From basic research on firefly bioluminescence to *Product Realization Research*

— Production of a multigene expression kit based on bioluminescent proteins —

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In the postgenome era, biological network analysis has become essential for elucidating animal physiology. We have developed a revolutionary tricolor reporter *in vitro* assay system that can be utilized for detailed analysis of a biological network. Bioluminescence has potential for visualizing the dynamics of living systems. To accomplish our vision, we are realizing new technologies through *Type 1 Basic Research.*

Keywords : Product Realization Research, bioscience, biotool, optical technology, gene analysis

1 Introduction

Bioluminescence refers to light produced by bioluminescent organisms such as the firefly. Light-emitting organisms possess a light source and an enzyme that catalyzes the luminescence of the light source. In light-emitting organisms, the light source is luciferin (meaning something that is luminescent or emits light) and the enzyme that catalyzes the reaction is luciferase. The French scientist Raphaël Dubois coined the terms luciferin and luciferase in the 19th century, and many researchers have studied bioluminescence science thoroughly. The field of bioluminescence science is not dominated entirely by European and American scientists, and the Japanese have contributed greatly to this field. For example, before the World War, Sakyo Kanda, who is often considered the second Minakata Kumagusu, wrote *Hotaru (Firefly)*, a work widely recognized both in Japan and overseas. Dr. Yata Haneda, the first director of the Yokosuka City Museum, was a world-class bioluminescence scientist, and Dr. Osamu Shimomura of the Woods Hole Marine Biological Laboratory is the discoverer of green fluorescent protein (GFP), which revolutionized bioimaging technology[1][2].

Since the 1990s, biotools using bioluminescence have been commercialized by companies in Japan, Europe, and the United States. However, because bioluminescence science is a composite discipline comprising wide-ranging fields including biology, chemistry, physics, biochemistry, and engineering, it failed to attract knowledge, technology, people, and money, and little innovative technological development has occurred. This was because of insufficient fusion of disciplines and the lack of a clear path through the "valley of death." Companies that have commercialized biotools using bioluminescence have not produced sufficient results from basic research to differentiate their products, to generate new products, and to communicate a positive message to the market.

In contrast, the Human Genome Project that started in the 1990s advanced rapidly by attracting knowledge, technology, people, and money, and decoding of the human genome was completed in less than 10 years. However, this advancement fell into the dilemma of sitting around waiting for the next discipline. The scientists initially believed that decoding the genome would provide groundbreaking basic information and great innovations would follow. Until then, it was believed that the human genome contained information for more than 1,000,000 proteins, and there was vague hope that this information would indicate the clear difference between humans and other animals. In reality, there are 21,600 genes in the lancelet, a chordate closely related to vertebrates, and the human has about 20,000 genes; thus, there is 2 % or less difference in genetic information between humans and monkeys. This raises questions such as, "Why are there differences between organisms?" or "Are humans really advanced?" There are now more issues in this postgenome era. Now, kinetic analysis and imaging of biomolecular groups that comprise life are gaining attention as methods to answer these questions.

Ohmiya, one of the authors, became interested in bioluminescence in the 1990s and engaged in research on the mechanism that determines the color of luminescence in the firefly, as part of the Sakigake 21 Project of the Japan Science and Technology Agency. He continued research on the biology and biochemistry of luminescence mechanisms of luminescent beetles and the sea firefly in the Faculty of Education, Shizuoka University. There, he succeeded in cloning the

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genes for red and green luciferase of Brazilian railroad worms and found that the 216th amino acid residue in firefly luciferase is involved in determining the color difference of luminescence[3][4]. Railroad worm (or glow-worm) is a luminescent beetle that survives only in Brazil, and expresses a luciferase that produces the strongest red-colored light on earth. Although these research results were new and may help produce biotools for bioluminescence systems, there was no direction that linked the results together. *Type 2 Basic Research* is not feasible for closing the gap between research and education within the university. In such background, Ohmiya and Nakajima started research at the new National Institute of Advanced Industrial Science and Technology (AIST), where they were given the opportunity to expand the knowledge gained in *Type 1 Basic Research* to *Type 2 Basic Research*. Development of biotools using the luminescent color differences started to take shape as an outcome, and a clear goal was set to link bioluminescence to kinetic observations and imaging of biomolecular groups that was being sought in 21st century biosciences.

2 What would be developed?

In 21st century bioscience after the completion of the Human Genome Project, it is understood that there are limited methods for pursuing a single biomolecule and that innovation would not happen by developing only measurement devices that are extensions of conventional methods. From this background, the "Development of Analysis Technology of Dynamism of Intercellular Network" Project started in 2002, lead by the Ministry of Economy, Trade and Industry, and New Energy and the Industrial Technology Development Organization (NEDO). The objective of this project was to measure efficiently the temporal and special kinetic changes in the intracellular biomolecular network that comprises the foundation of building and functioning of organic tissues in live cells and to establish the technology to allow this functional analysis. The project's goal was to clarify the information network created by several biomolecules. We participated in this project and suggested the use of luminescent proteins for the development of intracellular labeling technology of multiple types of biomolecules.

Our research concept was simple. Whereas traditionally, the amount of light was the only focal point in using firefly luminescence, we looked at the multicolor characteristic of firefly luminescence and studied how to transmit multiple bits of information from the cell. In other words, conventional tools using bioluminescence were an extension of black-and-white television, and we decided to use the color television characteristic, or color difference, to monitor the movement of multiple types of biomolecules in the cell. Our work would connect the results obtained in basic research conducted at the university in the 1990s. Figure 1 is a revised version of one of the slides used in the proposal and describes the research concept and strategy, and the use of luciferase for detecting diverse bioluminescence. Our powerful method was a first-in-the-world result of *Type 1 Basic Research* showing that the luciferase of railroad worms produces red light from the head and green light from the body.

The goal was to develop a multiple drug screening system that would emphasize clearly its difference from conventional technology (Figure 2). The product was ultimately commercialized in April 2006 as "TripLuc" from Toyobo Co.,

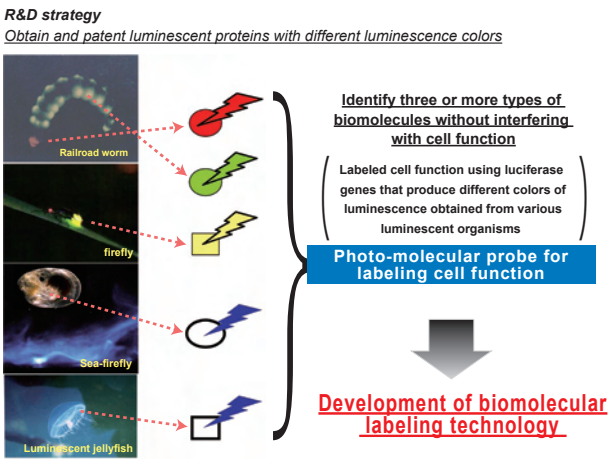

Fig.1 R&D strategy. Revised version of slide used at NEDO "Intracellular Dynamism Analysis" hearing.
The photographs on the left show, from top to bottom, railroad worm, Japanese firefly, sea-firefly, and luminescent jellyfish. The cell function labeling technology that we studied was accomplished using a photomolecular probe as the label that "identifies three or more types of biomolecules without interfering with cell function," based on luciferases (enzyme or catalyst for luminescence) with different colors obtained from various luminescent organisms.

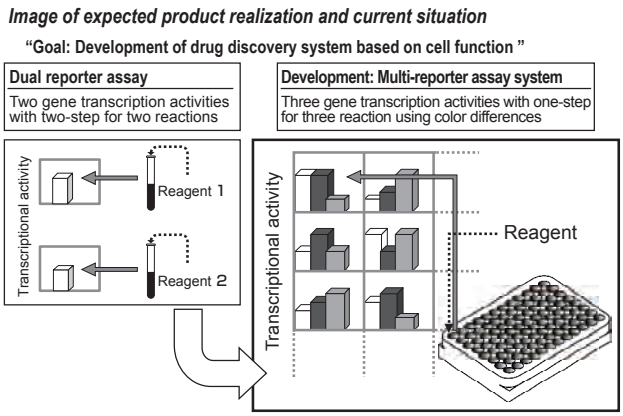

Fig. 2 Example of outcome of R&D. Revised version of slide used at NEDO "Intracellular Dynamism Analysis" hearing.
In conventional technology, two gene transcription activities were observed in two steps using two reagents, whereas in the newly developed multireporter assay system, three gene transcription activities could be observed in one step using one reagent. This allows the analysis of several samples at once (high-throughput analysis).

Ltd., and "MultiColor Luc" from Toyo B-Net Co., Ltd. We have sold the kits from these companies, as we had projected as an outcome of the proposal. The decision whether the company will sell the product depends on the demand for the products and the number of users and potential users that demand improvement in technology and expect the introduction of new technology. Japanese bioresearchers tend to take up technology they see in foreign journals rather than using their own judgment to introduce and adopt new technology. It is important for researchers to be alert to new developments and for those who produce new biotechnology to communicate the advantages of their products through as many channels as possible. Conducting T*ype 2 Basic Research* and practical research that starts from a patent will not lead to true product realization unless it is followed up by continued aftercare, which produces solid results from *Type 1 Basic Research* based on the new technology created.

The labeling technology for studying multiple types of biomolecules involves monitoring the expression of several genes in the cell. The expression of several genes is regulated by quick or slow responses to external stimulus in the cell, which produces various proteins. For example, when an environmental hormone reaches the cell, the cell produces a female hormone in response. To detect the expression of a certain gene, we developed a reporter assay using the firefly bioluminescence enzyme, luciferase. In this reporter assay, the promoter region of the gene sequence that regulates the expression of the gene is inserted into the firefly luciferase gene and this is then transduced into the cell. If the gene expression is induced, luciferase is synthesized accordingly. Because luminescence occurs when luciferin is added to the expressed luciferase, the extent of genetic expression can be assessed by the amount of luminescence. This method was already employed in the biomedical field and has become a standard chemical assay in the environmental field (approved as an official method by the Ministry of Environment) and in screening methods for drug discovery. In 2002, the method was part of a 500 million-yen market in Japan and 20 billion-yen market worldwide.

Is it possible that these companies will realize their products in this field? The reporter assay that was supported widely until 2002 was the dual reporter sold by Company P. This product used a two-step measurement method comprising two genes and two substrates, and Company P, which held the patent in the United States, dominated the market. Companies trying to break this domination had to search for other potential reporter assay systems. Users were dissatisfied because the method could assess expression of two genes only, and there were problems relating to the cost and the two-substrate two-step process. Potential users wanted to a reporter assay whose application could be extended to more genes. We were able to set a clear goal to develop a new "three-genes, one-substrate one-step reporter assay" using the principle of the diversity of luminescence color difference.

3 Patent construction for product realization from *Type 2 Basic Research*

The light emitted by the firefly occurs through an enzymatic reaction in which the oxidation of firefly luciferin is catalyzed by firefly luciferase. There families of luminescent beetles are the Lampyridae (firefly), Elateridae, Drilidae, and Rhagophthalmidae (Iriomote firefly) families. These are unique in that their colors of luminescence differ slightly and because the luciferin–luciferase reaction color may be changed depending on the pH of the reaction environment. We decided to detect the multiple gene expression using luciferases that produced different colors but that were not influenced by a pH change. We have already isolated the luciferase genes that produce red and green light from the South American railroad worm, which produces varied luminescence (i.e., the head emits orange to crimson colors while the abdomen emits green to yellow-green colors). We have succeeded in expressing luciferase in E. coli and in producing orange luminescence using this genetic information[3][4].

However, when applying the multiple gene expression detection kit in 2002, we were unable to produce the stable expression of luciferase in mammalian cells. Although we

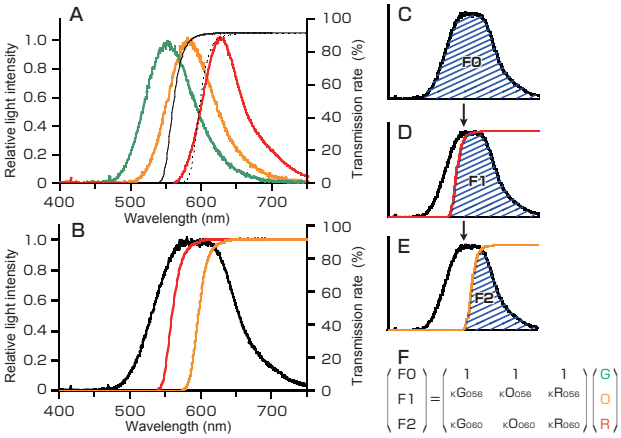

Fig.3 Outline of technology for dividing and measuring three luminescence colors using two filters.

A) Luminescent spectrum group of green, orange, and red luciferases.

B) Optical absorbance of two filters used and luminescence spectra when three colors are mixed.

C) F0 is the amount of light when all-optical output is measured without a filter.

D) Most green light is absorbed when passing through the first filter. F1 is the amount of light not measured.

E) Most green and orange light is absorbed when passing through the second filter. F2 is the amount of light not measured.

F) Equation to calculate the original amount of luminescence by calculating F0, F1, F2, and the transmission coefficient of the two optical filters, which had been predetermined.

had the idea for the new kit, we were unable to write the example for the patent. Nakajima *et al* considered it essential to improve the efficiency of transcription and translation processes when railroad worm luciferase is synthesized in the cell. They altered the arrangement of the genetic sequence and succeeded in altering the gene structure of the enzyme that could be used in mammalian cells in 2003[5]. Although the spectra were stable without being affected by pH, the next issue was how to divide and quantify the overlapping spectra. Obviously, the spectra that do not overlap could be divided by filters and quantified, but this would measure the extremes of green and red, which would mean that only two colors could be handled and most of the luminescence would be wasted. Through the joint effort of AIST, technologists at ATTO Corp., and Associate Professor Akiyama of the Institute of Solid State Physics, we developed a technology to divide and quantify three luminescence colors using two filters without wasting any of the light[6]. In the newly developed technology, luciferase is measured without a filter and with several optical filters to determine the transmission coefficient when various filters are used for each luciferase. The amount of luciferase is calculated from the transmission coefficients after the measurements using the various filters (Figure 3). This series of research was the result of *Type 2 Basic Research*. Although much may be gained through by a great leap of ideas, it is important to accurately grasp the current technology available and to use the "supreme knowledge" available at that point. Rather than producing supreme knowledge, to use it appropriately is the approach of *Type 2 Basic Research*.

The above description explained the success of commercialization in 2005, and although it seemed that the practical application went smoothly, it was not that easy. We wanted to patent the concept quickly, and we wrote the patent when we succeeded with the three-gene, two-substrate method using red and green railroad worm luciferase and existing sea pansy luciferase. Under the title "Genetic construction composed by integrating either of the two luminescent protein genes that emit different color luminescence in same luminescent substrate so there will be stable expression in mammalian cells," we applied for a patent in 2003[7] and published the concept[8]. At that point, we obtained a one-year grace period, which gave us time to study other luciferases. We were finally able to add to the patent of "the three-gene, one-substrate method" by combining the green luciferase from the Iriomote firefly, which was another subject at the time, and its orange mutant[6].

In the patent construction strategy, we received advice from the patent attorney to, first, take advantage of the one-year period after the patent application and, second, to ensure that examples for the core of the patent were written by as few people as possible and that patents were filed individually. The concept was emphasized in the patent, and although the patent describes a range of examples demonstrating such applications, it avoided details that may interfere with the companies' free research activities and product realizations. In particular, we did not include a description of the reagent of the multiple gene expression detection kit, and we left room so that companies can continue research to create reagents for the kit. The concept that formed core of the entire technology was known only to AIST, but the practical patent was constructed by placing priority on the joint patent application through joint research with companies or solo application by a company. As result, the aforementioned two companies worked on product realization and were able to sell the kits under their brands. Because these two companies were biotechnology companies, they were strong in software but weak in hardware. Therefore, we introduced Company A, a measurement instrument company, to ensure smooth product realization.

4 What can the multiple gene expression detection kit do?

We will use the MultiReporter Assay System Tripluc® from Toyobo Co., Ltd. as an example to describe our system. The name of the product, Tripluc, derives from "triple color" (luciferases for three colors and "trip" (to travel into different dimension past the conventional). It represents the general image and reflects the good taste of the company. I believe that the researcher should refrain from suggesting a name for the product because the company marketing department has the expertise to create meaningful product names.

The Toyobo kit is a package comprising green luciferase from Iriomote firefly (λ_{max} 550 nm), orange luciferase that is a site-specific mutant of green luciferase (λ_{max} 580 nm), red luciferase from railroad worm (λ_{max} 630 nm), and a promoter sequence that acts as a control for the three enzymes. Ultimately, the company will developed and marketed the reagent. The kit divides the tricolor luminescence spectra and quantifies these using optical filters, allowing the simultaneous measurement of two or three transcription activities. Because these luciferases all use D-luciferin as the luminescent substrate, the detection reaction can be done in one step, and all measurement is made by a luminometer equipped with optical filters.

Gene expression is regulated by bonding of transcription factors to promoters and cis elements that are present near the transcription start point of the gene. The reporter assay using luciferase can assess gene expression by joining the sequence that regulates gene expression, such as a promoter, to the luciferase gene, introducing this to the test cell, and then measuring the activity of the expressed luciferase. Using this reporter assay, it is possible to analyze the function of the transcription regulation region of a target gene and the mechanism regulating its transcription factor. Conversely, the luciferase gene can be joined to the transcription regulation region of a gene whose expression changes in response to

a certain phenomenon to analyze the signal transmission of that phenomenon or action as well as the mechanism of action of the receptors and ligands.

The multiple gene expression detection kit that we developed can simultaneously analyze the expression of several genes. The kit comprises vectors of tricolor luciferase genes to which a transcription regulation region can be inserted. The user can extract the transcription regulation region of the gene to be measured using some method and insert this in the vector of the kit. The inserted genes are chemically or electrically transduced into the cell or body of an animal or plant. For example, once the gene is transduced into the cell and the chemical substance of interest is added, the expression of the target gene is regulated by the stimulus, which changes the amount of luciferase synthesized. The cells are crushed, the luciferin solution optimized for the measurement condition is added, the luminescence of each color is measured, and the change in gene expression is calculated. Our patent covers the concept of analyzing three gene expression patterns using three colors and the tricolor luciferases. The optimized luciferin solution is covered by corporate patent.

Figure 4 shows the result of a model experiment that provided the measurements[6]. The roles of the bonding site of transcription activation factor (RORE sequence) within the clock gene *Bmal1* promoter sequence that exists in mammalian cells and the peripheral sequences were investigated in the model experiment. In the *Bmal1* promoter sequence, there is one RORE sequence and two similar sequences where the gene transcription activation factor RORα4 could bond, and we compared the roles of the *Bmal1* promoter sequence and an independent RORE sequence (Figure 4A). We created gene vectors with the RORE sequence and SV40 sequence to support the role of the sequence for red luciferase, the promoter sequence including the RORE sequence for orange luciferase, and the SV40 sequence, which acts as a control for green luciferase. These were genetically transduced into the cell (Figure 4B top). Adding the transcription activation factor RORα4 to the cell activated the *Bmal1* promoter and promoted gene expression in proportion to the amount of RORα4, but the luminescence of red luciferase, which represents the transcription activity of the RORE sequence did not increase and gene expression was not promoted sufficiently (Figure 4B). We had expected the sequence similar to the RORE sequence to play a major role in regulating the overall transcription. As seen in this example, the multiple gene expression analysis system enabled the simultaneous observation of the expression of three genes, which used to be a difficult process. We also succeeded for the first time in analyzing simultaneously the time course of expression of multiple genes within living cell[9]. The application of this system is not limited to biological clock analysis, which we investigated, and we expect this system to be applied in the fields of cell biology, pharmacology, and molecular physiology.

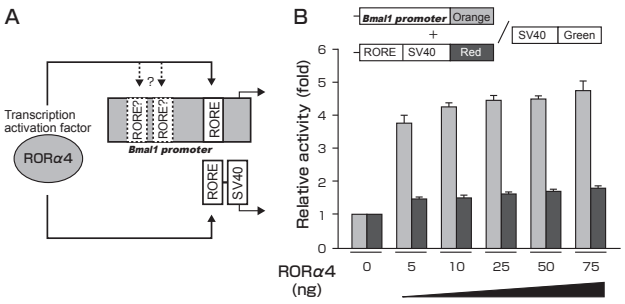

Fig. 4 Example of analysis of gene expression using multiple gene expression kit.

A) The clock gene *Bmal1* promoter sequence in mammalian cells contains one transcription activation factor-bonding site (RORE sequence) where the gene transcription factor RORα4 bonds and changes the amount of expression, and two similar sequences. The gene transcription activity of the RORE sequence can be assessed by extracting the RORE sequence individually and inserting it into the control promoter SV40 sequence.

B) Gene vectors are created with the SV40 sequence, which can support the role of the RORE sequence and the role of that sequence in red luciferase, with the entire promoter sequence including the RORE sequence in orange luciferase, and with the SV40 sequence that will serve as a control in green luciferase. These vectors are then transduced into the cell. Adding the transcription activation factor RORα4 to the cell promotes the expression of *Bmal1* in proportion to the amount of RORα4, whereas adding the RORE sequence only does not promote gene expression.

The multiple gene expression detection kit resulting from our study can be used to compare the responses of three or more genes. Highly reliable biological information can be obtained when assessing the toxicity of chemical substance as an alternative method to animal experimentation and for assessing efficacy in drug screening. We are currently working on a multicolor reporter system for toxicity assessment and trying to expand the use of this technology under NEDO's "Development of Hazard Assessment Method Using Cultured Cell" program. In the future, compatibility of data obtained from the simultaneous measurement of several samples will become important in toxicity assessment, and we are developing a new optical measurement device with high sensitivity and standardized optical metrology. Incorporation of new optical metrology technology will be very important for expanding the use of the multiple gene expression detection kit.

5 There is no end to the scenario of product realization

The basic concept of a multiple gene expression detection kit or "reporter assay for three-genes one-substrate using luciferase with different luminescent colors" has been put into practical use and realized as a product, and progress was made according to the scenario as a strategy. We are halfway

through the scenario, and we have not reached the endpoint. The three issues to address to complete the scenario and our solutions are as follows. 1) Is this concept correct? We shall solve this through *Type 2 Basic Research*. 2) Can the companies realize the products? This will be solved through dialogue between companies that can produce the products. 3) Will the concept be accepted by society? The answer to this is perseverance. It is vital for both researchers and companies to transmit information actively. Further ideas about addressing these issues follow.

1) Is this concept correct? Even if we think it is correct in the beginning, ideas can fail. Many researchers, including us, have experienced failure, although most are never published, so we never know. The importance of *Type 2 Basic Research* is to go beyond literature searches to gather information from other researchers through discussions such as at seminars. This involves making full use of personal connections as researchers and will provide a method to apply the concept in practical use by knowing about the best technology currently available. The important point is not to generate supreme knowledge through ideation, but to use supreme knowledge to expand our understanding to solve particular problems. It is also important to span different disciplines. Because our kit produces light or luminescence spectra using tricolor luciferase, we needed to find a method of quantifying individual colored light. We did not have the knowledge to solve this problem, but it was solved easily by a physicist. Collaborative research across disciplines is a key to promoting *Type 2 Basic Research*.

2) Can the companies realize the products? Product realization can be accomplished by companies with sufficient technological ability if the companies find the concepts and results attractive, feel the patent is worth secure, and perceive that there are potential users in the market. It is important to approach the researchers of a company and to convince the intellectual property division, legal division, sales division, and, of course, management. *Type 2 Basic Research* results from the clear communication of the difference, novelty, superiority, and justification of the new method compared with the conventional method (e.g., technology, cost, and users). However, researchers should not obstruct product realization by other companies but should file patent applications independently and make logical decisions and good judgment according to the contract.

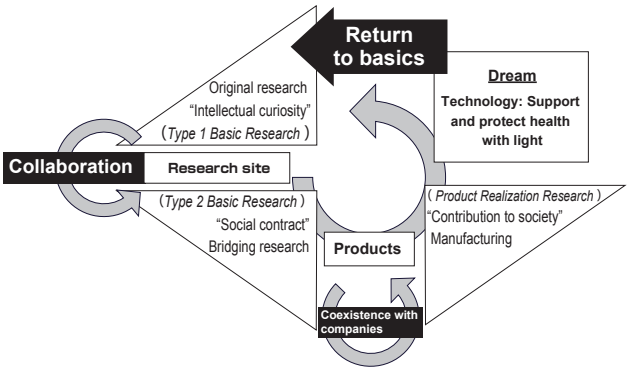

Fig. 5 Continuation of *Type 1 Basic Research*, *Type 2 Basic Research,* and *Product Realization Research* as perceived by the authors.
When *Type 1 Basic Research* develops into *Type 2 Basic Research*, the return from *Type 2 Basic Research* to *Type 1 Basic Research* is important. When *Type 2 Basic Research* develops into *Product Realization Research* with corporate collaboration, it is sometimes important to return to *Type 2 Basic Research* to shape the research results. Even after the product is realized, the research does not end, but returns to *Type 1 Basic Research*, which may take a new direction.

3) Will the concept be accepted by society? The researchers must focus on two aspects. First, they must work with the company to transmit information through, for example, lectures and reviews for seminars organized by the company. Second, the researchers must return to *Type 1 Basic Research* and create new knowledge that maximizes the technology they created. The researchers should help society understand and recognize the value of technology, and work should continue; that is, there is no end to the scenario.

Figure 5 summarizes these steps: *Type 1 Basic Research* develops into *Type 2 Basic Research*, the product is realized through collaboration with companies, and the researchers return to return to *Type 1 Basic Research*. In each step, two-way collaborations among researchers and companies are important.

6 The struggle to realize the dream

Our purpose is to use light to extract biological information. In reality, the information we obtain is a shadow created by light, but it is possible to obtain useful information if that information can be extracted smoothly using light. From such perspective, we are dreaming of developing technology "to support and to protect health with light." Practical use of the multiple gene expression detection kit is the first step toward, and it is now possible to obtain highly reliable biological information through a diversity of bioluminescence, for example, through a high-performance simple hazard assessment method. We expect that increasing the reliability of the biological information obtained at the cellular level will provide alternatives to animal experimentation. It may also be possible to develop technology "to protect health with light." Looking at several aspects of biological information simultaneously provides a view of the cellular dynamism through light, which may provide new information about life "to support health with light." This approach uses *Type 2 Basic Research* to produce new products with the underlying theme of collaboration between and coexistence with companies. The need to generate new knowledge returns to *Type 1 Basic Research* and satisfies our own intellectual

curiosity. These do not involve a planar rotation of research, but instead use a three-dimensional rotating ring, through which we continue our struggle by never returning to the starting point.

Acknowledgment

Type 2 Basic Research for developing the multiple gene expression detection kit was achieved by the staff of the Cell Dynamics Research Group of the Research Institute of Cell Engineering. Product realization was accomplished by the cooperation between Mr. Masayuki Ryufuku, Ms. Chie Suzuki, Mr. Toshiyuki Takeuchi, and others of Toyo B-Net Co., Ltd., and Mr. Tomomi Asai and Mr. Shigeaki Sakai of Toyobo Co., Ltd. Multicolor spectrum analysis was solved by cooperation between Associate Professor Hidefumi Akiyama of the Institute of Solid State Physics and Mr. Hidehiro Kubota, Mr. Toshiteru Emoto, Mr. Shuji Sekiguchi, and others of ATTO Corp. The research was funded by grants from the NEDO Intracellular Dynamism Analysis Project.

References

[1] O. Shimomura: *Bioluminescence*, World Scientific Publishing Co. Ltd. (2006).
[2] K. Imai and Y. Ohmia eds.: *Baio-kemiruminesensu handobukku* (*Handbook of Bio-Chemiluminescence*), Maruzen (2006) (in Japanese).
[3] V. R. Viviani, E. J. Bechara and Y. Ohmiya: Cloning, sequence analysis, and expression of active *Phrixothrix* railroad-worms luciferases: Relationships between bioluminescence spectra and primary structure, *Biochemistry*, 38, 8271-8279 (1999).
[4] V. R. Viviani, A. Uchida, N. Suenaga, M. Ryufuku and Y. Ohmiya: Thr-226 is a key-residue for bioluminescence spectra determination in beetle luciferases, *Biochem. Biophys. Res. Commun*., 280, 1286-1291 (2001).
[5] Y. Nakajima, T. Kimura, C. Suzuki and Y. Ohmiya: Improved expression of novel red- and green-emitting luciferases of *Phrixothrix* railroad worms in mammalian cells, *Biosci. Biotechnol. Biochem*., 68, 948-951 (2004).
[6] Y. Nakajima, T. Kimura, K. Sugata, T. Enomoto, T. Asakawa, H. Kubota, M. Ikeda and Y. Ohmiya: A multicolor luciferase assay system: One-step monitoring of multiple gene expressions with a single substrate, *Biotechniques*, 38, 891-894 (2005).
[7] Y. Ohmiya and Y. Nakajima: Patent Application 2005-506020 (H16/04/30), Multiple Gene Transcription Factor Assay System (in Japanese).
[8] Y. Nakajima, M. Ikeda, T. Kimura, S. Honma, Y. Ohmiya and K. Honma: Role of orphan nuclear receptor RORα in clock gene transcriptions demonstrated by a novel reporter assay system, *FEBS Lett*., 565, 122-126 (2004).
[9] T. Noguchi, M. Ikeda, Y. Ohmiya and Y. Nakajima: Simultaneous monitoring of independent gene expression patterns in two types of cocultured fibroblasts with different color-emitting luciferases, *BMC Biotechnol*., 8, 40 (2008).

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Postscript

After writing this paper, we received the wonderful news that Dr. Osamu Shimomura won the Nobel Prize in Chemistry for 2008 for the discovery of green fluorescent protein (GFP). Dr. Shimomura discovered the luminescent protein aequorin and GFP when he was attempting to clarify the luminescence mechanism of crystal jellyfish *Aequorea victoria*. The luminescent protein aequorin was realized as a product of the early stage in finding a reagent to detect calcium. At the time, no one believed that GFP would have practical application other than the fact that it was "pretty" protein. This was because no one believed that protein itself could emit fluorescence. Thirty years after the discovery, Dr. Prasher, who did not share in the Nobel Prize, isolated the genes for GFP in 1992 and discovered that the protein itself emits fluorescence. Dr. Chalfie, who received the Nobel Prize in Chemistry jointly with Dr. Shimomura, applied it to animal cells in 1994, and Dr. Tsien created GFP with different colors in 1998. Positioning the roles of theses researchers, Dr. Shimomura and Dr. Prasher engaged in *Type 1 Basic Research*, Dr. Chalfie conducted *Type 2 Basic Research*, and Dr. Tsien conducted *Product Realization Research*. We recognize the importance of *Full Research*, and we appreciate that *Type 2 Basic Research* and *Product Realization Research* have potential to change society.

Dr Ohmiya maintains contact with Dr. Shimomura and can consult him about the research. Dr. Shimomura often points out the importance of *Type 1 Basic Research* and urges those who engage in *Type 2 Basic Research* to continue studying the unsolved luminescent mechanism. He is aware that clarifying the luminescence mechanism may lead to new applications. For example, he points out that copper ion may be necessary for luminescence in certain luminescent shellfish and that a new metal ion sensor may become possible if this luminescent mechanism is clarified. *Type 2 Basic Research* does not evolve without *Type 1 Basic Research*. Conversely, *Type 2 Basic Research* and Product Realization Research are necessary to fully understand and apply *Type 1 Basic Research*. Dr. Shimomura is aware of these relationships.

This year's Nobel Prize in Chemistry reminds us of the importance of the full range of research activities. Our research group wishes to respond to Dr. Shimomura's expectation by working on *Type 2 Basic Research* and *Product Realization Research* on the luminescence of the sea-firefly, on which Dr. Shimomura worked previously, and to engage in *Type 1 Basic Research* for unsolved luminescence phenomenon (Ohmiya).

Authors

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Completed studies in endocrinology, Gunma University Graduate School of Medicine in 1990. Worked as researcher at Osaka Bioscience Institute, researcher of Original Independent Research "Sakigake 21: Light and Materials" of the Japan Science and Technology Corporation, and associate professor in the Faculty of Education, Shizuoka University. Joined the National Institute of Advanced Industrial Science and Technology in 2001. Currently dispatched as professor of photobiology, Lectures in Advanced Medicine, School of Medicine, Hokkaido University from October 2006. Group leader of Cell Dynamics Research Group, Research Institute of Cell Engineering. Worked partly on NEDO "Development of Hazard Assessment Method Using Cultured Cell." Council member of the Japanese Biochemical Society, person-in-charge of bioluminescence and chemical luminescence, and editor of international academic journal Luminescence. In this paper, worked on overall integration.

Yoshihiro Nakajima
Completed studies in production information science at the Graduate School of Science and Engineering, Saitama University in 1996. Worked as a researcher of Basic Special Science, RIKEN, and researcher of future pioneering at the Japan Society for Promotion of Science (Nara Institute of Science and Technology). Joined the National Institute of Advanced Industrial Science and Technology in 2001. Dispatched to the office of the Council of Science and Technology Policy, Cabinet Office, Government of Japan in 2007. In this paper, worked mainly on luciferase and construction and optimization of the measurement system.

Discussion with Reviewers

1 Patent construction for product realization

Question & comment (Hiroshi Kuriyama)

In chapter 3, you wrote that you "developed a method for dividing and quantifying luminescent color without decreasing light efficiency" in cooperation with Associate Professor Dr. Akiyama of the Institute of Solid State Physics, the University of Tokyo, and I think the content of that method is important point of *Type 2 Basic Research*. Can you please describe it in detail?

Answer (Yoshihiro Ohmiya)

We agree that this was not explained clearly. I added Figure 3 and the following sentence. "In the newly developed technology, luciferase is measured without a filter and with several optical filters to determine the transmission coefficient when various filters are used for each luciferase. The amount of luciferase is calculated from the transmission coefficients after the measurements using the various filters (Figure 3)."

2 Construction of multiple gene expression detection technology

Question & comment (Naoto Kobayashi)

I can understand very well that the authors wrote a clear scenario toward the goal of realizing multiple gene expression detection kit, and engaged in R&D along that scenario. In that sense, I can see the strategic effort toward the practical use of the result of simultaneous detection of three gene expressions for the first time in the world, and I think this paper is highly valuable. On the other hand, I think the synthetic description of "multiple gene expression detection technology using bioluminescence phenomena" that is the central issue of this research is lacking. Although the details of this technology have been published in separate papers, I think you should describe the details on how you were able to construct the multiple gene expression detection technology.

Answer (Yoshihiro Ohmiya)

The multiple gene expression detection kit that we developed is a system for analyzing simultaneously the expression of several genes. The kit comprises vectors of the tricolor luciferase gene to which a transcription regulation region can be inserted. The user extracts the transcription regulation region of the gene of interest using a particular method and inserts it into the vector provided with the kit. Transducing this vector into the cell and adding a particular chemical stimulates the expression of the target gene in the cell, and the amount of luciferase synthesized changes. The cell is crushed, the luciferin solution optimized for that measurement condition is added, the amount of luminescence for each color is measured, and the change in the gene expression pattern is assessed. Our patent covers the concept of analyzing the expression of three genes with three colors and tricolor luciferase. The optimized luciferin solution is covered by a corporate patent. The technological method for synthesizing the multiple gene expression detection technology was added to Section 4.

3 Improvements in optical metrology technology

Question & comment (Naoto Kobayashi)

I would like to ask about the optical metrology technology. You mentioned that you used a luminometer equipped with an optical filter for optical measurements. Are the technologies including measurement sensitivity, range, and SN performance complete? Please mention any improvements for the future optical metrology technology.

Answer (Yoshihiro Ohmiya)

Use of optical metrology technology is mandatory for the diffusion of our technology. From that perspective, we have added the following sentences. "In the future, compatibility of data obtained from the simultaneous measurement of several samples will become important in toxicity assessment, and we are developing a new optical measurement device with high sensitivity and standardized optical metrology. Incorporation of new optical metrology technology will be very important for expanding the use of the multiple gene expression detection kit."

4 Future development as an alternative to animal experimentation

Question & comment (Naoto Kobayashi)

The result of this research is greatly significant for the practical application, and I think you had better explain the future developments including whether this method can actually be used as an alternative to the animal experiment, or whether the observation using this method to observe the intracellular dynamics represents the actual *in vivo* reactions.

Answer (Yoshihiro Ohmiya)

We recognize that the technology for obtaining biological information using light is not all-powerful and that any errors in reading the light would lead to the wrong conclusion. When we say that biological information is obtained through light, we mean that the information is the shadow produced by light, so we cannot obtain useful information unless we can measure light accurately. This point has been added to the text along with the following sentences. "We expect that increasing the reliability of the biological information obtained at the cellular level will provide alternatives to animal experimentation. It may also be possible to develop technology "to protect health with light." Looking at several aspects of biological information simultaneously provides a view of the cellular dynamism through light, which may provide new information about life "to support health with light."

5 Improvement of precision

Question & comment (Naoto Kobayashi)

I understand that the "method for detecting the expression of three genes simultaneously using bioluminescence phenomena" is extremely effective. For the improvement of precision in the future, will you need more than three colors? Or do you think three colors are sufficient as a basic method?

Answer (Yoshihiro Ohmiya)

In terms of improving accuracy, I think it is important to perform simultaneous analysis with three or more colors. However, the light that can be made from firefly is green to red light with luminescence peaks at 540-430 nm. Human eyes can detect subtle differences in color, whereas a camera has only a limited ability to distinguish faint light. Because the light of bioluminescence is broad light, we have chosen a detection method that is applicable to broad light with 30 nm separation, and three colors is the limit at present. This points to the need for further *Type 2 Basic Research* to develop a new metrological system to broaden the applications. In addition, in gene expression analysis with more than three colors, the method for gene transduction into cells will become an important issue. Thus, we expect that this *Type 2 Basic Research* will lead to further *Type 2 Basic Research*.