Spotlight

Clinical, Medical & Diagnostic Products

The use of luminescence assays and reagents in drug discovery has increased significantly over the past ten years due to a combination of ease of use, very high specificity of assays and good sensitivity at low levels of screened compounds. Modern photometric instruments are able to accurately count very low levels of photon emissions from luminescence substrates and this has led to an increasing focus on the optical cross talk inherent in the design of SBS/ANSI standard microplates and the signal-to-noise ratio that can be experimentally obtained. This short paper compares the observable optical cross talk in three different manufacturers' micro well plates and finds that the use of a black supporting matrix can significantly improve sensitivity in microplate assays.

"Bioluminescence is a naturally occurring phenomenon exhibited by certain animal and plant species, which can emit light through one of two common mechanisms, the Aequorin and Luciferin pathways."

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Determination of well-to-well crosstalk in microplates used for luminescence measurements

PHOTOMETRIC METHODS

The study compares two different manufacturer's solid white polystyrene shallow well microplates with the patented Porvair Sciences design which combines white individual wells with a solid black plate matrix *(Figure 1)*. A luminescent assay using Firefly luciferase was used for the comparison. There are three basic methods of obtaining useful optical data from microplate-based samples. The simplest method is absorbance measurement. Following the Beer-Lambert law, concentration of a given compound in solution is directly proportional to the quanta of light absorbed at a given wavelength and at a constant optical path length. Thus a beam of monochromatic light can be directed into the well and the transmitted or absorbed light can be measured. A simple calculation gives the relative concentration in each well. The majority of laboratory microplate readers use this method.

Where greater sensitivity is required, fluorescence measurements are preferred. In this case an excitation beam of a given wavelength is directed into the well. The fluorophore absorbs photons from the incident light and re-emits photons of lower energy, which consequently appear at a longer wavelength. In a fluorimeter, simple cut-off filters can be used to remove reflected excitation wavelengths from the detected light, leaving only the emission wavelength. Sensitivity can be 10x greater than simple absorbance measurements. Greater sensitivity can be achieved by reducing the band width of both excitation and emission wavelengths using monochromators. Such spectrofluorimeters can offer another order of magnitude increase in sensitivity.

Bioluminescence is a naturally occurring phenomenon exhibited by certain animal and plant species, which can emit light through one of two common mechanisms, the Aequorin and Luciferin pathways. Firefly beetles (Lampyridae), the marine copepod Metridia Longa and the Sea Pansy (Renilla Reniformis) all contain enzymes in the class luciferase, which catalyse the oxidation of Luciferin substrate in the presence of ATP resulting in the emission of light. In luminescent jellyfish of the family Aequorea, the substrate Aequorin is excited in the presence of coelenterazine and Ca2+ ions. In returning to the ground state, blue light is emitted. These reactions are biological adaptations of a process, which can also be seen as purely chemically driven reactions, in which case it is referred to as chemiluminescence.

By transfecting cells with a generic construct that includes a lucieferase gene, luciferase can be used as a reporter to assess transcription activity within cells or provide a very sensitive assay for cellular ATP levels used for the determination of cell apoptosis in high throughput screening applications. In such assays, a continuous glow is emitted by the cloned luciferase enzyme system and the kinetics of this can be studied and linked to, for example, the concentration of available ATP or the propensity for a cell to undergo mitosis, remain stable or undergo cell death in response to chemical stimulus. Luminescence assays of this kind are marketed by several major Life Science companies under such names as AequoScreenTM, LucLiteTM, ATPLiteTM, OneGloTM. Detection is normally in a luminometer or a multi-label reader, which is a multiple purpose spectrometer capable of reading in absorbance, fluorescence or luminescence modes.

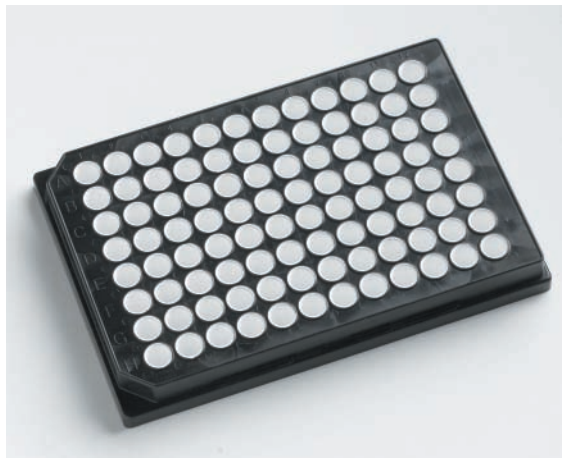

Figure 1: The unique Porvair black & white 96-well assay plate

The three optical methods of detection are shown schematically in *Figure 2*.

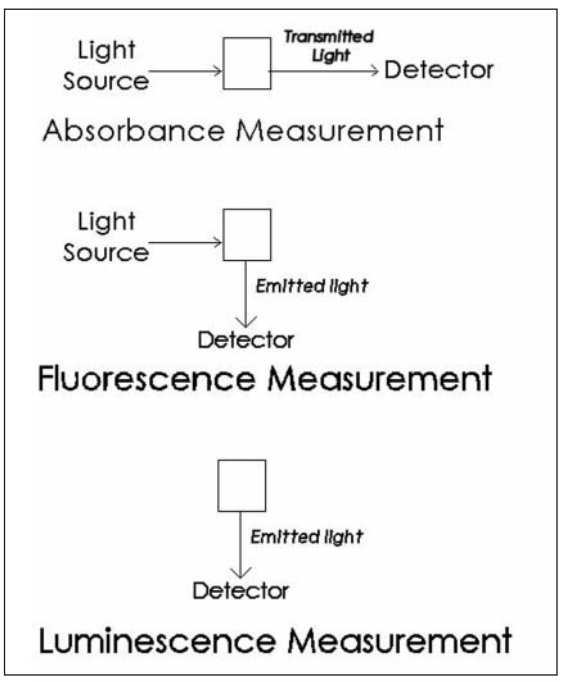

Figure 2: Photometric detection methods

MICROPLATE DESIGN CONSIDERATIONS

Microplates for use in assay development and High Throughput Screening are usually manufactured from polystyrene polymer. To this is added an optical brightener, such as titanium dioxide, to increase the reflectivity of the plate surface for white plates. In addition, the steel tools used to mould these plates are highly polished to give a very smooth bright surface inside the wells. These attributes ensure that emitted light within the wells is reflected back up towards the top of the micro well. This is where the sensitive optical detector of the measuring instrument is positioned during the reading cycle. Ideally a photomultiplier or photodiode detector will capture the majority of the light emitted and reflected in this way.

For fluorescence measurements, it is very important to 'quench' the natural auto-fluorescence of the polystyrene substrate. This is achieved by adding, typically, 1% carbon black to the polystyrene. This has been shown to be very effective in fluorescence assays and gives much better results than a white plate in these cases. Again all the internal surfaces are polished to increase reflectivity.

Difficulties can arise when using luciferase assays in screening where a high dynamic range is observed across the plate. In such cases, adjacent wells may exhibit very strong or very weak signals and this can lead to optical cross-talk between the wells and consequently to erroneous results. A certain amount of visible light can 'leak' through the white plastic walls of the plate and is erroneously detected in the adjacent cells as an additional, albeit low, signal. The error is worst when reading a low level signal adjacent to a far brighter well. Although plate designs using separated 'chimney' wells can help to reduce this,

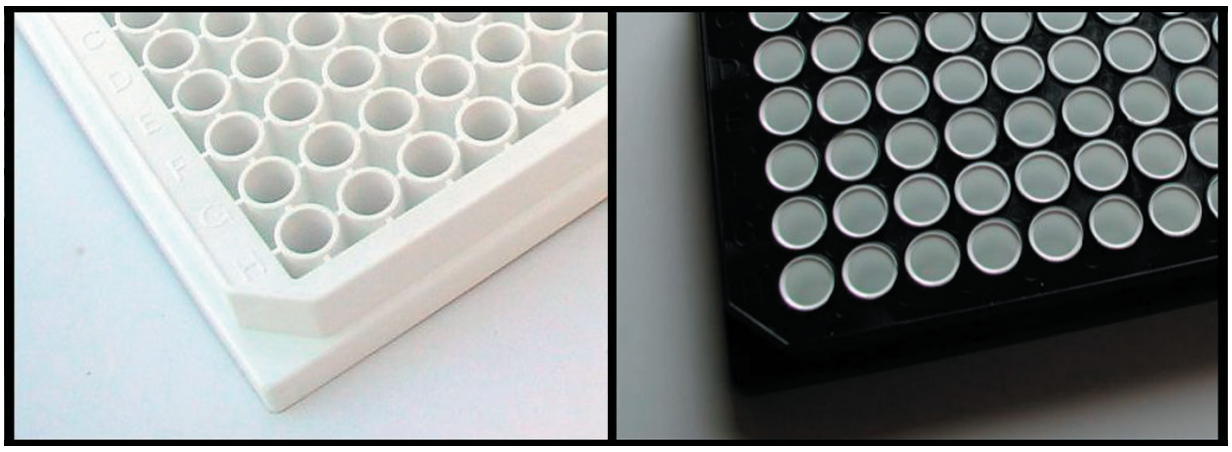

Figure 3: Left hand side - 96 well black plate with white wells, right hand side a solid white 'chimney well' plate

they cannot eliminate it. *Figure 3* shows a Porvair solid white 96- "chimney well" plate (left hand picture). Each well has individually formed well walls, which are separated from the plate matrix.

To overcome this problem of light piping by the white plastic, Porvair Sciences developed and patented the unique 96-well Black & White plate *(Figure 3 right hand picture)*. A black polystyrene plate matrix has individual white cells moulded into it at the time of production in what is known as a 'two shot' process. This two-shot tooling is also used to mould individual clear wells into either a white or black matrix to make Porvair Sciences Krystal 2000TM Zero-crosstalk plates for bottom-reading absorbance and fluorescence applications.

By incorporating bright white wells containing up to 18% TiO2 in their formulation into a 1% carbon black matrix, optical crosstalk is all but eliminated in the new plate. Indeed the data below shows that, when compared to two other manufacturers solid white assay plates, the black & white plate has respectively 82% and 56% lower average well-to-well cross talk.

RESEARCH METHODOLOGY

Readings were taken on a POLARStar Omega multi-mode spectrophotometer from BMG Labtech at 510nm using 5ul of a solution containing Luciferin and Firefly luciferase pipetted into well C4 of each 96-well plate (shown as 'P' in the data below). A 5uL aliquot of hydrogen peroxide was added to initiate the reaction. To allow for the luminescent signal decaying over time, four readings, each of 1 sec duration, were taken from each of the surrounding, empty, wells and averaged. These are shown in the data as 'Cycle 1, 2, 3, and 4'. Each cycle represents 1 minute of elapsed time since the first reading was taken. This is necessary as the Luciferase signal decays quickly over a period of 8-10 minutes and therefore readings are taken until the signal has dropped to almost 50% of initial levels.

Readings were taken from four different types of well:

X1 = wells which share a common well wall with the sample well

X2 = wells which share a common corner with the sample well

X3 = wells which are exactly one cell distant from the sample well

X4 = wells which are at the corner of the block, two cells distant from the sample.

Only one plate was prepared for each experiment and care was taken to select only a fresh clean plate from an unopened pack. This procedure was repeated for the other manufacturers' plates. An empty plate of the same type was used to take a background reading for the signal-to-noise calculation.

Results

The calculated signal-to-noise ratio for the Porvair 96-'chimney well' plate (right hand picture) was 192000:1, as opposed to 178000:1 for a solid white plate, showing a useful 7.5% increase in measurable signal over the background *(Figure 4)*. In the wells closest to the reaction mixture (Cells X1), the measured cross talk was just 0.026% in the Porvair Sciences black & white plate *(Figure 3, left hand picture)*, as opposed to 0.041% and 0.048% in the other two solid white plates, an improvement of 82%.

In luminescence crosstalk experiments it is not meaningful to run a 'blank' with only buffer or water, as no observable signal will be seen. If such an experiment were carried out, any reading from the photo-detector would be due either to stray light entering the sample compartment, or to the 'dark current', which represents continuous quantum noise from the electronics and elements of the detector that is corrected automatically by the instrument firmware.

CONCLUSION

The results clearly show that the Porvair Sciences composite black & white plate has much to offer for the determination of low-level luciferase based assays in screening and drug development. The combination of effective quenching by carbon black and increased reflectivity from titanium dioxide brightener yields an improved signal-to-noise ratio and better intra-plate dynamic range, giving screeners the opportunity to screen for weaker hits, at lower detection levels or with reduced concentrations of reagents.

The Author is Sales and Marketing Manager with Porvair Sciences Ltd. He has previously held sales and marketing positions with a number of spectroscopy and reagent companies including Philips-Pye Unicam, Shimadzu, Jasco, Gilford Systems, Packard Bioscience and PerkinElmer.

Average P	Average X1	Average X2	Average X3	Average X4
6.59E+05	172.75	3.5	1.6875	1.75
Crosstalk %	0.0262	0.0005	0.0003	0.0003
Cycle 4				
Average P	Average X1	Average X2	Average X3	Average X4
5.90E+05	155.25	3.25	1.9375	1.75
Crosstalk %	0.0263	0.0006	0.0003	0.0003
Cycle 5				
Average P	Average X1	Average X2	Average X3	Average X4
5.54E+05	145.75	3.25	1.6875	1.5
Crosstalk %	0.0263	0.0006	0.0003	0.0003
Average	0.0262	0.0006	0.0003	0.0003

% Increase in Sensitivity	
Porvair va Plate 1	56.75
Porvair vs Plate 2	82.34

Average P	Average X1	Average X2	Average X3	Average X4
6.68E+05	274.75	14.5	5.6875	3.25
Crosstalk %	0.0411	0.0022	0.0009	0.0005
Cycle 4				
Average P	Average X1	Average X2	Average X3	Average X4
6.48E+05	267	14	6.1875	4
Crosstalk %	0.0412	0.0022	0.0010	0.0006
Cycle 5				
Average P	Average X1	Average X2	Average X3	Average X4
6.45E+05	266.5	14	5.8125	3.5
Crosstalk %	0.0413	0.0022	0.0009	0.0005
Average	0.0411	0.0021	0.0009	0.0005

Average P	Average X1	Average X2	Average X3	Average X4
5.31E+05	253.75	32.25	3.875	2
Crosstalk %	0.0478	0.0061	0.0007	0.0004
Cycle 4				
Average P	Average X1	Average X2	Average X3	Average X4
4.66E+05	222.75	28.25	3.25	1.5
Crosstalk %	0.0478	0.0061	0.0007	0.0003
Cycle 5				
Average P	Average X1	Average X2	Average X3	Average X4
4.32E+05	207.5	26.75	3.1875	1.5
Crosstalk %	0.0480	0.0062	0.0007	0.0003
Average	0.0479	0.0061	0.0007	0.0004

	A	B	C	D	E	F	G	H
1	X4				X4			
2	X3	X3	X3	X3	X3			
3	X3	X2	X1	X2	X3			
4	X3	X1	SAMPLE P	X1	X3			
5	X3	X2	X1	X2	X3			
6	X3	X3	X3	X3	X3			
7	X4				X4			
8								

Schematic showing readings taken on each plate

Figure 4: Schematic showing the position of the sample and the various measurement positions around the sample.