MOLECULAR DEVICES

TECHNICAL NOTE

Spectral Fusion™ Illumination technology for an extended dynamic range on the SpectraMax i3x Multi-Mode Microplate Reader

Introduction

Many microplate readers and spectrophotometers use a photomultiplier tube (PMT) to detect fluorescence and luminescence signals. PMTs work by initially detecting photons from the sample that are emitted at specific wavelengths which are converted into electrons. The electron multiplier then amplifies the signal so it can be detected and expressed in relative fluorescence or relative luminescence units (RFU or RLU).

This technical note focuses on the unique patented Spectral Fusion™ Illumination and AutoPMT™ features found on the SpectraMax i3x Multi-Mode Microplate Reader. It explains how the system can normalize data with a combination of optical and electronic components to not only provide optimal sensitivity, but also maximize the signal range.

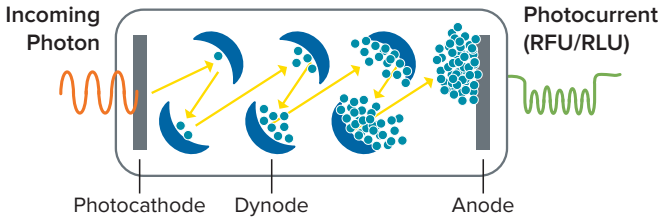

Figure 1. Diagram of a photomultiplier tube (PMT). PMTs convert photons into electrons and amplify the signal.

How does a PMT work?

A PMT counts incoming photons hitting the photocathode and converts them into electrons. First, the electrons are deflected to hit the primary dynode and are then amplified over a series of subsequent dynodes (Figure 1). The number of electrons generated is thus proportional to the number of incoming photons but also depends on the voltage or gain applied to the PMT; at lower PMT gains, fewer electrons are generated for a single incoming photon compared to a higher PMT gain.

Materials and methods

Fluorescein solutions were prepared with an initial concentration of 100 µM in PBS, then diluted in 3-fold steps to prepare a standard curve. The standard curve was made up of 14 concentrations ranging from 100 µM to 0.01 nM.

Alexa Fluor 430 dye solutions were prepared with an initial concentration of 10 µM in PBS, then diluted in 3-fold steps to prepare a standard curve. The standard curve was made up of 10 concentrations ranging from 10 µM to 0.1 nM.

For both fluorophores, 200 µL of solution was dispensed in triplicate wells of a solid black 96-well microplate, with PBS used as the plate blank. The plates were measured on the SpectraMax i3x reader according to the settings outlined below (Table 1). The signal was acquired and then analysed using SoftMax Pro Data Acquisition and Analysis software.

Fluorophore	Wavelength	PMT Gain settings
Fluorescein	Read mode: Fluorescence Read type: Endpoint Excitation: 485 nm Emission: 535 nm Optimization: Z height	Fixed at High (default)
Alexa Fluor 430	Read mode: Fluorescence Read type: Endpoint Excitation: 425 nm or 430 nm Emission: 540 nm Optimization: Z height	At 425 nm: AutoPMT At 430 nm: Fixed at High (default)

Table 1. Instrument settings used for the Fluorescein and Alexa Fluor 430 experiments. Note: for fluorescein (485 nm) and Alexa Fluor 430 (425 nm) excitation, the flash lamp is used, while for the 430 nm excitation the LEDs are used.

Spectral Fusion™ Illumination technology

The SpectraMax i3x reader uses a patented light source called Spectral Fusion™ Illumination. This technology combines a xenon flash lamp and light emitting diodes (LEDs) to cover the spectral range of the instrument (Figure 2). The xenon flash lamp is used to excite samples in the ultraviolet range (250 nm to 429 nm) and the near-infrared ranges (681 nm to 850 nm), while the LEDs cover the visible range (430 nm to 680 nm). The instrument selects the light source automatically according to the wavelength specified in the software. When the xenon flash lamp is used, the excitation light is kept constant and the PMT gain can be adjusted to measure the emission of the various samples. In this case, the SpectraMax i3x reader works as a flash-lamp based microplate reader as described in the supporting application note "The AutoPMT™ feature for an extended dynamic range in the SpectraMax iD and M series Multi-Mode Readers". The PMT gain can be set to manual, high, medium, low, or automatic (Figure 3A). As the AutoPMT setting permits an extended dynamic range without compromising data quality, it should be selected when available.

When the wavelength selected for excitation is between 430 nm and 680 nm, the range covered by the LEDs (see Figure 2), LED excitation will be selected automatically by the microplate reader. In this case, the LED intensity is adjusted while the PMT gain is fixed at a range where sensitivity is optimal (Figure 3B). Each LED offers four discrete intensity levels (Figure 4), and the SpectraMax i3x reader automatically pre-reads each well to determine the optimal LED intensity required to perform the measurement. During this pre-read, the instrument reads a well at each of the four available LED intensity levels, starting at the lowest level. The optimal LED level is the one that yields maximal signal without saturation while using the PMT at an optimal linear counting range. The result is that each well of the plate is read using an excitation with its own optimized LED intensity level.

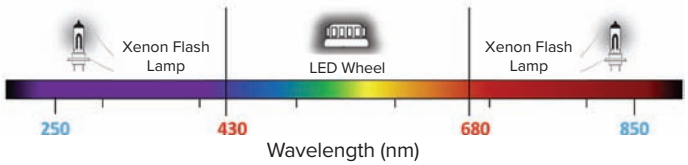

Figure 2. Spectral Fusion™ Illumination technology, a powerful combination of a xenon flash lamp (250-429 nm and 681-850 nm) and LEDs (430-680 nm), provides unmatched signal strength and superior sensitivity across the spectrum.

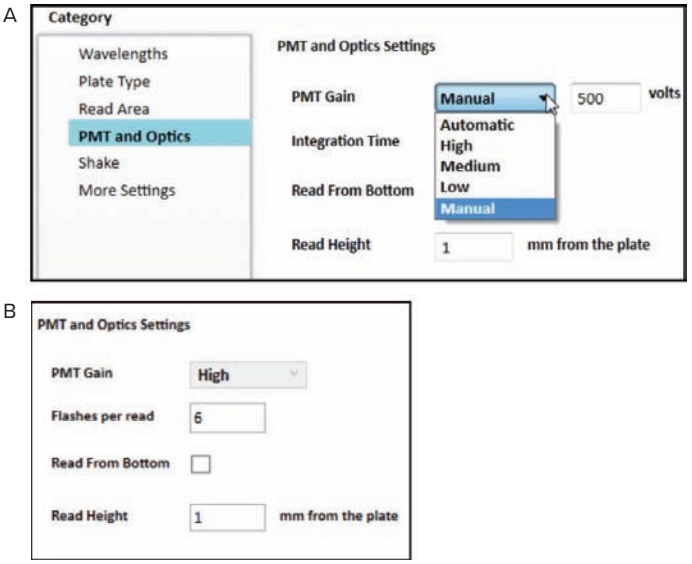

Figure 3. SpectraMax i3x PMT settings: (A) Different PMT levels are adjustable when the flash lamp is used. (B) PMT gain is not user-selectable when the LEDs are used ('High' appears by default).

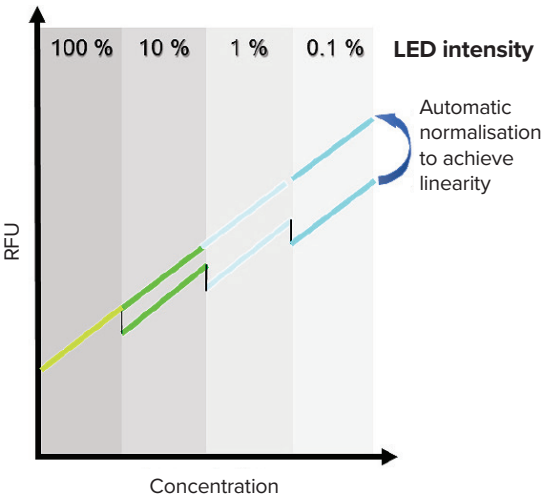

Figure 4. Visualization of the data normalization using LEDs on the SpectraMax i3x. For lower concentration samples, the highest LED intensity level (100%) is used for excitation, while a lower LED power will be used for more concentrated samples. The final RFU values are normalized and thus independent of the LED intensity level used. Note, the intensity levels indicated are just for illustrative purposes.

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Finally, resulting RFU values are automatically normalized to achieve linearity over a broad range of concentrations. With fluorescein, we were able to achieve up to a 6.5-log concentration range in a single microplate measurement (Figure 5).

LEDs vs xenon flash lamp

To compare data generated with the flash lamp and the LEDs on the SpectraMax i3x microplate reader, Alexa Fluor 430 dye was used. The plate was excited at 425 nm and 430 nm to make the instrument use either the xenon flash lamp or the LEDs, respectively. Data were analyzed and plotted on the same graph without manually normalizing the RFU values (Figure 6). Despite being excited with two different light sources, single concentrations gave very similar RFU values, and both curves overlapped closely. This is because there is an additional normalization of the signal between the xenon flash lamp and the LEDs, allowing data to be compared, as the signal measured is independent of the light source selected. This normalization allows the system to run a wavelength scan through the entire range of the instrument with no signal jumps or drops as the system switches seamlessly between the xenon flash lamp and LEDs.

In addition, as LEDs are a more powerful light source than the xenon flash lamp, more light will reach and excite the samples. Therefore, using LEDs instead of xenon flash lamp (where such a choice is possible) can increase the dynamic range and data reproducibility at low concentrations. With the Alexa Fluor 430 standard curve (Figure 6), we were able to detect two extra dilutions (one extra concentration log), with less variability (smaller %CV) at lower concentrations, by using the LEDs (ex 430 nm). For assays with excitation wavelengths just below or just above the LED wavelength range limits, choosing an excitation wavelength compatible with the LEDs will provide a clear advantage. Furthermore, running the wavelength optimization wizard for these assays will ensure that the reader selects the best excitation/emission pair as described in the supporting application note **"Optimized Wavelength Scanning of Fluorescent Proteins with the SpectraMax Paradigm Platform and TUNE Technology"**.

An important benefit of the LED light source is for kinetic assays, where the signal intensities change over time, from day-to-day or with different assay conditions. With instruments using the xenon flash lamp, the PMT gain must be fixed to a specific gain (high, medium or low) during a kinetic read. If the gain is too high for the well, some kinetic points will not be measured as the PMT saturates. The kinetic profile may miss important information, and resultant curves may not be plotted correctly. If the gain selected is too low for the signal, the system will not be as sensitive, and the resulting kinetic profile may not be optimally precise or accurate. With the SpectraMax i3x reader, the variable LED intensity with fixed PMT gain allows signal detection over an 8- to 9-log dynamic range in a single plate measurement for all read types, including kinetic reads. This allows the system to detect both very low and very high signals in a single plate without saturating or compromising on data quality.

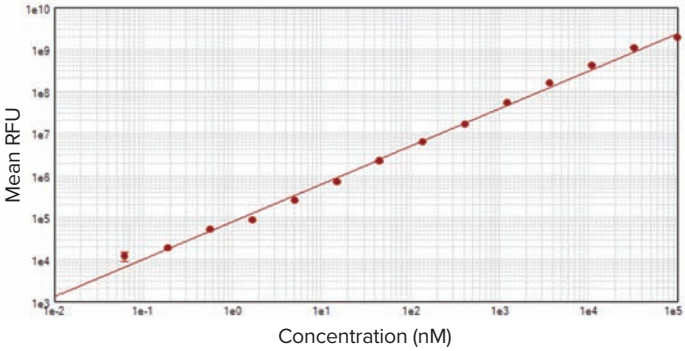

Figure 5. Fluorescein standard curve using the LEDs on the SpectraMax i3x microplate reader.

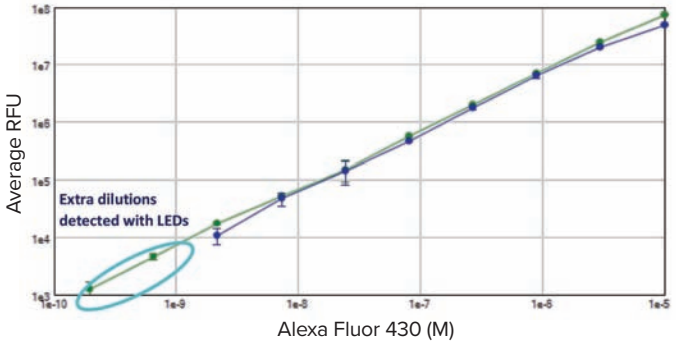

Figure 6. Alexa Fluor 430 Standard curve generated with the flash lamp (Ex 425 nm, in blue) or the LED (Ex 430nm, green) on the SpectraMax i3x reader. With LEDs, two extra dilutions (one extra concentration) were able to be detected.

Conclusion

It is commonly accepted that assay results may vary slightly from day to day or from instrument to instrument due to a variety of reasons, including assay set-up conditions, manufacturing tolerances and user inexperience, to name but a few. The patented Spectral Fusion Illumination featured on the SpectraMax® i3x reader can help compensate for these, and it allows researchers to obtain high-quality, reproducible fluorescence data time after time. This unique design allows the system to achieve full linearity over an extended dynamic range without compromising data quality. Spectral Fusion is available for all the fluorescence read types – endpoint, kinetic, well scan, and spectrum – available on the SpectraMax i3x reader.

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