Evaluation of the Spark® multimode reader for far-red fluorescence emission.

Technical Note

A COMPARISON REVEALS SUPERIOR PERFORMANCE FOR THE DETECTION OF THE FAR RED FLUORESCENT DYE ALEXA FLUOR® 647

INTRODUCTION

The fluorescence detection limit of multimode readers is normally determined using a green fluorescent dye such as fluorescein. However, many customers use dyes emitting in the red region, for example for multicolor detection or to reduce background fluorescence. Standard photomultiplier tubes (PMTs) are often less sensitive in the red and far red wavelengths. Consequently, detection limits determined using green dyes do not necessarily reflect the detection limits for other dyes in the far red range beyond 600 nm.

The Spark multimode reader, Tecan's flagship detection platform, displays excellent sensitivity for green fluorophores (1). With its unique Fusion Optics, it allows users to combine filters and monochromators (MCRs) on the excitation and emission sides within one instrument, and even within one measurement.

In this technical note, we demonstrate the superior performance of the Spark reader compared to two other multimode readers from different providers. The detection limit for the far red fluorescent dye Alexa Fluor 647 – which has a fluorescence spectrum comparable to Cy5 – was determined, operating each instrument in MCR mode.

Instruments A and B are both available with a red-optimized PMT. However, these PMTs do not guarantee optimal performance for green fluorescent dyes. For this study, both instruments were equipped with standard PMTs optimized for green fluorescence. In contrast, the Spark's PMT is optimized for the entire spectrum of visible light, so users do not have to choose between red- and green-optimized PMTs.

Figure 1: Spark next-generation multi-mode reader

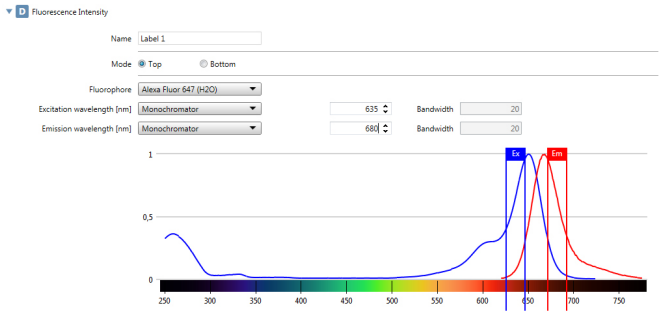

Figure 2: A list of 60 integrated fluorophores with precise spectra allows fast visualization and easy selection of excitation and emission wavelengths in the SparkControl software.

MATERIALS AND METHODS

- Spark multimode reader (Tecan, Austria) with standard fluorescence module
- Instrument A, using a monochromator combining linear variable short- and long-pass filters
- Instrument B, an MCR-based system
- Black 384-well plate, FLUOTRAC™ (Greiner® BioOne, Germany)
- Fluorescein (1 nM in 10 mM NaOH)
- 10 mM NaOH
- Alexa Fluor 647 (1 nM in H_2O, Life Technologies, USA)

To compare the detection limit of the Spark multimode reader with instruments A and B, fluorescein was diluted in 10 mM NaOH to a final concentration of 1 nM. Similarly, Alexa Fluor 647 was diluted in H_2O to a final concentration of 1 nM. A black 384-well microplate was filled with 100 µl per well of either the fluorophore or a blank according to the plate layout in Table 1.

<>	1	3	5	7	9	11	13	15	17	19	21	23
A	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
C	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
E	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
G	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
I	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
K	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
M	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
O	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl

Table 1: Plate layout for Fl top measurements. Bl (blank) = 10mM NaOH (fluorescein) or H_2O (Alexa Fluor 647); Fl (fluorophore) = 1 nM fluorescein or 1 nM Alexa Fluor 647.

Measurement parameters

Each plate was measured three times with the Spark reader, and instruments A and B using the settings in Table 2. All measurements were performed using the instruments in MCR mode. For optimal results, the flash number for all devices was set to 100.

Parameter	Setting
Spark	
Measurement mode	Fluorescence intensity top
Fluorescein	
Excitation	485 nm
Emission	535 nm
Alexa Fluor 647	
Excitation	635 nm
Emission	680 nm
Flashes	100
Gain	Optimal
Dichroic mirror	Auto
Z-optimization	Calculated from well
Settle time	100 msec
Instrument A	
Measurement mode	Fluorescence intensity top
Fluorescein	
Excitation	483 nm
Emission	530 nm
Alexa Fluor 647	
Excitation	635 nm
Emission	680 nm
Flashes	100
Gain	Optimal
Dichroic mirror	No
Z-optimization	Calculated from well
Settle time	100 msec
Instrument B	
Measurement mode	Fluorescence endpoint
Fluorescein	
Excitation	485 nm
Emission	535 nm
Alexa Fluor 647	
Excitation	635 nm
Emission	680 nm
Flashes	100
Gain	Calculated from well
Dichroic mirror	No
Z-optimization	Calculated from well
Settle time	100 msec

Table 2: Measurement parameters used for fluorescence measurements with the Spark reader and instruments A and B.

The detection limit (DL) was calculated for each individual measurement as shown in Equation 1. The average of the three detection limits was used to determine the sensitivity of the instruments for each fluorescent dye.

$$\textit{Detection limit} = \frac{\textit{Concentration[Fl]}}{\textit{(mean[Fl] – mean[Bl])}} * 3 * \textit{Stdev[Bl]}$$

Equation 1: Calculation of the detection limit.

Concentration[Fl]:
Final concentration of fluorescein or Alexa Fluor 647 in pM
mean[Fl]: Average RFU value of wells filled with fluorophore
mean[Bl]: Average RFU value of wells filled with blank
Stdev[Bl]: Standard deviation of wells filled with blank

RESULTS

Table 3 shows the detection limits for fluorescein and Alexa Fluor 647 measured with the Spark, and instruments A and B. Lower detection limits represent increased sensitivity of an instrument.

The detection limit of instrument A is comparable to the Spark reader for fluorescein, while for Alexa Fluor 647, the detection limit is increased more than four-fold. The detection limit for fluorescein is slightly increased (<2 fold) for instrument B compared to the Spark, and is significantly higher (~3.6 fold) for Alexa Fluor 647 (Figure 3).

		Spark MCR	Instrument A	Instrument B
Fluorescein	**DL (pM)**	**0.416**	**0.440**	**0.804**
	St dev	0.033	0.044	0.060
Alexa Fluor 647	**DL (pM)**	**1.140**	**5.144**	**4.189**
	St dev	0.059	0.853	0.295

Table 3: Average detection limits for fluorescein and Alexa Fluor 647.

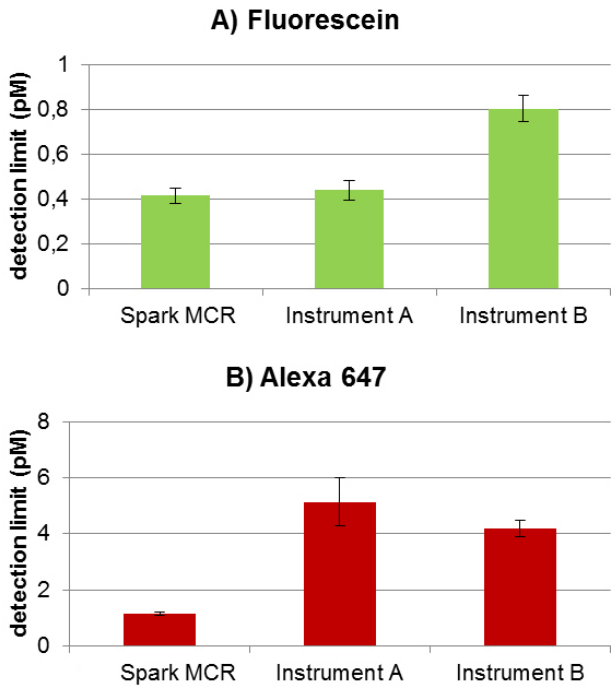

Figure 3: Detection limits of fluorescein (A) and Alexa Fluor 647 (B) measured with the Spark and two comparative multimode readers. Error bars represent the standard deviations

CONCLUSION

PMTs are often less sensitive for red and far red emission than in the green wavelength range. Consequently, detection limits generated for green dyes do not necessarily reflect the detection limits of other dyes. The results presented in this technical note demonstrate the superior performance of the Spark compared to two competitor multimode readers for the far red fluorophore Alexa Fluor 647. Using monochromators, the Spark shows lower detection limits for Alexa Fluor 647 than the other two instruments, and is therefore more sensitive. Detection limits for fluorescein are comparable between the readers. This demonstrates that the Spark reader, with its unique Fusion Optics, is an excellent choice for fluorophores with green as well as red and far red emission spectra.

ABBREVIATIONS

DL	detection limit
MCR	monochromator
PMT	photomultiplier tube

REFERENCES

(1) Technical Note: The ingenious Fusion Optics in the Spark multimode reader. 398568 V2.0 03-2017

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398695 V3.0, 2018-04

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