Tweaking fluorescence scans

Guidelines to recording excitation and emission spectra on the Infinite® M200 PRO and Infinite M1000 PRO

Introduction

The Infinite M200 PRO and Infinite M1000 PRO are Tecan's latest generation of Quad4 Monochromators™-based multimode microplate readers, packed with application-focused functions and technical innovations to offer exceptional speed and sensitivity for academic, biotechnology and pharmaceutical research.

The Infinite M200 PRO is a robust, basic research-oriented reader with multiple innovative features such as the Gas Control Module (GCM™) to regulate the CO_2 and O_2 environment in the reader. Complementing the Infinite M200 PRO is the Infinite M1000 PRO, a multimode reader with unrivalled flexibility and a clear focus on sensitivity and throughput that is tailored for assay development and screening applications.

Both the Infinite M200 PRO and the Infinite M1000 PRO are capable of recording excitation (230 to 850 nm) and emission spectra (280 to 850 nm) of fluorescent compounds. Based on Tecan's patented technology, the Infinite M1000 PRO is the only multimode reader on the market that allows fine-tunable and precise bandwidth adjustment in fluorescence, as well as offering highly acclaimed absorbance, luminescence and 3D fluorescence scanning capabilities.

Fluorescence measurements in general depend on various parameters which can all be optimized to achieve better results in terms of signal intensity and quality.

This Technical Note describes the relevant instrument parameters to acquire optimal fluorescence excitation and emission spectra on the Infinite M200 PRO and Infinite M1000 PRO.

Materials and Methods

Reagents

200 µl of 5 nM fluorescein in 0.01 M NaOH was measured in black 96-well microplates, using 0.01 M NaOH as blank.

Instruments

- Infinite M1000 PRO premium Quad4 Monochromators-based multimode reader
- Infinite M200 PRO Quad4 Monochromators-based multimode reader

Microplates

- Greiner® Bio One 96-well black (Cat. No. 655900, GRE96fb)
- Reagents
- 5 nM fluorescein (Sigma-Aldrich, Cat. No. F6377)
- 0.01 M NaOH (Sigma-Aldrich)

Measurement parameters and instrument settings

Excitation and emission wavelength

While the excitation spectrum demonstrates how efficient it is to excite the fluorophore at a specific wavelength, the emission spectrum describes how efficient it is to detect the emitted light at any given wavelength. In general, peak values describe the wavelength of greatest efficiency for the excitation and measurement of fluorophores. However, depending on the size of Stokes shift[1)] and the bandwidth, or in a spectrum scan, excitation and measurement at peak values might cause an overlap of excitation and emission spectra. In this situation, the excitation and emission wavelength must be moved away from the peak values (Figure 1).

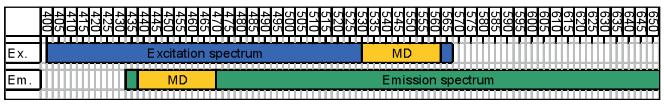

Figure 1 Schematic drawing of the wavelength selection for the excitation (blue) and emission (green) spectra on the Infinite M200 PRO. The minimum distance between the end of the excitation spectrum and its corresponding emission wavelength, as well as the beginning of the emission spectrum and its corresponding excitation wavelength, is shown in yellow.

1) Stokes shift is the wavelength distance between the excitation and emission maximum in nanometres.

Theoretically, a fluorophore can be excited at any wavelength of the excitation spectrum and measured at any wavelength shown by the emission spectrum. The drawback of this flexibility is a reduced excitation or emission efficiency. For instance, fluorescein (Figure 2) can also be excited at 450 nm, accepting a 50 % loss in emission intensity. The same is true for the emission spectrum; the detection wavelength can be shifted to 540 nm resulting in a 50 % loss of signal intensity. However, the use of a higher gain can easily compensate for this. In general the excitation and emission wavelength should be chosen to be as close as possible to the peak spectrum values, but offset as far as necessary to avoid any overlap.

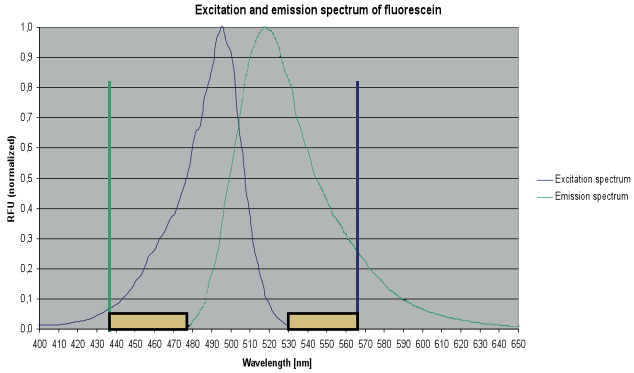

Figure 2 Excitation (blue) and emission (green) spectra of 5 nM fluorescein in 0.01 M NaOH. The vertical, similarly colored bars represent the corresponding fixed excitation/emission values. Yellow spacers show the minimum distance between
1: the beginning of the emission spectrum (green; 470 nm) and its excitation wavelength (green bar; 436 nm) and
2: the end of the excitation spectrum (blue; 530 nm) and the emission wavelength (blue bar; 564 nm) used for detection.

Bandwidth and minimum distance (MD)

Based on the selected bandwidth, it is important to keep a minimum distance between the excitation and emission wavelengths to prevent excitation light from the flash lamp reaching into the detector. Bandwidths for the Infinite M200 PRO are fixed, so its minimum distance remains constant at 34 nm (9 nm excitation bandwidth + 20 nm emission bandwidth + 5 nm safety factor). Due to its high precision components, the Infinite M1000 PRO's minimum distance is only dependent on the selected excitation and emission bandwidths and can therefore vary between 10 nm (5 nm Ex/5 nm Em) and 40 nm (20 nm Ex/20 nm Em), for example if the excitation bandwidth is 8 nm and the emission bandwidth is 10 nm, the resulting MD would be 18 nm.

Recording full excitation and emission spectra

In an excitation spectrum, the emission wavelength is held constant while a range of wavelengths is scanned to determine the best possible wavelength for excitation of a fluorophore (the excitation peak). The upper and lower limits of the scanned range are user-defined, but for the corresponding emission value, the excitation and emission bandwidths must be taken into consideration in the form of the minimum distance. For fluorescein, the excitation spectrum was recorded from 400 to 530 nm. The nearest possible emission wavelength that can be used is calculated by adding the MD to the last excitation wavelength of the spectrum.

Infinite M200 PRO

End of Ex spectrum (530 nm) + MD (min. 34 nm) → 564 nm

Infinite M1000 PRO

End of Ex spectrum (530 nm) + MD (Ex + Em bandwidth)

In an emission spectrum, the excitation wavelength is held constant while a range of wavelengths is scanned in order to determine the wavelength at which light is emitted with the highest energy (the emission peak). The upper and lower limits of the scanned range are user-defined[2)], but for the corresponding excitation wavelength, once again the minimum distance needs to be considered. The emission spectrum was recorded from 470 to 700 nm, and the closest excitation wavelength that can be used is calculated by subtracting the MD.

Infinite M200 PRO

Start of Em spectrum (470 nm) - MD (min. 34 nm) → 436 nm

Infinite M1000 PRO

Start of Em spectrum (470 nm) - MD (Ex +Em bandwidth)

	Excitation spectrum	Emission spectrum
Excitation	400 - 530 nm	436 nm
Emission	564 nm	470 - 700 nm
Step size	1 nm	1 nm
Bandwidth (Ex/Em)	Infinite M200 PRO: (9 nm/20 nm) Infinite M1000 PRO: 5 - 20 nm variable	

Table 1 Measurement parameters for recording the excitation and emission spectra of fluorescein.

2) Literature resources might be used to get an initial idea of where to set the limits.

Gain

Depending on the strength of the signal coming from the sample, the gain (amplification factor) of the detector must be adapted. In most cases, the gain for the intensity scans can be automatically calculated from the microplate well. It is particularly important that the minimum distance is maintained, otherwise the calculation will be based on excitation light that irradiates into the detector, and the signal peak will be lost. For more experienced users, manual gain mode may be used (linear range between ~60 and ~225 nm). Depending on the size of the spectrum, automated gain calculation might considerably extend the scanning time.

Z-position

The Z-position determines the vertical position of the detector with respect to the sample. If it is calculated automatically, the MD must be considered when selecting the Ex and Em wavelengths for its determination. Alternatively, Table 2 provides a tool for manual determination.

Microplate format	Volume (µl)	Z-position (µm)
96-well	300	22781
	250	21844
	200	21374
	150	20723
	100	19912
	75	19412
	50	19163
384-well	30 - 100	23300
1536-well	3 - 10	22775

Table 2 Reference values for the Z-position. For a 384-well microplate with filling volumes between 30 and 100 µl, the respective Z-positions are all in the same range. The same is true for 1536-well plates with filling volumes between 3 and 10 µl.

Measurement mode and microplates

To reduce background, samples were measured in black, flat bottom, 96-well plates using fluorescence intensity top mode.

Flash number and flash frequency

In general, a flash number of 50 and flash frequency of 400 Hz is recommended as the standard setting for fluorescence scans. However, using a higher flash number and the 100 Hz flash frequency option can help to improve the accuracy of the analysis.

Results and discussion

Data trimming

Signals and blanks were recorded (n = 10) using the kinetic cycle option and averaged. The average blank values were subtracted from the average signal values. As excitation and emission spectra generally have different amplitudes, values were normalized by dividing them by their maximum RFU value to fit both spectra onto one single diagram[3)]. After normalizing, values were plotted using a Microsoft Excel® XY (scatter) plot. Figure 2 shows the recorded full excitation and emission spectrum of fluorescein.

Conclusion

Using the guidelines as described for this simple test study will help to optimize the acquisition of fluorescence intensity spectra using the Infinite M200 PRO or Infinite M1000 PRO. As well as selecting which range to scan, it is important to consider the minimum distance between the excitation and emission wavelengths. The rule of thumb for the Infinite M200 PRO is to consider a minimum distance of 34 nm between the excitation and emission wavelengths and the end/beginning of their corresponding spectra. For the Infinite M1000 PRO, the minimum distance only depends on the selected excitation and emission wavelengths.

Abbreviations

Ex/Em	Excitation/emission wavelength
RFU	Relative fluorescence units

3) Maximum values in Microsoft Excel can be determined by: =max("data range")

Austria +43 62 46 89 33 **Belgium** +32 15 42 13 19 **China** +86 21 2206 3206 **Denmark** +45 70 23 44 50 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
Italy +39 02 92 44 790 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 174 **Singapore** +65 644 41 886 **Spain** +34 93 490 01 74 **Sweden** +46 31 75 44 000
Switzerland +41 44 922 89 22 **UK** +44 118 9300 300 **USA** +1 919 361 5200 **Other countries** +41 44 922 8125

397522 V1.0, 06-2012