido who was deeply influenced by the professor, knew the professor very well, and was happy to answer their questions. This person was Dr. Yoshihiro Ohmiya*1, a professor of Hokkaido University.

He mentioned that he visited Shimomura on various occasions, and that "although Shimomura criticized my studies harshly, he also gave me words of encouragement about the basic research in which I engaged". Shimomura's success story as the discoverer of GFP made the headlines in Japan, because it was a lesson that taught us the "importance of basic research". This was what inspired Ohmiya, who did not directly study under Shimomura, to continue studying "glowing mechanisms in living organisms" from the perspective of basic science. In this regard, Ohmiya is surely one of the successors of Shimomura.

The difference between fluorescence and luminescence – the roots of the misunderstanding

Bioluminescence is basically caused by a reaction between a luminescent substrate and its corresponding enzyme. Although there are several variations in the mechanism of luminescence, the fundamental mechanism is largely common among different organisms. In the case of the firefly, firefly luciferin and firefly luciferase serve as luminescent substrate and luminescent enzyme, respectively.

When GFPs are used, cells transfected with a GFP gene are illuminated with excitation light to detect fluorescence.

On the other hand, luminescence is detected by adding a substrate into cells transfected with a luciferase gene (Figure 1). Hereafter, fluorescence represents a fluorescent-based system using GFPs and other substances, and luminescence represents a luminescence-based system using luciferase for simplicity.

Now, let's consider the "misunderstanding" that is regarded almost as "an established theory." It is true that a very small amount, even a molecule, of GFP expressed in cells can easily be observed. In contrast, it is difficult to detect light emitted by commercially available luciferase expressed in cells.

On the other hand, Ohmiya strongly believes that it must be possible to make light emitted by luciferase bright enough for easier detection, because "the light produced by a firefly is so bright." His explanation was simple. "The light emitted by luciferase isn't weak. It is only because there is no available detection system suitable for measuring the light of luciferase."

Basically, current measuring systems for bioluminescence assays, such as microscopes, have been sophisticatedly designed to measure GFP fluorescence. The system design itself needs to be drastically revised for the effective measurement of luciferase-based luminescence. Ohmiya, again, proposed a new system to solve this problem.

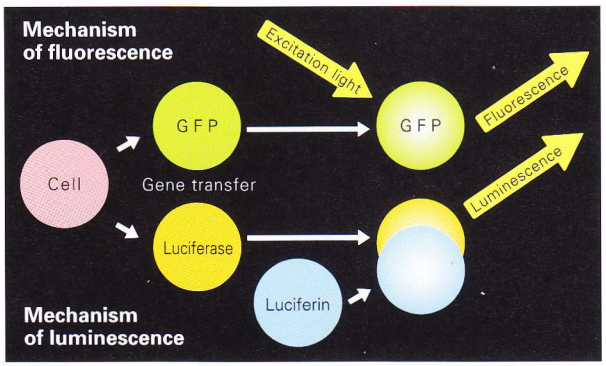


Figure 1 Light-emitting mechanisms of the fluorescence- and luminescence-based systems

The key characteristics of the system are as follows. The fluorescence-based system, which employs an excitation light applied externally to cells, allows us to easily observe the resulting emitted fluorescence. However, this is a highly invasive approach to the cells because of the use of strong excitation light used for detection. Any researcher who has tried to observe living cells transfected with a GFP reporter gene for a long period of time must have felt it very hard to keep the cells alive, as they gradually weaken during the observation due to strong excitation light. This is a drawback of the fluorescence-based system.

On the other hand, the light emitted from the luciferase-luminescence system is relatively faint. Although this has been recognized as a shortcoming of bioluminescence observations, the system is much less invasive because it is not necessary to apply any external light to cells to be investigated. This is the most prominent advantage of the experimental system using luciferase luminescence. The challenge, however, was to develop a method to detect the faint light. To achieve this, extra light from the surrounding environment must be eliminated as much as possible. In other words, it is important to prepare a good "black box" to enclose the material to be observed.

GFP and Luciferase

One of the reasons why GFP has been used more widely than luciferase as a marker for the detection of genes is the fact that GFPs need no luminescent substrate. Thus, once the GFP gene has been introduced into cells, the expression and localization of the gene can be readily determined.

However, one limitation involves the high stability of GFP: their intracellular half-lives usually exceed 24 hours. It is difficult to quantitatively track a change in gene expression over time following treatment with a drug using GFP due to residual GFP remaining in the cell. This has prompted the development of GFP variants with shorter half-lives.

In addition, autofluorescence from cells may be a problem during GFP detection. Removing such background autofluorescence has been a challenge in the application of GFP as a quantitative indicator for gene expression. However, the most crucial issue involving GFP use is the fact that the excitation light applied externally to observe GFP fluorescence causes serious damage to cells over time, resulting in cell death.

The characteristics of luciferases differ from those of GFP, presenting both advantages and disadvantages. Although the requirement of a substrate for light emission is a disadvantage, the high specificity of the reaction between a luciferase and its substrate presents the advantage of a reduced background from autofluorescence and non-specific reactions, leading to superior quantitativity.

In addition, the half-lives of luciferases are inherently very short, at only three hours (note that luciferases with even shorter half-lives have also been developed). Luciferin is considered to have low cytotoxicity. Prior addition of luciferin to cell culture allows a change in the level of light-emission to be tracked over a long period of time.

Making full use of the properties of the luminescence assay system

Developed based on these properties of luciferase, the "luminescence" assay system has excellent advantages different from those of the "fluorescence" assay system using GFP. Ohmiya believes that different approaches making full use of the advantages of both fluorescence and luminescence will be developed in the future. Figure 2 shows a schematic diagram of this concept.

A strong advantage of the fluorescence assay is that fluorescence can be detected even at the level of a single molecule simply by applying a strong excitation light. That is not the case in the luminescence assay, because the light emitted from a single molecule is too weak to detect. On the other hand, the luminescence system is an extremely non-invasive method suitable for long-term observation. Thinking of the hierarchy of biological units (i.e., molecules, cells, tissues, organs, and the whole body) in the field of life-science research, the fluorescence system may cover the structural units from molecules to tissues, while the luminescence system is capable of imaging the higher-level structural units, from cells to the whole body.

How are the differences between these two assays effectively used for "your" experiment? An excellent experimental tool stimulates the creative imagination of the researchers who use it. On the other hand, current state-of-art instruments for life-science research are complicated and thus not intuitive at a glance.

Next, let's review the development processes for the "Luminescenser MCA," "Kronos," and "Cellgraph," which were designed based on the idea of using the properties of luminescence, in order to gain an understanding of the features and potential of these devices. It is you who decide for what purpose these devices are used. However, an understanding of "how were these devices developed" could help to inspire some new ideas regarding how you might use them for your research.

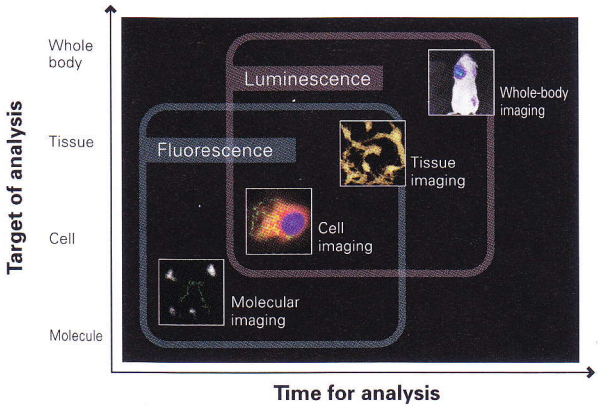


Figure 2 Individual features of the fluorescence- or luminescence-based assay

Door to successful tri-color luminescence assay

Discovery of the railroad worm —a new idea from field work

Ohmiya devotes himself to field work and cherishes the ideas that spring to his mind during field work. He sometimes leaves his laboratory when he has spare time between his experiments to travel around the world to search for interesting organisms. During one such journey, he encountered a very strange and intriguing insect in Brazil.

It was the "railroad worm (Phrixothrix hirtus)," an insect belonging to the Omethidae Family. While the worm's head shines orange to red, it also has many dots lining the two sides of its abdomen—like train windows—which shine green to yellow (Figure 3). An organism which glows red is rare, to say nothing of one glowing in multiple colors. True to its name, the worm runs here and there through the jungle like a night train, while illuminating the jungle darkness with its red and green light.

Ohmiya was absolutely fascinated with this worm. This encounter occurred in 1991, the year when the human genome project began. In response to the start of this project, many new studies have started with an eye towards the "post-genomic era" after the completion of the human genome project.

In the post-genomic era, molecular biology research, which started half a century ago, has finally reached its peak, and greatly promotes studies on cells and individual organisms by returning the outcomes of molecular biology to next-generation research. In this era, tools for tracking the expression status of genes and the intracellular behavior of proteins will be more important.

It was not long before Ohmiya came up with an idea to apply the characteristics of the railroad worm described above to the "simultaneous measurement of three luminescence colors within cells of a single living organism."

ATTO develops instruments and equipment that meet the needs of researchers

"I want multi-colored luminescence to be used in experiments by freely controlling it." The person who Ohmiya approached with his ambition was Hidehiro Kubota, an engineer and assistant manager of the

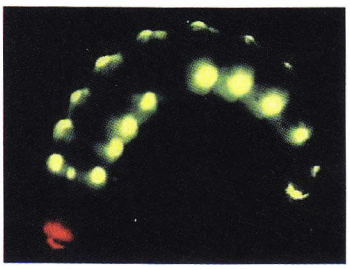


Figure 3 Railroad worm

*2 Setsuro Ebashi (1922-2006) Held various important posts successively, such as Professor at the School of Science, the University of Tokyo and the President of Okazaki National Research Institutes (the present National Institutes of Natural Sciences). He tried hard to improve the quality of research in the fields of molecular biology and physiology in postwar Japan to internationally-recognized levels by elucidating the role of calcium ions on the mechanism of muscle contraction.

Technologies Department at ATTO, which manufactures scientific instruments.

Ohmiya and Kubota had first met when Ohmiya purchased the first of "Luminescencer," a luminometer developed by ATTO, as an experimental device designed to stably measure luminescence from firefly luciferase in order to investigate the light-emitting mechanism of fireflies.

"I'm a user frankly telling my comments and opinions on products to their manufacturers. For example, as I become more familiar with a device, I start saying that, 'the device could be better if it can do something like this and like that'" Ohmiya laughed.

To accomplish the precise measurement of multi-colors, there were numerous problems such as reaction repeatability and background absorbance derived from the measurement vessels. Ohmiya never hesitated to tell Kubota any problems that he had found.

"All the staff of ATTO sincerely listened to my requests and suggestions, and discussed possibilities on how to solve these challenges. Because of this, I could rely on the company as a user," he said.

ATTO has been a leading company in the industry of analytical instrument manufacturing since its establishment 40 years ago, while keeping its open-minded frontier spirit corporate culture unchanged. This corporate culture has been cultivated by President Shigemitsu Yamada, the founder of ATTO, who has been engaged in listening to the needs for experimental devices from young and energetic scientists including the late Professor Setsuro Ebashi*2, and Dr. Makoto Asashima*3, a professor emeritus at the University of Tokyo, and giving their novel ideas a concrete form.

Ohmiya then proposed a more ambitious project to Kubota: "Would it be possible to use different luminescent enzymes that can catalyze reactions resulting in different colored lights? It would enable researchers to simultaneously investigate the behaviors of multiple genes and proteins inside living cells." Kubota willingly agreed with this idea, saying that it sounded interesting. With support from the project, "PRESTO (Precursory Research for Embryonic Science and Technology)" by the Japan Science and Technology Agency (JST), a new project based on the idea from Ohmiya was launched. However, many challenges were waiting to be overcome, before a practical machine could be rolled out of the laboratory.

Simultaneous observation of tri-color luminescence

The first difficulty was to obtain luciferases generating different luminescent colors. Space does not permit a close description of all of the details of the process for overcoming this problem. In 1999, Ohmiya succeeded in cloning two luciferase genes with different luminescent colors from railroad worms.

Ultimately, three luciferase genes expressing different colors were obtained: a red-colored luciferase derived from railroad worms, and orange- and green-colored luciferases from fireflies indigenous to Iriomote Island (Rhagophthalmus ohbai). All three enzymes reacted with the same luminescent substrate (D-luciferin), making it possible to build a platform for a system for simultaneous observation of three differently-colored lights*4.

Development of "Luminescenser MCA" and "Kronos"

Once the luciferase enzymes producing three different colors were prepared, Kubota began to develop a device capable of separately detecting these colors. The problem that Kubota faced was extremely difficult: the lights emitted by the system based on these luciferases were unexpectedly weak. The luciferases that should be expressed in the cells

*3 Makoto Asashima (1944-) achieved a great leap in molecular developmental biology by fixing actipin, a differentiation-inducing substance, for the first time in the world in 1988.

*4 This multi-color assay system is now available under product names of TriplucTM (Toyobo) and MultiColorLuc (TOYO B-Net Co., LTD.).

were not detected at all, when Kubota tried to directly observe the light produced by these luciferases with conventional microscopes, which were not designed to detect luminescence.

After Kubota had reached a dead end, Ohmiya introduced an associate professor at the Institute for Solid State Physics at the University of Tokyo, Dr. Hidefumi Akiyama, who was a fellow researcher of Ohmiya in a JST research project in the "Light and Matter" field of PRESTO. Akiyama was a specialist in the field of spectroscopy. Thus, research collaboration was established via the participation of Akiyama in the team of Kubota and Ohmiya.

Kubota recalled that, "We were in perfect harmony." Ohmiya dealt with biological issues such as the modification of genes and proteins, Kubota produced devices for which the resulting proteins and reagents could be used, and Akiyama helped to solve various problems related to theoretical (application) physics faced by Kubota in developing the appropriate devices. In general, such collaboration among researchers and engineers in different areas rarely works well. "I think that our success derived from the fact that we always respected each other for our expertise and specific experience," Kubota said.

The most challenging issue was to recover the faint light of the luciferases without loss, and decompose each component of the multiple colors. Advice about these difficult problems obtained from Akiyama was extremely useful. To begin with, he proposed that they needed to accurately measure the intensity of the luminescence and identify the characteristics of the luminescence spectra. At that time, however, ATTO's wide range of luminometers capable of detecting faint light could only measure the intensity of a single light source, during a single run.

For the purpose of detecting faint light emitted by luciferase, the light obtained from a cell population should be measured in a black box, in which all external light is completely eliminated. In an effort to overcome this critical issue, the first device was developed. The collaboration team finally created the "Luminescenser MCA*5," a modified version of the luminometer that could segregate multi-colored light and separately measure the light intensity of each colour component, and "Kronos", a device for the real-time measurement of gene expression that accomplished data output on a change in expression over time.

Figure 4 (Left) Kronos Dio [AB-2550] (Right) Elements on a larger scale

Like changing from monochrome to a multi-color

The Luminescenser MCA is a product for measuring three-color luminescent light from luciferases. Conventional luminometers were able to obtain only information on the intensity of light. In contrast, the Luminescenser MCA enables a researcher to simultaneously and separately measure the intensities of three different colors by using the red-emitting luciferase from railroad worms and the orange- and green-emitting luciferases from Iriomote fireflies. Using this

*5 The former "Luminescenser MCA" has been improved to have a higher sensitivity and is commercially available as "Luminescenser Octa".

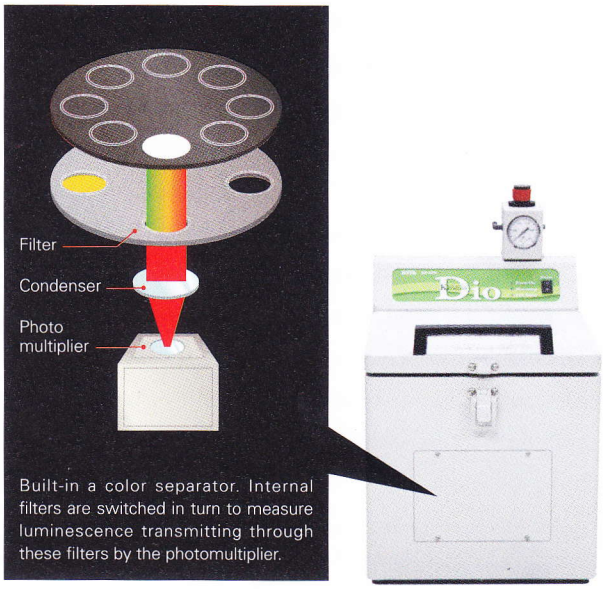


Figure 5 Measuring mechanism of Kronos Dio

system, the expression of three genes can be simultaneously and separately confirmed with one luminescent substrate, D-luciferin. The tri-color detection of gene expression should have an impact on the science community similar to that of the paradigm shift from monochrome to multi-color photographs.

Kronos is equipped with a cabinet in which eight 35 mm-culture dishes used as measuring vessels can be set, all mounted on the top of a turn-table (Figure 4). Cells transfected with a target gene fused with a luciferase gene as a reporter are grown on the culture dishes. After adding luminescent substrate to the dishes, they are set onto the turn-table. Light emitted from the cells can be measured at a pre-defined time interval specified by computer control.

Kronos provides adequate function as an incubator to maintain the optimal conditions for cell culture. The early-type of Kronos machine featured a function for constant temperature, but not a CO_2 gas chamber. A CO_2 regulator was added in an improved version, Kronos Dio [AB-2550]. This ensures the maintenance of appropriate culture conditions—for example, 37°C and 5% CO_2 concentration. The incubator of the Kronos Dio is capable of returning to a pre-defined condition within a few minutes even after the opening and closing of cabinet hatch for reagent addition, minimizing changes in temperature and CO_2 concentration that may cause experimental errors.

Of course, the most appealing feature of the Kronos Dio is that it can detect multi-color luminescence. It is equipped with a color separation system using internal filters that allows a simultaneous multi-color assay (Figure 5). This means an extreme increase in the amount of information, again like a paradigm shift from monochrome to multi-color TVs.

Advantages brought about by the simultaneous tri-color assay

The simultaneous measurement of three luminescent colors in viable cells or tissues is very meaningful in the field of life-science. What does this increase in the power of expression and information obtained due to the multi-color luciferase detection system mean for us?

One possibility is that it allows us to track intracellular signalling cascades. It is difficult to fully understand the actual intracellular changes caused by numerous interactions among a wide range of molecules only by tracking the gene expression related to a single factor. However, the combination of three factors will allow researchers to determine which pathway is selected among the many branches within the signalling cascade. Using this system, we can investigate the status of a disease that is difficult to identify from morphological characteristics of cells and the selection process of cell fate during cytogenesis.

Such signal cascades have been previously analyzed by extracting mRNA from cells to determine the expression level of a target gene. However, it has well known that the amount of extracted mRNA and its translation level are not necessarily correlated. For this reason, it was impossible to compare the amounts of two different mRNAs in cells. Kronos can solve this problem at a single stroke, because it can compare transcription activities between two or more promoters (they can be measured based on difference in the levels of luciferase expression, i.e., color emission) in living cells.

The luminescence substrate luciferin is water-soluble and also dissolves easily in the cytoplasm. Therefore, the multi-color luciferase assay can also be used to investigate plant cells, which require a considerably complex procedure compared to animal cells in general. When the luminescent substrate, luciferin, is added to culture medium, a plant can absorb it from the root and transport the substrate throughout the cells of the plant. The luminescent reaction resulting from this process is noninvasive, causing no damage to the plant body.

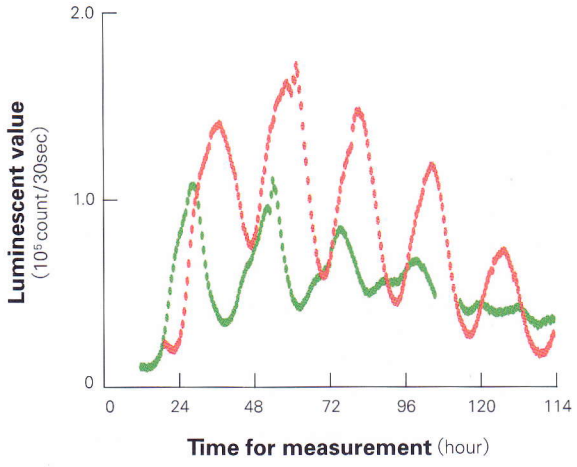


Figure 6 Dual-continuous measurement of transcriptional activity using red- and green-color luciferases from railroad worms in Cyanobacteria.

Specific applications

Various studies are currently being performed by applying the features of the luminescence assay system using luciferase. One of these attempts is to produce a "plant factory," where plants make pharmaceuticals. The introduction of a luciferase reporter gene to a gene encoding a target protein for drug use or a gene governing plant growth will enable researchers to easily investigate the best growing environment for such plants to efficiently produce pharmaceuticals. Thus, literally, a plant grown in the best environment will "vividly brighten".

In other projects, luciferase genes are applied to research studies on the "use of plants as an indicator of environmental stress." These projects are attempting to detect chemicals contained in soil and water that may have adverse impacts on the human body, by using plants as an indicator. For example, plants transfected with a gene responsive to dioxins will emit light under the presence of dioxins, telling us the level of dioxin pollution of the soil or water in which the plants are grown.

Here, we present another example of long-time luminescence monitoring using Kronos. Figure 6 shows the results of continuous dual measurement of transcription activity using red and green luciferases derived from cyanobacteria determined by Ohmiya and a team from Nagoya University. Cyanobacteria are known to have a 24-hour cycle circadian rhythm. The graph clearly shows that the rhythm was accurately measured by the system

Exploring even brighter luminescence

Discovery of another curious insect

Despite being heavily involved in many research projects, including the joint study with ATTO, Ohmiya still managed to find time for field work overseas. In February 2003, he planned to visit the Yunnan province in China to collect fireflies. However, because he was prohibited from traveling to China due to the SARS outbreak, he decided to change his destination to Sao Paulo, Brazil. After he again met with Dr. Viviani, who had taught Ohmiya about railroad worms, he found another interesting insect in the jungle.

This insect was the Brazilian click-beetle, Pyrearinus termitilluminans (Figure 7). Similar to the Japanese Pyrearinus species, when the beetle is turned upside down, it can bend its body at the segments to give a push sufficient to set itself upright. On the other hand, it has characteristics greatly different from those of Japanese species, including a long body length (5 cm) and illuminating organs located on the both sides of its head.

Ohmiya' first impression of the insect was "What a big, quick, and above all, incredibly bright insect this is!" The luminescence from the click-beetle plays a role as a tool for communication for mating. Thus, when Ohmiya flashed a light with his torch to mimic the mating signal, many insects with green light began speeding to him from the depths of the jungle, like a "super fastball glowing green." The brightness of the light produced by the beetles was so intense that South American natives used to illuminate the dark by tying the insects to their feet.

It was the beetle's incredible brightness that strongly caught his attention: "The luciferase of this beetle might be extraordinary", he thought. After offered the luciferase gene of the click-beetle from Dr. Viviani, Ohmiya firmly believed that it must change something.

Figure 7 Brazilian click-beetle

New arrival of "Cellgraph": observing luminescence from the inside of cells

Unfortunately, although it had already been cloned, the luciferase gene from Brazilian click-beetles could not be used in mammalian cells. Therefore, Ohmiya needed to make steady efforts to modify the codons of the gene with his team members, and in collaboration with an industrial company. As a result, a new luciferase assay system was developed, called the "EmeraldLuc (ELuc)," which is commercially available from Toyobo Corporation.

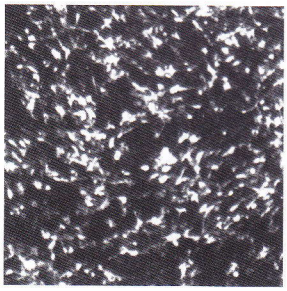
The light of the ELuc system was not only much brighter than those of other luminous beetles' luciferases, but also showed improved stability in living cells (Figures 8A and 8B). In addition, the extremely intense brightness also made possible the observation of the light emitted from a single cell, which had been difficult in the system using previous luciferases.

Following the development of the ELuc system, and due to enthusiastic persuasion by Ohmiya, ATTO finally began to develop a new device for the detection of luminescence on a cell basis. After much trial and error, the "Cellgraph" was completed—a system for microscopically observing cells transfected with the ELuc gene. This was the moment at which the long-time efforts by Ohmiya to "observe luminescence in a single cell" bore fruit.

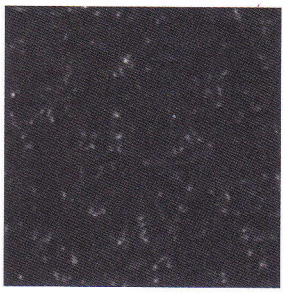
Cellgraph is equipped with a cell-culturing stage and a supersensitive CCD camera with absolute sensitivity enabling a single photon counting within an incubator. It also has color filters to separate three elements of light. However, while the luminescence intensity of ELuc was intense, it was still considerably weaker than GFP fluorescence, resulting in difficulty in focusing the target under a microscope. To solve this problem, a transmitted illuminator with a wavelength of 480 nm was also built into the instrument so that light form GFP and luciferase can be observed on the same disc. This combination allows quick focusing on the target cells using GFP fluorescence as an indicator before taking a photo of luciferase luminescence.

Using the Cellgraph system, organelles such as peroxyzomes that are generally difficult to see in living cells can be observed over time (Figures 8C and 8D).

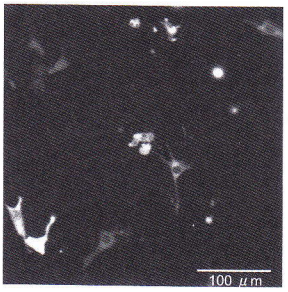
Figure 8 Luminescence imaging of mammalian cells transfected with luciferase



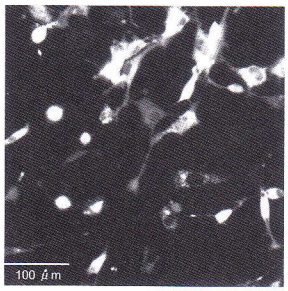
A. Cells transfected with Eluc



B. Cells transfected with luciferase derived from American fireflies.



C. Cells transfected with cytoplasm-localized Eluc.



D. Cells transfected with peroxisome-localized Eluc.

Future of luminescence assay systems

Choosing "fluorescence" or "luminescence" according to purpose

The luminescence assay devices have been created based on the fact that luciferase presents greater advantages than GFP in specific research areas where GFP fluorescence could not be used as a reporter. In Japan, however, there have been few examples of use of these luminescence-based systems in scientific research. Interestingly, overseas researchers, rather than Japanese researchers, want to contact with Kubota to purchase the system or inquire about the luminescence-based system. This trend may reflect the "researcher disposition" specific to Japan to hesitate to use totally new techniques or methods unless they follow the preceding examples in Western countries.

For ATTO, as a private company pursuing profitability, it would be best if our devices or systems sell well. On the other hand, much to sorrow of Kubota and his partners, the advantages of the systems that they developed with enthusiasm have not yet been understood and used widely, only because these are not known to researchers.

Let me summarize the features of the luciferase-based luminescence system which are superior to those of the fluorescence system which mainly uses GFP.

One of the important properties of the luciferase assay is its non-invasiveness, as no excitation light is necessary to detect emitted luminescence. In addition, for the fluorescence assay there is the problem that when the expression level of a target gene labelled with GFP is low, the light emitted from cells might be weak enough to be obscured by background light due to the self-fluorescence from the cells. However, the level of self-"luminescence" generated by cells can be almost neglected. In addition, luciferase has a relatively short half-life and low cytotoxicity. It enables researchers to continuously observe cells for a long period of time using it as a marker.

On the other hand, luminescence methods also present some disadvantages. As the signals derived from luciferases are still weaker than those from the GFP-fluorescence system, the luminescence system requires a longer exposure time to capture images than fluorescence. Luminescence imaging requires an exposure time from a several tens of seconds to several tens of minutes to detect light from cells, while GFP-fluorescence imaging requires only a time in the order of milliseconds. This is a considerable difference between the two systems.

Based on these features of the two systems, the GFP-fluorescence system would be best for use in short-time (seconds to hours), high resolution imaging, while the luciferase-luminescence system would be suitable for long-term observation (minutes to days) with low background interference ensured.

Of course, the applications above described show only part of the possibilities of what the two systems can accomplish. In fact, there are numerous options, which may be explored by applying biochemical or genetic-engineering knowledge and detection tools. Researchers will be tested, and require wide-ranging

knowledge and creative imagination to develop new applications.

As described above, Cellgraph features a system that can measure the light derived from both GFP and luciferase. With the use of this system, we can observe cells based on four differently-colored signals with a single run via full use of the advantages of GFP suitable for short-term observation and three different luciferases for long-term observation.

We always overcome any challenge

As described above, the luminescence-based assay presents a wide range of possible applications. What challenges will arise on the system in the future? In consideration of today's needs in scientific research fields, Ohmiya and Kubota believe that the realization of "individual imaging" might be one of the most challenging issues. An example is the introduction of cancer cells labelled with a luminescence tag into a mouse, followed by the real-time tracking of the development of the disease by observing the behavior of the cancer cells within the living host animal. This will allow us to investigate the metastatic process of the cancer on the real-time basis, and thus, help to develop new anti-cancer drugs. This in vivo, real-time imaging will have a substantial impact on the progression not only of medical research but also of basic research.

In addition, combination with computer tomography (CT) scanning would be useful. But, there have been many complicated issues to work in this area, due to the number of patents concerned. Therefore, different approaches not related to these issues are now required.

There are a huge number of problems, said Kubota. "We don't know at all what we should do next. But, in any case, the improvement of the very-weak luminescence signal in our system may play a key role in opening the door to the next step." Yet, his words were never conveying the feeling of giving up, rather quivering with excitement in anticipation of future challenges.

He continued, "There are other requests from clients. For example, some clients want to detect active oxygen species present at high levels in cells. However, the activity of active oxygen species is extremely weak and they decay rapidly. It is logically impossible to detect them" he said, and after a little pause, he added while smiling, "so far."

His words might have indicated his pride as an experienced engineer. In his collaborations with researchers outside of ATTO, including Ohmiya and Akiyama, Kubota has learned that such challenges generally emerge to bring a fresh point of view, and eventually lead towards a solution.

Epilogue: "Playing" with science

The interviews with Ohmiya and Kubota were carried out at Hokkaido University and ATTO headquarters in Tokyo, respectively. In the interviews, I was very impressed with a word commonly used by both persons. This keyword was "playing."

Kubota reminisced. "Luckily for me, we could devote all our energies to the studies on luminescence without worrying about a deadline, because ATTO has been positive about this idea. On the other hand, we never had a large budget for our study, so I had to spend my spare time looking for external funds. But even so, I really enjoyed the experiments and discussions with professor Ohmiya and professor Akiyama...we were just playing with science, I'd say" he said.

Ohmiya compared their product development process to a game like "pitch and catch." "At first, I played catch with Mr Kubota, and then professor Akiyama came along. We were throwing balls of ideas—among us. This process somehow led us to develop a range of products," he said.

The word "collaborations between businesses and universities" is commonly misunderstood, in particular by scientists, as something involving monetary interests. However, in reality, it is a highly creative action accomplished by mutual relationships and activities among researchers and engineers using only a common language, science, to create a product as a concrete outcome.

Why not "play" with ATTO's products? ATTO is always prepared to help you to promote your research.

ATTO's products are "devices to promote basic research"

"Playing" with ATTO's products brings forth creative research solutions.
To making it all possible, collaboration between researchers and ATTO development engineers will be important.
Finally, let us discuss what an ideal relationship between scientists and companies is.

We have never had any technology for which we could say, "we are second to none in this technology"

In 1964, ATTO started business as a company dealing in imported laboratory instruments. Then, in response to market demands, it began to customize these imported devices and equipments, and started selling these improved instruments as their own products. It was an era when the idea of protecting intellectual property rights had not yet become publically predominant. As time went by, ATTO continued improving their engineering techniques and expertise.

Small-and-medium businesses like ATTO are often asked to produce a product with relatively-low market demand. "Can you make something like this?" The manufacture of many of these products had been rejected by industrial giants, because they were often highly specific and attracted little market attention. However, ATTO has rejected hardly any such requests. "We have taken on any job, whenever we thought it to be interesting," said Masamichi Usuda, who is the Director of Technology Development at ATTO. He said that they never showed any reluctance in accepting these tough requests "because we didn't know that how much trouble would occur." They didn't understand that it was extremely difficult to develop a product which met the demanded requirements, until they had actually started working. However, this was a quite reasonable consequence, because all of the projects offered to ATTO had been otherwise given up as hopeless.

On the other hand, responding to these near-impossible requests has helped ATTO to grow. "When we start developing a new product, we might only have only 20% of the skills needed to solve the problems related to the development of the target product. Nonetheless, we knew that even if we are unable do it, there is someone who could. We never have had any technology for which we confidently could say, "we are second to none in this technology," Usuda laughed. Although this comment is apparently self-mocking, it is supported by ATTO's long list of achievements and the resulting mighty pride.

ATTO's real value lies in its flexibility. After taking on a new project, the first thing that ATTO's engineers do is to draw up blueprints, purchase mechanical parts from Akihabara, and then begin building a prototype by repeated trials and errors. If the technology to tackle a problem does not exist at ATTO, they go out and find someone who does have the skills, and try to collaborate with them. This agile footwork is ATTO's true worth.

The era when "All the technologies required for conducting a project can be developed by a single company by itself" has finished. With current highly-developed technologies, all the organizations engaged in scientific

research are required to know how to construct "an external network" in order to "collect and organize an enormous amount of knowledge and technologies scattered throughout the community" to accomplish their goal. ATTO has put this external networking into its technological development.

The difference between research and corporate activity is the targeted goal

When a researcher purchases equipments for research from a company and uses them to perform research activity, this relationship can be also seen as a form of industry-academia collaboration. For the past 10 years, industry-academia collaborations have become increasingly common, leading to both successes and failures. For example, the collaboration between Ohmiya and ATTO can be regarded as a good example of remarkable success. To discuss the opposite case, "Who is a partner whom ATTO finds "difficult to work with?"

"Someone who doesn't understand how business works, I suppose," Usuda said. Although it seems obvious, a company cannot survive unless it makes a profit. This is the principle which divides definitely corporate activities from academics.

In the scientific world, a failure as a scientist is to write no papers at all that would present outcomes of the study for all the cost and time spent. In other words, the most important thing to a scientist is to produce a valuable result before their research grant runs out. In industry, however, product development is merely a small part of a larger process. The real challenge is to turn the product into something that can pay off all the cost and time used to develop it. The small but definite differences in motivation and pressure between scientists and corporate engineers may lead a collaboration project to failure.

This does not mean that researchers should understand and follow basic economics, or that engineers need to put more care into the result itself. It is the process where everything lies. Usuda pointed out that if a researcher who comes to ATTO asking for research collaboration thinks that "ATTO must cooperate to achieve my research goal," he/she has already made a mistake. While accomplishing a scientific goal is a good thing, it can never be a company's main priority. "It is easier to work with someone who understands this point, and is willing to work with us and our equipment," he said.

The future of collaborations

In the current research environment with highly-advanced experimental instruments, it is not easy for researchers to produce a fruitful result, even though they purchase sophisticated, high-performance equipment or devices and learn how to use them. In this context, collaboration between researchers and engineers would be essential to achieve some meaningful outcomes. In doing so, it has become important to have a mutual understanding of each other's way of working, while pursuing individual advantages that are different in nature on the same project.

When the author asked a slightly mean question, as to whether there were researchers who have looked down on engineers, Usuda honestly answered, "Yes, I think that there have been such experiences. However, we have taken a certain pride in knowing that they could do nothing without our products."

"We don't see researchers as our rivals; in fact, it is quite the opposite," Usuda said, laughing. "What we provide is more than a service; it is a product that promotes basic research. It doesn't bother us when we get complaints from partners or customers who understand our values. Such comments or suggestions just motivate us to meet their expectations," he said.

Building up network links that are valuable for each party might provide the advantages required to accomplish individual goals.

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