Real Time Gene Expression Measurement in Living Cells

AB-2550 Kronos Dio

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ATTO

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Monitoring the gene expression of living cells

35mm culture dishes

Luminescence measurement device for real-time reporter assay

AB-2550 Kronos Dio

AB-2550 Kronos Dio is a luminescence measurement device (luminometer) that equips a photomultiplier tube as a detector. This device is suitable to monitor gene expression of culture cells and cultured tissue slice at fixed intervals over a period of several hours to several days using 35mm diameter culture dish as a sample container.

For the long term cultures, it is possible not only to control temperature in the cabinet by the air circulation but also to introduce CO . Additionally, because an optical filter is built in, it is possible to monitor up to three kinds of gene expression using luciferase with different colors of luminescence.

Flow of sample measurement using Kronos Dio

Kronos Dio measures luminescence of the cells in the culture dish.

①Sample preparation

Stimulate the cell line or the tissue slice to which the reporter vector has been transfected, and add the luminescent substrate luciferin to the medium. Set the culture dish in Kronos Dio.

Kronos Dio measures luminescence at regular intervals.
Temperature and CO2 control
With built-in filter and color separation function
PC control
Culture conditions can be monitored. (temperature and CO2 density)

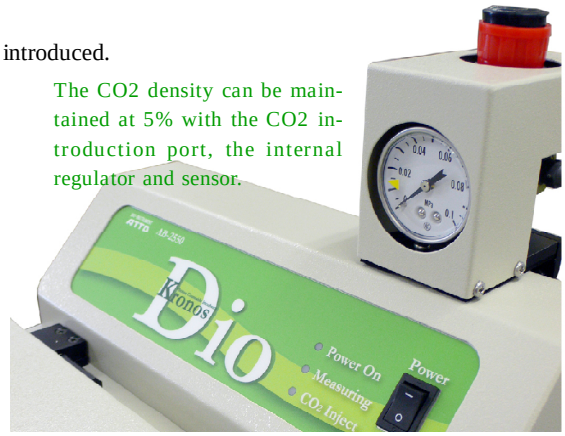
②Real-time measurements

The interior of the Kronos is kept at a constant suitable temperature for culturing. CO2 can be introduced as necessary. Luminescence is measured at regular intervals, and the expression of the target gene is monitored.

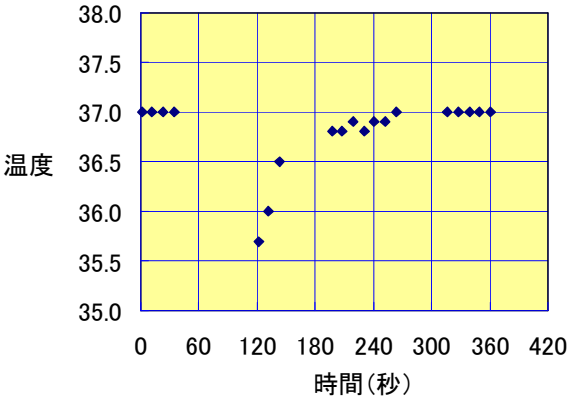
To measure living cells

In the chamber, the temperature is kept at a constant level and CO2 gas can be introduced.

For general cultures of cells, constant temperature conditions of 30 to 40 degrees are essential. AB-2550 Kronos Dio is equipped with a constant temperature device using Peltier device, so it is possible to maintain the temperature in the chamber at a constant level (25 to 45 degrees:room temperature 25 degrees). Moreover, for cells that need to be cultured with CO2 introduced, it is possible to maintain CO2 density at 5% (CO2 sensor and regulator equipped).



The CO2 density can be maintained at 5% with the CO2 introduction port, the internal regulator and sensor.



The time taken to recover was measured when the door of the chamber was opened and closed while maintaining a constant temperature of 37 degrees. The top part of the chamber is designed to be opened fully, however, it recovered to 37 degrees in 2 to 3 minutes (room temperature 25 degrres).

There is a turntable in the chamber, and eight 35mm diameter culture dishes can be set. There is a detector (condenser lens + photomultiplier tube) under position A in the photo.

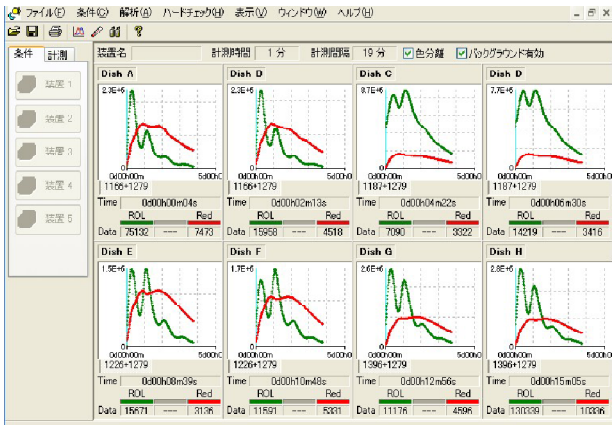
Applicable to long term measurements

and multicolor luminescence measurement

Measurement function

Kronos Dio can hold up to eight 35 mm diameter culture dishes in the chamber. The turntable has a structure which no stray light can cause interference between each dish. Because dishes can be exchanged easily by opening the door of the chamber, stimulation with drugs can easily be performed. After the door is closed, temperature rises promptly, so a stable culture condition can be obtained.

Luminescence is measured for long time at regular intervals. This is applicable to multicolor assay measurement to obtain luminescence of three colors using one substrate because a color separation mechanism is built-in.



The inner filters are switched sequentially and luminescence permeating through the filter is measured by the photomultiplier tube.

Gene expression analysis and bioluminescence

Pacific Jellyfish
Reprinted from Bioscience Saizensen(1998) ATTO Corp."Discovery of aequorin and GFP" by Osamu Shimomura.

The mutual adjustment of the expression of various genes within a cell a fundamental control process at the work within all living bodies, which is to say that there is a gene expression network. To analyze this kind of adjustment mechanism, the amount of mRNA, which is a transcript product or the amount of protein, which is a translation product can be masured directly. However, the reporter gene assay using luciferin-luciferase reaction is widely used because the expression can be quantitatively monitored more easily and more sensitively.

Firefly (Photo provided by: Dr.Kazuki Niwa)

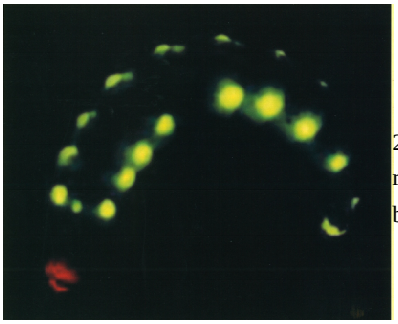
GFP and firefly luciferase are used as a reporter protein.
GFP is fluorescent protein and it is widely used as a reporter of the gene expression.
The search results of PubMed in 2005 are shown in the table above. One characteristic point is that in comparing total number of hit, the

PubMed Result	luciferase	GFP
All	2,220	3,976
& Gene Expression	1,076	1,234
& Transcription Factor	1,307	524
& siRNA	159	183
& G-protein	67	228

number of GFP is larger but, luminescent protein is more frequently used for studies related to transcription factors. GFP is an extremely stable protein that absorbs light of 470 to 490 nm and emits green fluorescence. It does not require a luminescent substrate such as luciferase, so it is advantageous in terms of cost, and it is a very convenient reporter when confirming the presence or localization of expression over time.

However, when measuring fluctuations of expression over time, the fact that GFP is a stable protein becomes a disadvantage and it becomes difficult to monitor subtle difference. (It has become possible to obtain GFP with a shorter life in order to compensate for this disadvantage.) Moreover, for measuring the expression amount of the gene over time, it is necessary to assume that excitation light irradiation may easily damage the cell. Firefly luciferase is a protein with a molecular-size of approx. 62kDa, and it is normally considered as having a shorter half-life of around 3 hours. Luciferase with an even shorter life can also be used. Luciferin, the luminescent substrate is sold as an item enclosed in a kit together with a crystal product, cytolysis agent and reactive reagent, so it is utilized according to different needs.

Luciferin shows specific luminescent reactions with luciferase and has few nonspecific reactions, therefore its S/N is satisfactory and it has excellent quantitativeness. It is also considered that cell toxicity from luciferin is small, so that it is possible to monitor fluctuation of transcript revitalization real-time for extend periods by just resolving luciferin in the medium.



2 color luminescence of railroad worm (photo provided by: Dr.Vadim Viviani)

As mentioned at the beginning, the method of measuring the amount of mRNA is also used normally. When this method is used, a cell needs to be crushed to extract RNA, so when measuring temporal changes, it is necessary to prepare cells of the same quality for the amount equivalent to the number of measurement times. Besides, RNA needs to be extracted within a limited time, so it becomes quite a serious task. It is also known that there is not necessarily a correlation between the amount of RNA and the amount of the translation when comparing the amounts of the two kinds of RNA.

Luminescence reporter protein list used

Emitted light protein	Origin animal	Luminescence substrate	Usage	Luminescence wavelength	Remarks
Luciferase	Firefly	D-luciferin	Reporter gene assay	560～620nm	Luminescence yield changes by the pH when reacting.
	Railroad worm	D-luciferin	Reporter gene assay	620nm	There is no pH dependency and it emits light red.
	Click beetle	D-luciferin	Reporter gene assay	540nm	There is no pH dependency and it emits light to green.
	Rhagophthalmus obai	D-luciferin	Reporter gene assay	550、580nm	There are two kinds that the pH- independent emits light to green & orange
	Seapansy(Renilla)	Coelen terazine	Reporter gene assay	470nm	
	Photobacterium	FMN	Reporter gene assay	495nm	
Secretion Luciferase	Sea firefly	Sea firefly luciferin	Reporter gene assay	462nm	
	Copepod	Coelenterazine	Reporter gene assay	495nm	
Others	Apoaeguorin	Coelenterazine	Measurement of density of calcium	470nm	

■ Various luminescence measurement devices

For a device to monitor gene expression using bioluminescence, highly sensitive luminescence detector is used. A typical units are a PhotoMultiplier Tube (PMT) and a coolded CCD. AB-2550 Kronos Dio is equipped with a PhotoMultiplier Tube.

PhotoMultiplier Tube (PMT)

PMT's detectable wavelength area is determaind by the material of a photoelectric surface that converts the incident light to electron and the material of the window that keeps it in a vaccum state. In the case of bioluminescence, the luminescence is in the visible region, so the detection efficiency is decided mainly by the material of the photoelectric surface. In a photomultiplier tube equipped device, when the luminescence has a different wavelength even if the amount of light is the same (the number of photons is the same in this case), the signal value obtained various. This is due to the wavelength dependency of the electronic conversion efficiency (quantum efficiency) on the photoelectric surface.

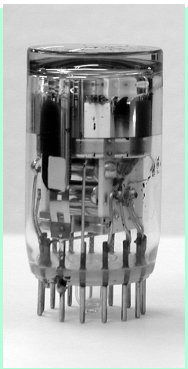
Firefly luciferase changes the color of its luminescence from yellow green to red according to a change in pH. Care must be taken when trying to assay using this luciferase without lysing a cell because the signal intensity changes according to changes in pH in the cell.

CCD (Charge Coupled Device)

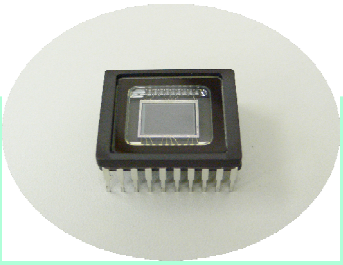
Along with the popularization of digital camers, CCDs have become well known optical sensors, which no longer require explanation. This sensor's quantum efficiency is not low in the red luminescence though PMT is low in red. Although it is expensive, a back illuminated CCD has a high quantum efficiency of about 90% and is suitable for measurement of weak light.

Instantaneous luminescence and stable luminescence

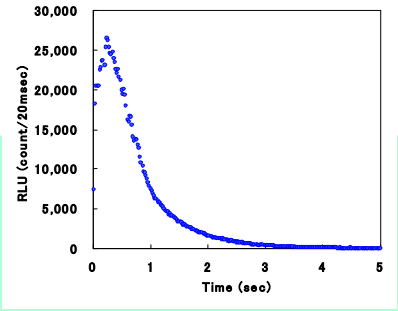
Depending on the reaction conditions, light emission may finish within a few seconds or it may be relatively stable for about 60min. In the case of stable emission, the amount of luminescence can be measured with the luminescence measurement device after starting a luminescent reaction, so it is not necessary for the device to be equipped with a injection pump. In AB-2550 Kronos Dio, the luciferase assay that uses a stable luminescent reaction with a luminescent protein as a reporter will be the main target.
On the other hand in the case of instantaneous emission, luminescence at dispensing reagent is often the strongest, therefore to measure the amount of luminescence just after dispensing, a injection pump must be equipped. So AB-2550 Kronos Dio is not suitable for this usage.



PMT



CCD



30mM Ca(NO) 200 L was dispensed to 0.1ng/ μ L aequorin 10 μ L using a pump and luminescence was measured for 20 seconds (using Lminessensor JNRII). The graph above shows kinetics for 5 seconds.

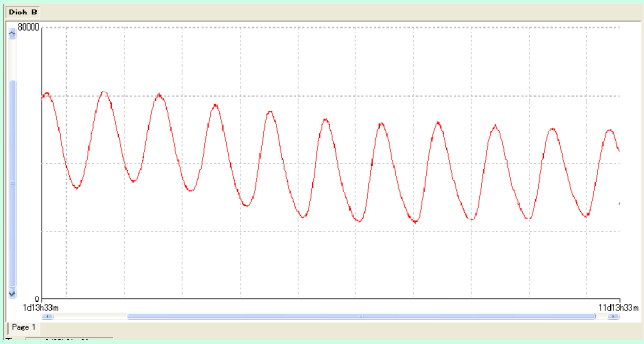
Example of gene expression measurment

The firefly luciferin permeates easily into the cell. When using luciferase as a reporter protein, real-time reporter assay is achieved by adding luciferin to the medium.

Real Time Reporter Assay for long term

Luciferase is used to analyze the function of the clock gene. The study progresses from various aspects including the relationship with disease, chronotherapy, etc.

A transgenic mouse including luciferase gene with a promoter of clock gene (*Per 1*) related to circadian rhythm was added with luciferase gene was kept with the period from 06:00hrs to 18:00hrs as the light period, and its suprachiasmatica nucleus (SCN) was cut into 300 m slices using a micro slicer and the slices were placed on culture inserts (Millicell CM) and monitored for 11days. Extremely stable circadian rhythm was detected.



<Data Supported> Dr. Kenichi Honma

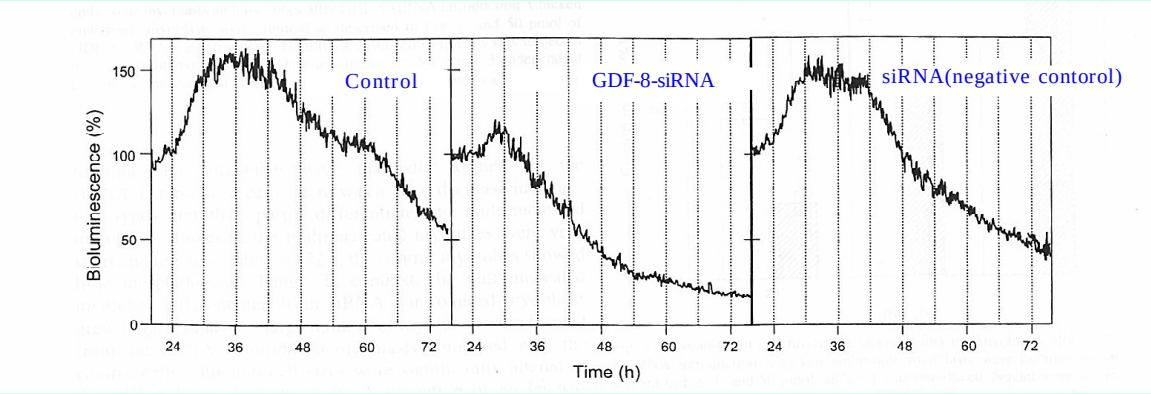
Dept. of physiology, Hokkaido University Graduate School of Medicine.

[Reference] Shin-ya Nishide , Sato Honma , Yoshihiro Nakajima , Masaaki Ikeda , Kenkiti Baba , Yoshihiro Ohmiya and Ken-ichi Honma (2006) New reporter system for *per1* and *Bmal1* expressions revealed self-sustained circadian rhythms in peripheral tissues. *Gene to Cells*, **11**, 1173-1182

Measurement of RNA interference (siRNA) effect

The effect of prepared siRNA can be examined. Because this effect is maintained for a long time, when luciferase is used as the reporter protein, the effect in the period can be easily measured.

After introducing siRNA of Growth/Differention Factor 8 (Myostatin) to Myoblasts of a chicken with Lipofectamine, promoter activity of GDF-8 was compared for 3 days and the effect was detected.



[Data supported] Dr.Akira Hattori

Dept. of Animal and Marine Bioresource Sciences, Faculty of Agriculture, Kyushu University.

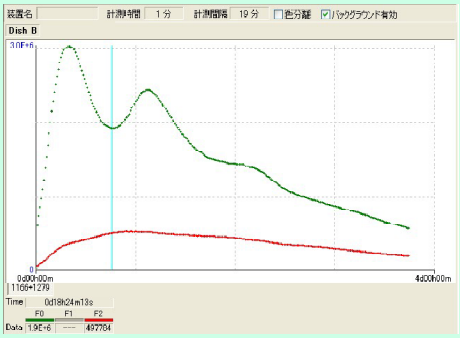
[Reference] Fujimori Sato, Masatoshi Kurokawa, Nobuhiko Yamauchi and Masa-aki Hattori (2006) Gene silencing of myostatin in differential of chicken embryonic myoblasts by small interfering RNA. Am J Physiol Cell Physiol , 291, C538-C545

Example of measuring gene appearance

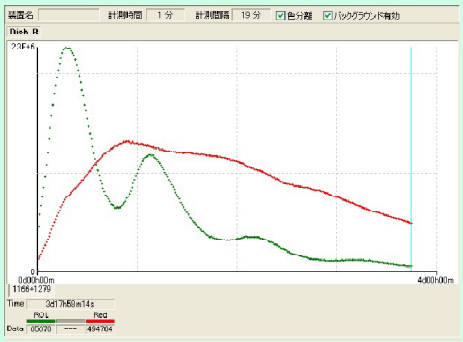
Real Time Reporter Assay of two or more genes

If you use multicolor luciferases, two kinds of gene expressions can be monitored in real time.

Vector 1μg in which firefly green emitted luciferase is inserted in the downstream of mouse clock gene *Per2* promoter and, vector 0.2μg in which the railroad worm red emitted luciferase is inserted in the downstream of the SV40 promoter, was cotrausfected to mouse fibroblast NIH3T3 by the lipofection method. After it cultures for 24 hours in the CO2 incubator, it was exposed for one hour to Dexamethasone of 0.1μM. And it was exchanged to DMEM including 200 M D-luciferin and 10% FBS, the luciferase activity was measured in 19 minutes intervals with F0(no filter) and F2(R62 filter).



Two kinds of luciferase activity
data before calculation



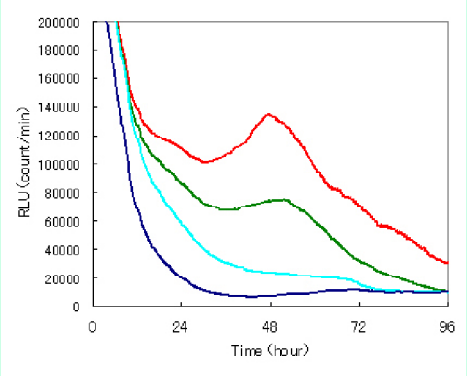
Two kinds of luciferase revitalizations
after separation is calculated

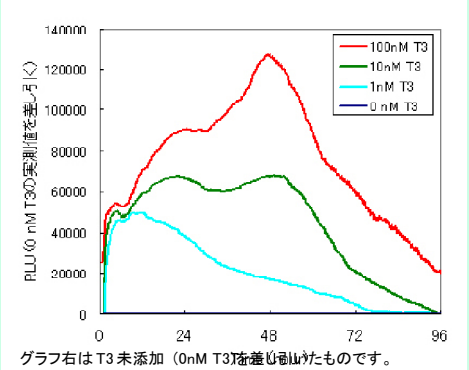
＜Data Support＞Dr.Yoshihiro Nakajima
National Institute of Advanced Industrial Science and Technology, cell dynamics research group

Drug stimulation response

The influence of the drug on the gene can be measured in time course.

The reporter vector including the firefly luciferase gene with the downstream of growth hormone (GH) promoter was transfected into the GH3 cell. ① This cell is cultured with the medium, ② it exchanges for OPTI-MEM, ③ cultures it for three hours, thyroid hormone (T3) is added, ④ the transcription activity of GH promoter was measured in real-time. It can be confirmed that there is no difference in the effect duration and the transcript activity of the GH promoter depends on the concentration of T3.





グラフ右はT3未添加（0nM T3）を差し引いたものです。

Specification

20070119MAS

Model/Name	AB-2550 Kronos Dio
Mesurement container	35mm culture dish
Throughput	8 dishes
Incubation	Peltier device and air circulation
Temperature setting	(Room temperature -5℃) ～45℃ 1℃ Step
Temperature accuracy	±0.5℃ (at room temperature 25℃)
CO2 gas	Control with sensor and regulator(5% in density)
CO2 sensor accuracy	±0.1%
Detector	Photomultiplier Tube
Measurement method	Photon counting method with Photomultiplier Tube
Range of detection wave length	350nm～670nm
Luminescence amount measurement time	1～60sec／1～60min
Filter	F0：Non、F1：056 filter、F2：R62filter
Control	PC (Windows XP/2000) with the control software.
Connection with PC	USB
Size and weight	280(W)×400(D)×330(H)mm ・ 16.0kg
Power supply and power consumption	AC100V・Maximum150W

Composition	Main body
	CO2 gas regulator
	Kronos control software USB (Windows XP version) cable AC cable
	USB cable
	AC cable
	Manual

※ PC is not included in AB-2550Kronos Dio

※ Recomended PC specification is Windows XP, 512MB RAM, 1GB hardward space