Tech Note

Use of the Fluorescence Optics to Measure Luminescent Reactions in High Well-density Microplates

Measuring Luminescence in 1536-well Microplates

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Luminescence based assays are one of the mainstays for gene expression studies. The development of long lasting glow luminescent reactions has allowed this technology to be used as a means to screen large numbers of compounds for their ability to regulate the expression of specific gene targets. The dedicated luminescence optics of the Synergy reader product line has not been designed for the use of high density 1536-well microplates. Here we describe the use of the fluorescence optical pathway in the Synergy™ 4 Multi-Mode Microplate Reader to measure luminescence in high density microplates.

Introduction

Luminescence reactions have been classified into two general categories, flash and glow, based on their duration of light output. Flash luminescence, as the name implies, are reactions that are short-lived. These reactions usually reach a peak intensity within seconds and rapidly dissipate. Thus flash luminescent assays usually require liquid dispensers integrated with the reader to add reagents such that the short-lived detection signal can be recorded, usually kinetically. Glow luminescent reactions occur at a much slower rate and often produce a stable signal for decades of minutes to hours. These reactions do not require integrated injectors and because the signal is stable for long periods of time, numerous microplates can be processed for reagent addition before detection in the reader. These attributes make the glow luminescent reactions ideal for screening assays of large numbers of samples. This attribute has also allowed for the miniaturization of luminescent assays to 1536-well densities, to increase sample throughput and reduce assay costs per well.

The Synergy 2 and 4 line of readers offers a dedicated luminescence probe that captures emitted light and routes it through the emission filter wheel and subsequently to the photomultiplier tube (PMT) detector. While this dedicated light guide is extremely efficient at channeling the light to the PMT, the probe size is too large to accommodate the small size of the wells of a 1536-well microplate.

When this optic is employed a warning message is displayed indicating to the user that the probe size is too large (Figure 1). However, the fluorescence optics, which uses different apertures for different well densities, has the ability to measure fluorescence in 1536-well microplates. Here we describe the use of the fluorescence optical path to detect and quantitate glow luminescence in 1536-well microplates.

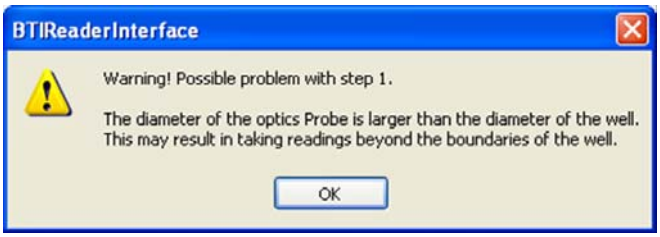

Figure 1. Warning message displayed when the Luminescence optics are selected to read 1536-well Microplates.

Materials and Methods

Kinase Glo Assay kit (catalog Number V3772) and ATP (catalog number V222A) were purchased from Promega Corporation (Madison, WI). Solid white 96-well (Catalog number 3912) and 1536-well (catalog number 3937) microplates were obtained from Corning (Corning, NY). A series of dilutions of ATP ranging from 0 to 500 µM were made using distilled water as the diluent. Aliquots of each dilution (100 µL) were pipetted in replicates of eight into wells of a 96-well microplate.

An equal volume (100 µL) of Kinase Glo reagent, made fresh by mixing the provided diluent with the lyophilized reagent, was then added and the plate was allowed to incubate for 5-10 minutes. After incubation, 10 µL of the reaction was pipetted into a 1536-well microplate, with subsequent luminescence determination.

Luminescence measurements were made using a Synergy 4 Multi-Mode Microplate Reader. Determinations were made from the top using both luminescence or fluorescence read modes. The PMT sensitivity was set to 135 for all measurements. The default luminescence collection parameters in Gen5 were used for data collection. In addition, with fluorescence mode, different top optics such as the 50% mirror, a 510 cut off dichroic mirror and no mirror were compared for the collection of luminescence from the 1536-well microplate.

Results

Different read modes were tested for their ability to measure luminescent samples in 1536-well microplates. As demonstrated in Figure 2, both luminescence and fluorescence mode can be used to detect a luminescent signal in 1536-well plates. Not surprisingly, the dedicated luminescence optic is the most efficient means to capture the light signal.

Fluorescence optics can also be used with good success in measuring luminescence. Using either the generic 50% mirror or a dedicated dichroic mirror for the wavelength of emitted light, the luminescent signal can be quantitated. Inherent inefficiencies of this optical path relative to the dedicated luminescent optic are evident in the signal returned. Because the 50% mirror is reflective of some of the light regardless of the wavelength it is less efficient than a dedicated dichroic mirror.

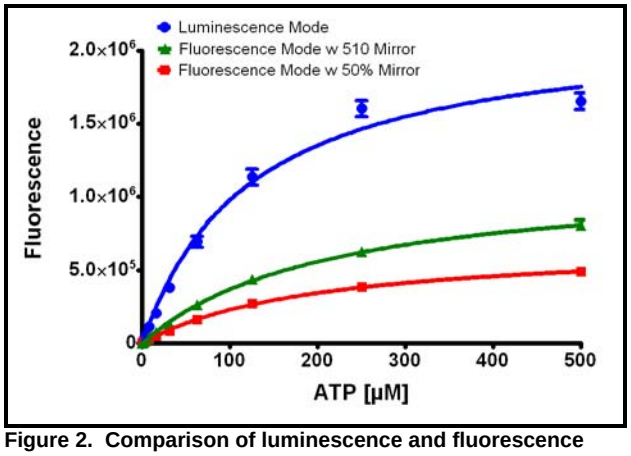

Figure 2. Comparison of luminescence and fluorescence modes of detection with high ATP concentrations.

When significantly lower levels of ATP are examined, markedly less light is emitted. As expected the dedicated luminescence optic is the most efficient in regards to light collection. In order to reduce the light lost as a result of using the fluorescence light path, the dichroic mirror was removed from the light path. As demonstrated in Figure 3, an approximate 5% improvement in the signal is observed when there is no mirror present in the top probe optics when measuring a luminescent signal.

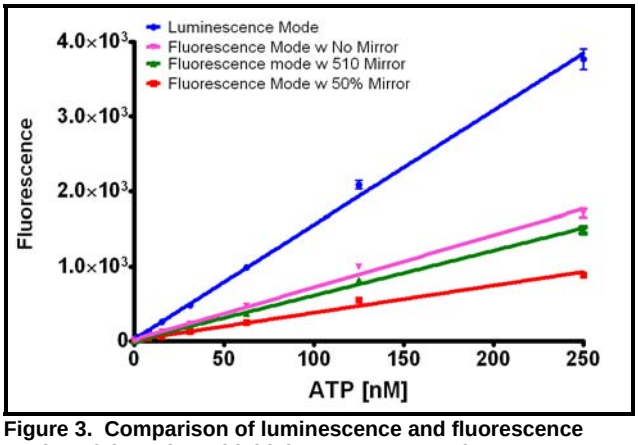

Figure 3. Comparison of luminescence and fluorescence modes of detection with high ATP concentrations.

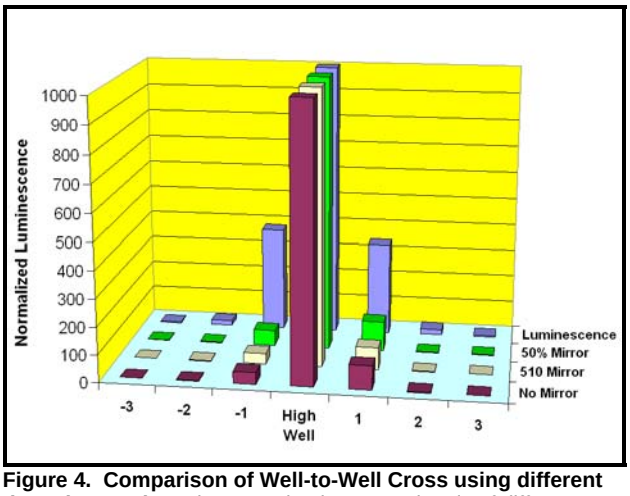

Figure 4. Comparison of Well-to-Well Cross using different detection modes. The mean luminescent signals of different detection modes were normalized to 1000 for the high wells. Each data point represents the mean of 8 determinations.

Crosstalk from nearby wells was assessed by measuring the signal of a very bright signal and measuring the increase in luminescent signal in adjacent wells. The large dedicated luminescent optic results in significant crosstalk from wells with a high signal. As demonstrated in Figure 4, the signal in adjacent empty wells can approach 40% of the actual experimental well. This increase in signal can be observed as far away as 2-3 wells in any direction. This crosstalk is not evident using 384-well or lower density microplates. The fluorescence optics showed little crosstalk in adjacent wells (4-8%) with background levels being observed in wells located 2 positions away from a very high signal. Of the different mirror configurations used with the fluorescence optics, the “no mirror” combination and the 510 nm dichroic had identical results, while the 50% mirror resulted in slightly higher crosstalk values in adjacent wells. Interestingly, the luminescence optics showed a slight skewing of the crosstalk to the left, while the fluorescence optics were slightly skewed to the right. This most likely represents differences in positioning relative to the center of the well.

Discussion

While the Synergy™ 2 and Synergy™ 4 Multi-Mode Microplate Readers were not specifically designed to measure luminescent reaction in a 1536-well format, these data indicate with some limitations the readers are capable of doing so. The choice of top optics when using the fluorescence mode of detection needs to be made carefully. Removal of a mirror from one of the three mirror positions in the mirror block results in the best performance. However Gen5™ Data Analysis Software does not directly support not having a mirror. One has to create a custom mirror at that position in the software look up table. Selection of the faux-mirror will result in measurements through the open space of the mirror block. If one is familiar with the light output emitted by the luminescence reaction the correct dichroic mirror can be used with little loss in signal. For example, the luminescent reaction resulting from the interaction of firefly luciferase, luciferin and ATP emits light at a wavelength of 570 nm. The standard 510 nm cut off dichroic used for fluorescein will work at these wavelengths. Much like any true fluorescent reaction, selection of the appropriate mirror is necessary. The generic 50% mirror also produces very adequate results. Selection of this mirror type is wavelength independent.

There are differences between fluorescence and luminescent measurements that the user needs to be aware of when using the fluorescence optics for luminescence measurements. The luminescence read automatically turns the lamp off, then defaults to a “Hole” or open aperture for the emission filter, but allows the user to use a filter if desired during the read step set up. When using the fluorescence mode the lamp is not turned off and the user decides on what filter positions to use. When using the fluorescence optics to measure luminescence, it is critical that a “Plug” be selected for the excitation filter. Use of a filter at this location would result in light illumination of the sample, which is undesirable. As with luminescence, the user has the option to use either a “Hole” or a wavelength specific filter.

Typical PMT sensitivity settings for luminescence and fluorescence mode are different. When measuring luminescent reactions, the PMT sensitivity setting used is higher than typically used for fluorescence. Recommended PMT settings in fluorescence are generally between 35 and 120, while those employed with luminescence are between 100 and 255. While there is some overlap between the two reading modes, the default value of 135 for luminescence is recommended when using the fluorescence optics to measure luminescence. This will automatically trigger a warning message in the Gen5™ Data Analysis Software (see Figure 5), which can be ignored by selecting the “No” button when asked if you wish to return to the Read Step dialog.

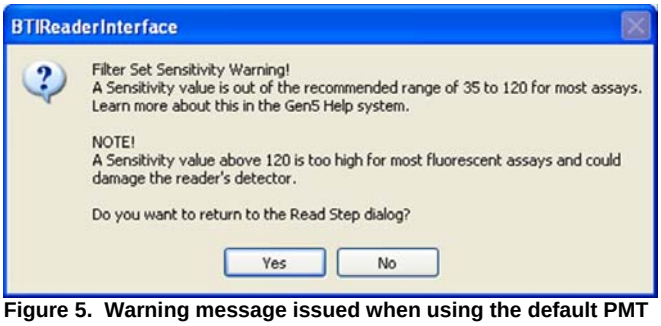

Figure 5. Warning message issued when using the default PMT setting for Luminescence when readings are made with the Fluorescence optics.

Fluorescence optics can be employed for luminescence measurements that must be made from the bottom of the plate. The dedicated liquid light guide is only available for top read measurements. Under some circumstances it is desirable to read luminescent reactions in 96- and 384-well plates from the bottom of the plate. This detection mode requires clear bottom plates for light transmission, as well as the selection of the fluorescence optics to accomplish the read. The selection of PMT sensitivity, excitation and emission filter wheel positions and fluorescence read mode would be similar to that described for a top read. While the 5 mm bottom probe will work for most 96-well plates, its size relative to the well diameter of a 384-well plate requires that solid opaque sided clear bottom plates be used to prevent well to well cross talk. While clear–bottom solid sided 1536 microplates are not available; this measurement mode would not be recommended for 1536-well plates even if they were.

Here we have described the undocumented use of the fluorescence optics to measure luminescence in 1536-well microplates. Using the fluorescence optics allows the investigator to take advantage of the smaller aperture size available with these optics that are not available with the dedicated liquid light guide normally employed for luminescence measurements. While the amount of light captured using the fluorescence optics is less than that of the luminescence optics, the degree of well-to-well crosstalk is considerably less, making it a much more desirable option for the high density plates.

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