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Research paper



Comparison of versatile vs. dedicated detector as a luminescence detection platform

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Abstract

The measurement of light emission signals in various experiments is a routine task to evaluate numerous biological events quantitatively. To accomplish these tasks, two types of detectors have been employed both EnVision (EnVision® Multilabel Plate Reader, Xcite) and Victor (VICTOR™ Luminescence Counter, Light). EnVision is a multimode instrument with complicated detection chamber to measure UV-Vis absorbance, fluorescence intensity, time-resolved fluorescence, fluorescence polarization and luminescence. However, Victor is a simple dedicated platform for the luminescence detection. To test the luminescence detector functionality, a functional assay system for the G-protein coupled receptor was utilized. The aequorin based functional assay system for the calcium sensing receptor with HEK293-aeq/hCaS cells presented higher signal-to-noise ratio with Victor than EnVision for both of the endogenous and targeted receptors because of decreased background values. The dynamic range and data variation associated with the signal measurements were similar in both of instruments. In conclusion, both types of instrument provided comparable results for the luminescence detection that can be applied in variety of light emission experiments. However, the dedicated luminescence detector, Victor Light, with a simple optical path and jet-direct injectors provided extremely low background which is a real benefit for the flash-type luminescence applications.

Keywords: *Aequorin; Calcium mobilization; Detector platform; Drug screening; G protein coupled receptor; Luminescence detection*

1. Introduction

G protein coupled receptors (GPCRs) are the most important drug target classes [1] accounting for about 40% of current drugs on the market and involved in various physiological processes [2]. Drug development efforts have been focused on these molecular classes as they are located on the cell surface making them easier to target and modulate cellular activities. According to the recent market analysis by Insight Pharma Reports, GPCRs are favored for approximately one-third of approved drugs and for hundreds of drugs currently in development. Many GPCRs trigger, upon binding of an agonist, a transient increase in intracellular calcium concentration [3, 4]. This variation acts as an internal secondary messenger and is an important modulator of many physiological mechanisms [5]. Among hundreds of GPCRs, the calcium sensing receptor was set as a model assay system for the current study [6].

Calcium ions (Ca^{2+}) are of vital importance to many physiological processes for humans and other mammals. It plays a pivotal role in a myriad of crucial physiology and biochemistry of organisms and the cell [7]. Intracellular calcium is essential for controlling various cellular processes such as differentiation, proliferation, secretion and mobility, serving as a second messenger for a large number of receptor-mediated event [8]. The level of free calcium within the cytosol, while dependent on the presence of extracellular calcium, are much lower than the latter (100 nM vs 1.1-1.3 mM) [9]. Extracellular calcium is maintained within a narrow range, whereas the level of internal calcium fluctuates rapidly and over a broad range (10-fold or more) in response to hormonal and other stimuli [8].

The calcium sensing receptor (CaS) is a member of the G protein-coupled receptor superfamily and was first cloned from a bovine parathyroid gland cDNA library in 1993 [10]. This receptor is highly expressed in tissues involved in regulating extracellular calcium concentration, including the parathyroid, calcitonin-secreting cells of the thyroid (C-cells), and several regions of the kidney [11, 12]. The physiological function of CaS is to coordinate calcium homeostasis by regulating the release of parathyroid hormone (PTH) and cell proliferation in the parathyroid glands [13]. Activations of CaS by increased extracellular calcium leads to an inhibition PTH secretion, whereas its presumed inactivation by lowered extracellular calcium leads to an increase in PTH secretion. As an alternative approach to the PTH analogues, and yet achieve similar anabolic effects on bone, is based on the use of CaS antagonist which block the parathyroid cell CaS and stimulate secretion of endogenous PTH [14]. This may result in transient increase in circulating PTH levels which in turn may stimulate bone formation [12, 15, 16]. With growing interests on potential therapeutics [17, 18] based on the CaS receptor physiology, experimental systems for the search of CaS antagonists have drawn strong attention [19].

Measurement of intracellular calcium concentration in cells expressing a GPCR can thus be used to monitor the efficiency of activation of a GPCR by ligand [20-22]. When the cells exposed to an agonist of the GPCR, the intracellular calcium concentration increases and these changes have been detected by fluorescence or luminescence technology [22]. The luminescence detection is regarded as a better

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option as it can lower the light interference. For luminescence platform, calcium increase leads to the activation of the catalytic activity of aequorin, which oxidizes coelenterazine and yields apoaequorin, coelenteramide, CO_2 and light [23].

Aequorin from the jellyfish, *Aequorea Victoria*, is a calcium-sensitive photoprotein and has been almost exclusively for monitoring intracellular calcium concentration [24]. The apo-enzyme (apoaequorin) is a 21 kD protein, which requires a hydrophobic prosthetic group, coelenterazine, to be converted to aequorin, the active form of the enzyme. The binding of calcium ions to the three highly conserved calcium binding sites of the active complex induces a conformational change, resulting in the oxidation of coelenterazine to coelenteramide and the emission of photons in the visible range (λ_{max} = 470 nm) [25]. In this way, aequorin oxidizes coelenterazine into coelenteramide with the production of CO_2 and emission of light [26].

The consumption of aequorin is proportional to the calcium concentration within a physiological range (50 nM to 50 μM) [27, 28]. Therefore, measurement of the light emitted upon oxidation of coelenterazine is a reliable tool for the measurement of intracellular calcium flux and furthermore generates results comparable to those obtained with traditional fluorescent dyes [23]. This technical scheme was confirmed that the intensity of light emission is proportional to the increase in intracellular calcium in the physiological range [29]. Thus, in this system, measurement of light emission following agonist addition reflects its ability to activate the GPCR. Because light is emitted for only 20 to 30 seconds after activation of the GPCR, recording of the emitted light must be performed during the few seconds following agonist addition to the cells. This flash-type signal reflects the transient increase in calcium concentration following GPCR stimulation and a flash type luminescence reaction can only be measured with an instrument equipped with an automatic dispenser [30, 31]. Some of major instruments were listed in Table 1.

Table 1: Commercial detectors for the luminescence detection

List of detector	Vendor
Lumax ultra-sensitive flash luminescence reader	Cybio
FDSS kinetic fluorescence plate reader	Hamamatsu
ViewLux ultraHTS microplate imager	PerkinElmer
FLIPR-TETRA fluorescence imaging plate reader	Molecular devices
MicroBeta LumiJET	PerkinElmer
Centro luminometer	Berthold
PHERAstar microplate reader	BMG LABTECH
Victor X light luminescence plate reader	PerkinElmer
EnVision multilabel reader	PerkinElmer

Together with these detectors, growing number of instruments for the luminescence detection are on the market. Among them, an important set of machine, EnVision (EnVision® Multilabel Plate Reader, Xcite) and VICTOR (VICTOR™ Luminescence Counter, Light), is selected as a realistic comparison for the detector with implemented injectors to measure the flash luminescence. The reason for this simplified study is that they are both from same company so that detector components are regarded homogeneous except for their complexity and functionality. In addition to the difference in its optical design, the injector system is quite different. Therefore, this type of comparison can give us an insight for the choice of detector between the versatile with multiple functionality and dedicated with limited application, especially for real-time kinetic readings that is essential for the flash-type luminescence application.

EnVision is a multi-detector instrument, designed with modular label-specific optical mirror modules, high energy flash lamps, and detectors. However, VICTOR is a simple luminescence counter for microplate applications, including various cell-based assays. As a small benchtop unit, it is cost effective detector with a reliable integrated injector system dedicated for luminescence detection. These two detector platform would be an ideal example of photomultiplier based detectors to compare for their sensitivity and overall performance except for those CCD based detectors.

In this report, both types of instrument were evaluated with the aequorin functional assay system for the model GPCR, calcium sensing receptor (CaS) [31], to help research communities to decide which detector can be the best fit for their luminescence applications.

2. Materials and Methods

2.1. Materials

The aequorin parental cell line (ES-000-A30) was licensed from PerkinElmer life and analytical sciences (Boston, USA). cDNA for CaS receptor (GenBank Acc# U20760) were from ORIGENE (Rockville, USA). The calcium chloride was from BIOSESANG Inc. (Bundang, Korea). Eagle's minimum essential medium was from Lonza group Ltd. (Basel, Switzerland). Fetal bovine serum and antibiotics were purchased from Invitrogen Corporation (Carlsbad, USA). 96-well black flat bottom polystyrene microplates (cat# 3915) were from Corning life science (Corning, USA). The chromophore cofactor, coelenterazine h, for calcium mobilization assay was from DiscoverX (cat# 90-0084, USA).

The luminescence signal was measured to compare in EnVision (EnVision® Multilabel Plate Reader, Xcite) and VICTOR (VICTOR™ Luminescence Counter, Light) both from PerkinElmer life and analytical sciences (Boston, USA).

2.2. Cell culture

The aequorin parental cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml zeocin. Cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2.

To establish HEK293-aeq/hCaS, a stable cell line for the human CaS receptor, the cDNA for CaS (GenBank Acc# U20760) was transfected to aequorin parental cells (ES-000-A30) with 4D-Nucleofector system (LONZA, Switzerland) according to the manufacturer's instructions. During the clonal selection for the search of maximum calcium responses in transfected cells, the concentration of blasticidin S was kept at 5 μg/ml. To obtain reproducible calcium responses, cells were split every 5 days before they became confluent. Cells were discarded after 2-3 months of continuous growth with splitting, and new cells were prepared from a frozen stock.

2.3. Measurements of intracellular calcium mobilization

The functional assay based on the luminescence of mitochondrial aequorin following intracellular calcium release was performed using HEK293-aeq/hCaS cells generated from the aequorin parental cell line (ES-000-A30). Briefly, HEK293-aeq/hCaS cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml zeocin and 5 µg/ml blasticidin S. Cells were grown at mid-log phase without antibiotics 18 hours before the day of experiment and washed with phosphate buffered saline followed by gently detaching with Accutase™.

Collected cells from culture plates were centrifuged and resuspended in assay buffer (DMEM/HAM's F12 without phenol red, with L-Glutamine, 15 mM HEPES, pH 7.0 and 0.1% BSA) at a density of 2×10^6 cells/ml. HEK293-aeq/hCaS cells were incubated for 4 hours at room temperature in the dark with 5 µM of coelenterazine h in a constant agitation. After the coelenterazine loading, cells were diluted in assay buffer at a concentration of 2×10^5 cells/ml and incubated for 60 min as described above.

For the agonist response, 50 µl of cells (i.e., 10,000 cells) were injected over 50 µl of agonists (calcium chloride, 0.1M – 45.7 µM) plated in 96 well plates. For the antagonist response, the mixture of 50 µl of cells (i.e., 10,000 cells) and 50 µl of antagonists (NPS-1, 3µM 40(AUC).

2.4. Statistical analysis

The data from dose-response experiments were analysed using the nonlinear curve fitting functions in PRISM version 5.0 (GraphPad Software Inc., San Diego, USA). The classical equation for one site-competition binding for the nonlinear regression analysis was used. Other statistical data were expressed as the mean ± SEM and analysed using Microsoft Excel (Microsoft Corporation, Redmond, USA). In all comparisons by unpaired t-test, the difference was considered to be statistically significant at $p < 0.05$.

Z' factors were utilized as assay coefficients that are reflective of both the assay signal dynamic range and the data variation associated with the signal measurements, and therefore is suitable for assay quality assessment [32]. For representations of the quality of assay itself, Z' factor was calculated as the following equation (1).

$$Z' = 1 - ([3 \times SD_{c+}] + [3 \times SD_{c-}]) / (Mean_{c+} - Mean_{c-}) \quad (1)$$

SD_{c+} and SD_{c-} were denoted for the standard deviations of the positive control and negative control signals. $Mean_{c+}$ and $Mean_{c-}$ were represented for the means of the positive control and negative control signals.

3. Results

The aequorin photoprotein undergoes a luminescent reaction in the presence of calcium ions, producing a flash of light peaking at 700 nm. During 20 to 30 seconds of light emission after the receptor activation, the emitted light was measured by EnVision and Victor representing a versatile multimode detector and a simple dedicated luminescence detector. Measurement time/interval and injection controls were kept identical in both of EnVision and Victor.

3.1. Agonist response

Agonist response of endogenous and recombinant receptor was assessed by the aequorin-based functional assay. In terms of signal-to-noise ratio (S/N), HEK293-aeq/hCaS cells resulted quite high assay windows as shown in Fig. 1. With the acetylcholine activation, range of S/N values were from 3.52 to 16.07 in transient transfected cells. This acetylcholine response was for one of the endogenous receptor in HEK293-aeq/hCaS cells detected by EnVision as an internal control. To compare the detector performance of Victor and EnVision in the condition of stable receptor expression, transfected cells were through the clonal selection process against the target receptor. As a result of this clonal selection, maximum signals for the endogenous receptor were increased more than three times comparing for their transient expression.

Using the established stable cells, HEK293-aeq/hCaS, acetylcholine responses in the range of S/N values were recorded as 28.94 to 58.47 and 4.40 to 11.98 in Victor and EnVision, respectively. As expected, the acetylcholine response in transient cells was lower than stable cells. Interestingly, the endogenous receptor signals from Victor were more than four times higher than those from EnVision. However, this difference in S/N values between EnVision and Victor was due to the extremely low noise data rather than the maximum endogenous receptor response.

For the response of target receptor, CaS, presented as 507.15 to 1366.63 and 28.94 to 58.47 in Victor and EnVision, respectively. The response with transient cells was still much lower than those of stable cells (S/N: 5.73 - 39.00). Comparing the maximum CaS signals in transient and stable cells, CaS receptor signals in stable cells increased more than 30 times and this amplification was present in both of detectors, representing results from cell conditions not the detector platform.

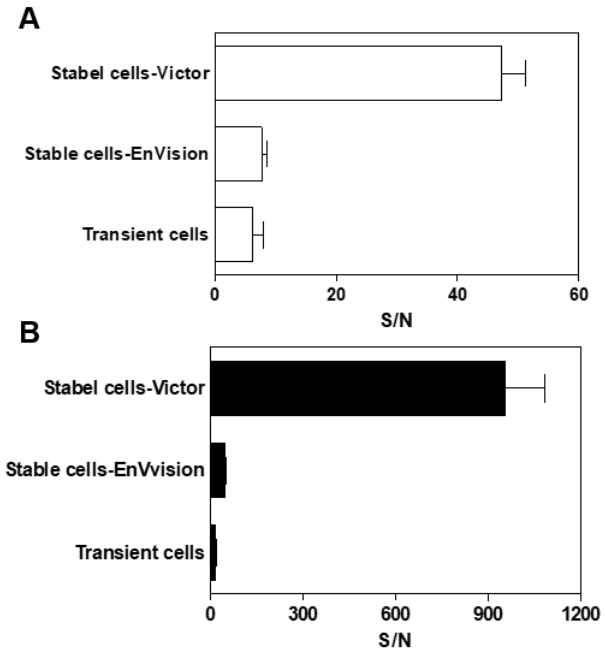


Fig. 1: Endogenous and recombinant receptor expression detected by EnVision and VICTOR. Cell surface receptor expression was assessed by the aequorin-based functional assay. Presented data were a representative data set from three separate experiments performed in triplicate, and each point was expressed as mean ± SEM (standard error of mean). A) agonist response by acetylcholine, B) agonist response by calcium chloride, S/N: signal-to-noise ratio

3.2. Antagonist response

In addition to the agonist response by acetylcholine and calcium chloride, cells were pre-incubated with the antagonist (NPS-1) followed by agonist activation. Luminescence signals were recorded as in the agonist response and expressed as S/N (Fig. 2).

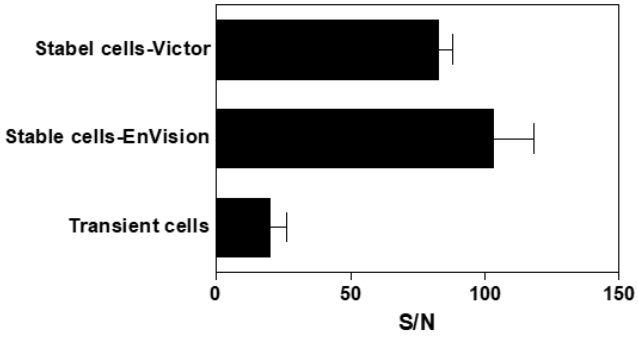


Fig. 2: Recombinant receptor expression detected by EnVision and VICTOR. Cell surface receptor expression was assessed by the aequorin-based functional assay. Presented data were a representative data set from three separate experiments performed in triplicate, and each point was expressed as mean ± SEM (standard error of mean). Antagonist (NPS-1) responses in transient and stable cells were expressed as S/N (signal-to-noise ratio).

The range of S/N values were from 8.10 to 53.40 in transient transfected cells. After cells were selected for the target receptor, the S/N range of stable cells was increased to 63.10 - 91.60 and 47.70 - 136.70 in Victor and EnVision, respectively. The assay signal dynamic range and the data variation associated with the signal measurements were higher in EnVision, however, further analysis showed that differences in those conditions were not statistically significant.

To measure the ability of inhibition on reference agonist response of calcium chloride at 4 mM, with or without 1 μM of NPS-1. A specific assay window for CaS was calculated from the negative (with 1 μM of NPS-1) and positive control points (without 1 μM

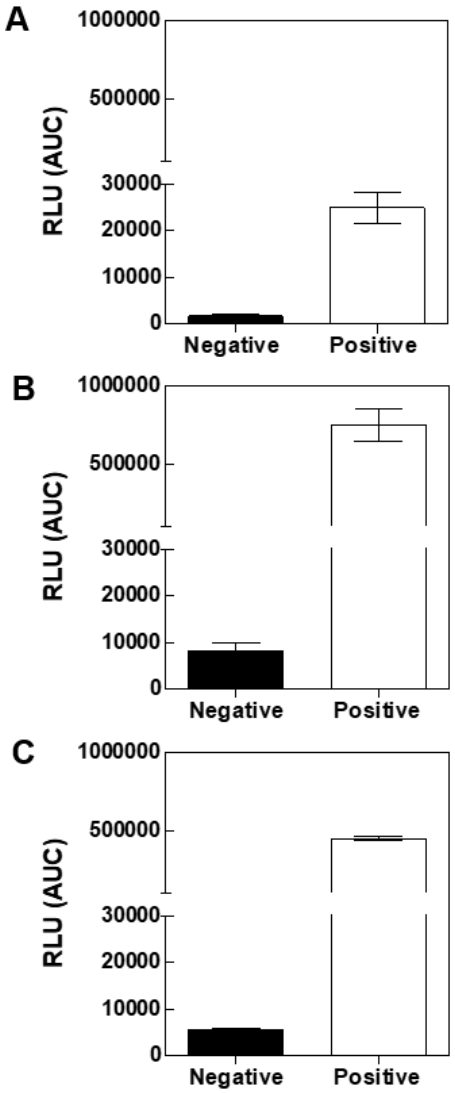


Fig. 3: Antagonist response of transient and stable cells detected by EnVision and VICTOR. The negative control was conditioned with 1 μM of NPS-1 before to inject 4 mM of calcium chloride as an agonist, and the positive control was treated with assay buffer instead of high concentration of NPS-1 so that covered the maximum signal range within the assay. Presented data were a representative data set from three separate experiments performed in triplicate, and each point was expressed as mean ± SEM (standard error of mean). A) NPS-1 response in transient cells, B) NPS-1 response in stable cells detected by EnVision, C) NPS-1 response in stable cells detected by Victor

of NPS-1), then antagonistic abilities were measured with the decrease of assay window by test materials. Therefore, the negative and positive control points are important parameters in this experimental design (Fig. 3).

Control signals in transient cells were measured with EnVision and those levels were lower than stable cells as expected. In this case, assay window reached at >20,000 RLU(AUC) with 13-20% SEM in each of the negative and positive control points. Stable cell signals measured with EnVision (assay window: >740,000) were higher than transient cells but with similar variations (13-20%), suggesting that the signal variation is more like dependent to the detector type along with experimental conditions. However, stable cell signals measured with Victor (assay window: >440,000) presented less variation in both control signals (3-7%). From this comparison in EnVision and Victor, control results were more reliable with Victor and the amplitude of signals were not significantly different.

3.3. Comparative analysis of the receptor response

To compare control signals for antagonist screening, the negative and positive control points in endogenous and recombinant receptors were detected by EnVision and VICTOR. In all comparisons by unpaired t-test, the difference was considered to be statistically significant when $p < 0.05$ by two-tailed p value.

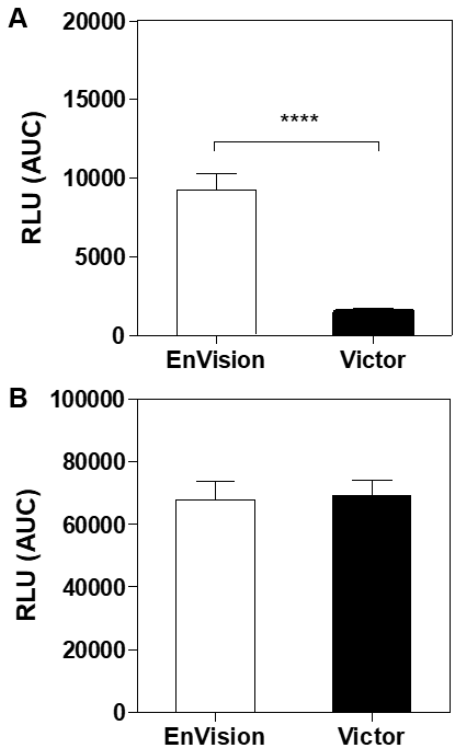


Fig. 4: Comparison of endogenous receptor signals detected by EnVision and VICTOR. Cell surface receptor expression was assessed by the aequorin-based functional assay. Presented data were a representative data set from three separate experiments performed in triplicate, and each point was expressed as mean ± SEM (standard error of mean). A) negative control data of the endogenous receptor, ****: $p > 0.0001$, B) positive control data of the endogenous receptor

As shown in Fig. 4, negative and positive control data for the endogenous receptor were measured in EnVision and Victor. Interestingly, negative control values for the endogenous receptor were 6 times lower with Victor ($p > 0.0001$). However, positive control values for the endogenous receptor were similar in EnVision and Victor. In addition to control signals in the endogenous receptor, the assay window was calculated as 58,653 with EnVision and 67,798 with Victor. These similar assay window resulted from equivalent distribution of negative and positive control data from both of EnVision and Victor. Percent of SEM relative to average data of the negative and positive control points for the endogenous receptor were 22-30% with EnVision and 18-29% with Victor.

Because negative control values for the endogenous receptor with Victor were significantly different to the data with EnVision, the signal-to-noise ratio (S/N) value of the endogenous receptor is also statistically different. S/N ratios of the endogenous receptor were 47 in Victor and 8 in EnVision. This difference was due to the extremely low values of the negative control for the endogenous receptor.

Fig. 5 presented negative and positive controls for the target receptor (CaS) that were measured in EnVision and Victor. A specific assay window for CaS was calculated from the negative (with 1 μM of NPS-1) and positive control points (without 1 μM of NPS-1). It was 1,507,285 with EnVision and 1,826,935 with Victor. Same as the endogenous receptor data, negative control values for CaS receptor were about 6 times lower with Victor ($p > 0.0001$), and positive control values for CaS receptor were similar in EnVision and Victor. Percent of SEM relative to average data of the negative and positive control points for CaS receptor were 25-37% with EnVision and 25-38% with Victor.

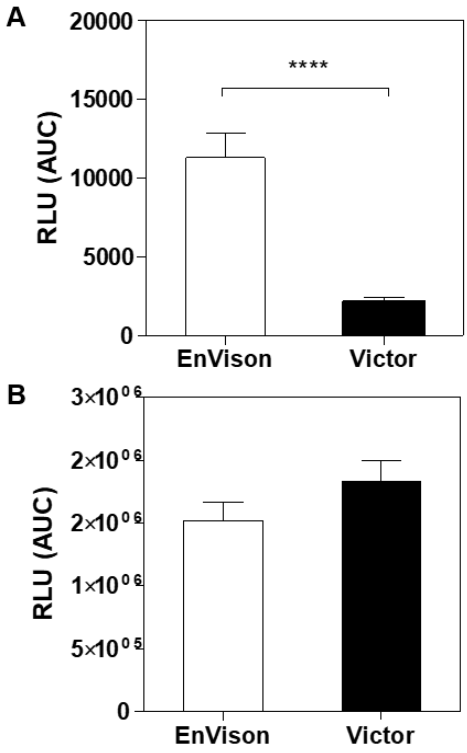


Fig. 5: Comparison of target receptor (CaS) signals detected by EnVision and VICTOR. Cell surface receptor expression was assessed by the aequorin-based functional assay. Presented data were a representative data set from three separate experiments performed in triplicate, and each point was expressed as mean ± SEM (standard error of mean). A) negative control data of CaS, ****: $p > 0.0001$, B) positive control data of CaS

Because negative control values for CaS with Victor were significantly different to the data with EnVision, the signal-to-noise ratio (S/N) value of CaS is also statistically different. S/N ratios of the endogenous receptor were 955 in Victor and 153 in EnVision. This difference was due to the extremely low values of the negative control for CaS receptor.

4. Discussion

As a disease target, the CaS belongs to the family C of GPCR, triggering a calcium mobilization upon activation [13]. The biologically active, cell surface CaS, upon binding calcium, that is preferentially coupled to G_q. This stimulate the coupling of CaS to phospholipase (PLC) results in hydrolysis of poly-phosphoinositides, accumulation of inositol 1,4,5-trisphosphonate (IP_3), and release of calcium from its intracellular stores followed by stimulation of calcium influx [33, 34].

To test the luminescence detector functionality, a functional assay system for the GPCR was utilized [35]. The aequorin based functional assay system for the CaS with HEK293-aeq/hCaS cells produced flash-type and short-living luminescence signals that are specific and dependent on the calcium influx as in the GPCR activation. As reported previously [28] [30, 36], These type of luminescence signals were well captured with various detector systems.

Both EnVision (EnVision® Multilabel Plate Reader, Xcite) and Victor (VICTOR™ Luminescence Counter, Light) employ photomultiplier tube detection and measure one well at a time. Victor is a dedicated luminescence counter for microplate applications, including cell-based assays, toxicology screens. As a small benchtop unit with a simple optical path, Victor provides easy access to waste and syringes, and features a dead volume less than 500 µL. EnVision Multilabel Plate Reader is fast, sensitive and versatile benchtop readers that deliver optimized performance for versatile applications including accepting microplates from 1- to 3456-wells and features dedicated LASER excitation modules. It has modular label-specific optical mirror, high energy flash lamps, and high speed detectors. The versatile EnVision can perform fast kinetic measurements, enzyme assays and numerous other cell-based drug discovery assays.

In this paper, EnVision and Victor were adopted for the comparison of versatile vs. dedicated detectors as a luminescence detection platform and the aequorin based CaS functional assay system was utilized as a model system for both of detector platform.

Table 2: Comparison of agonist data values determined for CaS by EnVision and VICTOR

	EnVision		Victor	
Agonist	**Acetylcholine**	**Calcium**	**Acetylcholine**	**Calcium**
Bottom	9260 ± 2727	11286 ± 4211	1538 ± 460	2115 ± 824
Top	67913 ± 15035	1518571 ± 383473	69336 ± 12730	1829050 ± 448826
S/N	7.78 ± 2.42	152.8 ± 69	47.2 ± 10.2	954.5 ± 344.4
Hillslope	1.09 ± 0.23	2.34 ± 0.15	0.98 ± 0.17	2.47 ± 0.32
EC_{50}	3.23 ± 0.80 (µM)	1.49 ± 0.30 (mM)	4.46 ± 3.42 (µM)	1.76 ± 0.23 (mM)
Z' value	0.61 ± 0.11	0.88 ± 0.06	0.70 ± 0.15	0.86 ± 0.12

* S/N (signal-to-noise ratio) represents the height of detection signal compared to the background. Listed data were expressed as mean ± SD (standard deviation).

Table 2 summarized the overall performance of EnVision and Victor in CaS functional assay system. With 5,000 cells/well condition, the transient calcium signals by acetylcholine were measured with both of machines and the resulted EC_{50} were 3.23-4.46 μM. Together with the calcium response (EC_{50}: 1.49-1.76 mM), the luminescence output was robust and reproducible. Nonlinear regression analysis of the sigmoidal dose response represented around 1 for acetylcholine and 2.4 for calcium as the Hill slope. Especially, Z' factors from EnVision and Victor presented similar values ranging from 0.61 to 0.88.

Although the dynamic range and data variation associated with the signal measurements were similar in both of instruments, higher signal-to-noise ratio with Victor than EnVision for both of the endogenous and targeted receptors. This dramatic difference was caused by the decreased background values in Victor.

Table 3: Comparison of antagonist data values determined for CaS by EnVision and VICTOR

	EnVision	Victor
Bottom	8185 ± 3897	5523 ± 914
Top	750779 ± 229603	450464 ± 27783
S/N	103 ± 34	83 ± 11.4
Hillslope	1.27 ± 0.37	0.83 ± 0.17
IC_{50}	0.66 ± 0.079 (μM)	1.24 ± 5.33 (μM)

* S/N (signal-to-noise ratio) represents the height of detection signal compared to the background. Listed data were expressed as mean ± SD (standard deviation).

As shown in Table 3, the CaS antagonist measurements in EnVision and Victor were similar in terms of an assay window, S/N and IC_{50} values.

5. Conclusion

The aequorin based functional assay system for CaS receptor with HEK293-aeq/hCaS cells represented higher signal-to-noise ratio in Victor than EnVision for both of the endogenous and the model receptor, CaS. The assay signal dynamic range and the data variation associated with the signal measurements were similar in both of instruments. In conclusion, both types of instrument provided comparable results for the luminescence detection that can be applied in variety of light emission experiments. However, the dedicated luminescence detector, Victor Light, with a simple optical path and jet-direct injectors provided extremely low background which is a real benefit for the flash-type luminescence applications. More instruments from various vendors would need to assess the actual performance for this type of luminescence applications in future.

Acknowledgement

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References

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