The ingenious Fusion Optics™ in the Spark® multimode reader.

Technical Note

COMBINING FILTERS AND QUAD4 MONOCHROMATORS™ IN SPARKS STANDARD FLUORESCENCE MODULE FOR FLUORESCENCE APPLICATIONS

INTRODUCTION

Multimode readers can be equipped with either filters or monochromators (MCRs) to define excitation and emission wavelengths in fluorescence applications. MCRs allow the user to select the exact wavelength desired, offering excellent flexibility for a variety of applications. Additionally, only MCRs enable precise spectral scans with small stepsizes. However, as less light is transmitted through MCRs than through filter-based systems, users have to compromise on sensitivity (1). In contrast, filter-based readers offer higher sensitivity, but are limited to certain, defined wavelengths, and are therefore only suitable for certain assays.

There are hybrid systems available which allow for selection of filters or MCRs within one device. However, these systems still require users to choose between the benefits of the two optical technologies, as MCRs and filters cannot be combined within one experiment. In addition, some fluorescence applications such as TRF, TR-FRET and FP are limited to the filter-based optics in hybrid multimode readers, offering no wavelength flexibility at all. Some systems do not even support filter-based fluorescence bottom-reading, which is a significant drawback when working with cells emitting a very weak fluorescent signal.

The Spark reader is equipped with Tecan's unique Fusion Optics, which combine the benefits of filters and MCRs in a single multimode reader. The Spark can be equipped with filters, Quad4 Monochromators or a combination of both using the ingenious Spark Fusion Optics. This allows users to independently choose between filters and MCRs for both excitation and emission independently from each other. So in addition to standard filter or MCR based measurements, it is now possible to measure with filter on excitation and MCR on emission site or vice versa (Figure 1). The new SparkControl™ software facilitates easy selection of filters or the MCR wavelength.

Thus, with the innovative Spark Fusion Optics, the user gets the ideal reader offering optimized performance for every application, without the compromises of standard hybrid systems.

To demonstrate the advantages of Fusion Optics, results with filters, MCRs or combinations enabled by the Fusion Optics were compared in standard fluorescence intensity (FI) end-point measurements, fluorescence spectral scans, and HTRF® exemplary for TR-FRET, a technology from Cisbio Bioassays combining the principles of time resolved fluorescence (TRF) and fluorescence resonance energy transfer (FRET), thereby requiring a high level of instrument sensitivity.

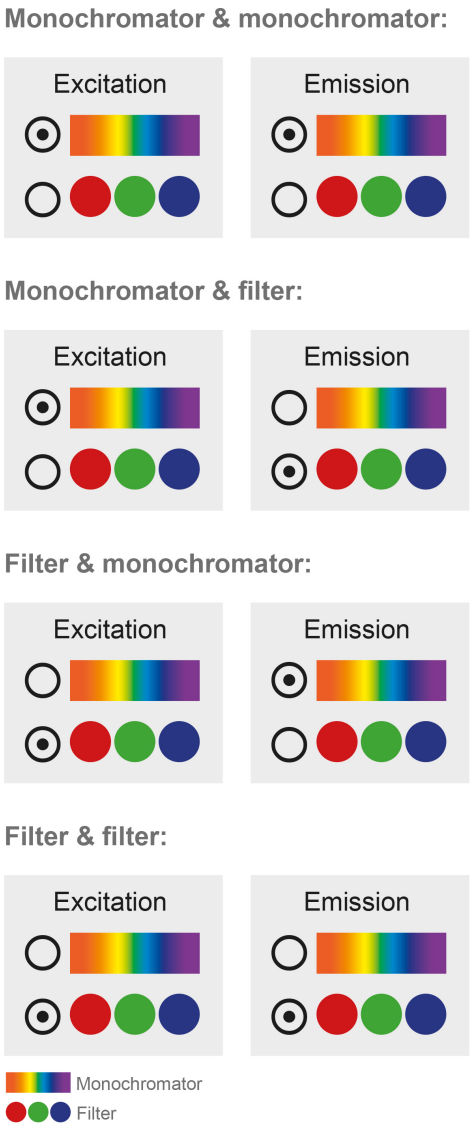

Figure 1: Scheme of the innovative Spark Fusion Optics and the combinatory possibilities. Users can chose between filters and MCR on excitation and emission site within one measurement. This also allows for measurements with filters or MCR on both sites, but also MCR and excitation site and filter on emission site or vice versa.

SPARK FUSION OPTICS FOR FI END-POINT MEASUREMENTS

MATERIAL AND METHODS

- Spark multimode reader with standard fluorescence module (Tecan, Austria)
- 384-well plate, fluorotrack, black (Greiner Bio-One, Germany)
- 1 nM fluorescein in 10 mM NaOH
- 5 nM Alexa Flour® 555 (Life Technologies, USA) in 10 mM NaOH
- 10 mM NaOH
- DELFIA® 1 nM europium standard solution
- #(Perkin Elmer, USA)
- DELFIA® enhancement solution (Perkin Elmer, USA)
- Heraeus™ Labofuge™ 400e (Thermo Scientific, USA).

PROCEDURE

For comparison of the Spark Fusion Optics in standard FI measurements, fluorescein or Alexa Fluor 555 were diluted in 10 mM NaOH to a final concentration of 1 nM and 5 nM, respectively. A black 384-well microplate was filled according to the plate layout Table 1 with fluorescein, Alexa Fluor 555, or europium, respectively. To remove air bubbles, the plate may be briefly spun down at 500-2,000 rpm (Heraeus Labofuge 400e).

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl
B	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
D	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
F	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
H	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl	Bl	Fl

Table 1: Plate layout for FI top measurements. Bl (blank) = 10 mM NaOH or europium enhancement solution; Fl (fluorophore) = 1 nM fluorescein, 5 nM Alexa Flour 555, or 1nM europium.

MEASUREMENT PARAMETERS

The plate was measured three times using the instrument settings in Table 2. For optimal results the flash number was set to 100.

Parameter	Setting
Fluorescein	
Measurement mode	Fluorescence Intensity Top
Excitation	Filter: 485 (20) nm MCR: 485 nm
Emission	Filter: 535(25) nm MCR: 535 nm
Flashes	100
Lag time	0 µs
Integration time	40 µs
Gain	Optimal
Mirror	510 nm dichroic
Z-optimization	Calculated from well
Settle time	0 ms
Alexa Fluor 555	
Measurement mode	Fluorescence Intensity Top
Excitation	Filter: 510 (10) MCR: 510 nm
Emission	Filter: 560(20) nm MCR: 560 nm
Flashes	100
Lag time	0 µs
Integration time	40 µs
Gain	Optimal
Mirror	Wide-band
Z-optimization	Calculated from well
Settle time	0 ms
Europium	
Measurement mode	Fluorescence Intensity Top
Excitation	Filter: 340 (35) nm MCR: 340 nm
Emission	Filter: 612(10) nm MCR: 617 nm
Flashes	100
Lag time	100 µs
Integration time	400 µs
Gain	Optimal
Mirror	510 nm dichroic
Z-optimization	Calculated from well
Settle time	0 ms

Table 2: Test Parameters used for fluorescence measurements with different combinations of the Fusion Optics.

Data reduction

To exclude results of wells which were significant outliers, the Grubbs' outlier test (also known as the maximum normed residual test) was used. This statistical test is used to detect outliers in a univariate data set assumed to come from a normally distributed population (2). The test was performed using the GraphPad online calculator with a significance level of 0.05 (3). After removing outliers, the detection limit (DL) was calculated as shown in Equation 1.

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

Equation 1: Calculation of the detection limit.
Concentration[Fl]: Final concentration of fluorescein 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

The Grubbs' test and calculation of the detection limit were performed for each individual measurement. The average of the three detection limits was calculated and used to determine the sensitivity of the instrument. The resulting absolute detection limit was then transformed to a relative detection limit with the filter/filter combination set to 100% (Figure 2).

RESULTS

Lower detection limits stand for increased sensitivity of an instrument. The end-point FI measurements of the two fluorescent dyes demonstrate that using filters or a combination of filter and MCR of the Fusion Optics gives comparable results, with no major differences in the detection limits. Using MCRs on both sides, an increase of approximately two-fold in the relative detection limit for fluorescein and Alexa Fluor 555 was observed (Figure 2), equivalent to half the sensitivity of filter-based measurements. For TRF measurement of europium, only marginal differences can be seen.

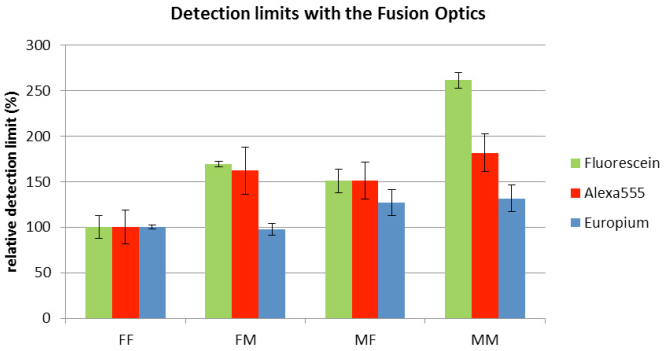

Figure 2: Relative detection limits of fluorescent dyes comparing different combinations enabled by the Fusion Optics. Error bars represent standard deviations. FF = filter on both sites; FM = filter on excitation, MCR on emission site; MF = MCR on excitation, filter on emission site; MM = MCR on both sites.

Yet, using all combinations of the Spark Fusion Optics show very low detection limits thus very high instrument sensitivity, indicating that for standard FI endpoint measurements, all combinations enabled by the Spark Fusion Optics of the Spark can be used without compromising on performance (Table 3).

Measurement No.	Mode FF	FM	MF	MM
1	0.117 pM / 11.7 amol/well	0.225 pM / 22.5 amol/well	0.219 pM / 21.9 amol/Well	0.336 pM / 33.6 amol/well
2	0.129 pM / 12.9 amol/well	0.220 pM / 22.0 amol/well	0.185 pM / 18.5 amol/well	0.344 pM / 33.4 amol/well
3	0.151 pM / 15.1 amol/well	0.228 pM / 22.8 amol/well	0.195 pM / 19.5 amol/well	0.358 pM / 35.8 amol/well
Average	**0.132 pM / 13.2 amol / well**	**0.225 pM / 22.5 amol/well**	**0.200 pM / 20.0 amol/well**	**0.346 pM / 34.6 amol/well**
StDev	0.0168 pM / 1.68 amol/well	0.0040 pM / 0.40 amol/well	0.0173 pM / 1.73 amol/well	0.0112 pM / 1.12 amol/well

Table 3: Representative detection limits for fluorescein using the Spark reader with optimized conditions. The plate was measured three times with each combination of the Fusion Optics. FF = filter on both sides; FM = filter on excitation, MCR on emission; MF = MCR on excitation, filter on emission; MM = MCR on both sides.

FUSION OPTICS FOR FLUORESCENCE SCANS

MATERIAL AND METHODS

- Spark multimode reader with standard fluorescence module (Tecan, Austria)
- 384-well plate, fluorotrack, black (Greiner Bio-One, Germany)
- 1 nM fluorescein in 10 mM NaOH
- 5 nM Alexa Flour® 555 (Life Technologies, USA) in 10 mM NaOH
- 10 mM NaOH
- DELFIA® 1 nM and 0.5 pM europium standard solution (Perkin Elmer, USA)
- DELFIA® enhancement solution (Perkin Elmer, USA)
- Heraeus™ Labofuge™ 400e (Thermo Scientific, USA)

MEASUREMENT PARAMETERS

Excitation and emission spectra for fluorescein, Alexa Fluor 555 and europium were acquired using the same gain for filters and MCRs. Detailed measurement parameters are depicted in Table 4.

Parameter	**Setting**	
Measurement mode	**Excitation scan**	
	Fluorescein	**Alexa Fluor 555**
Excitation	440 – 496 nm (MCR)	480 – 570 nm (MCR)
Emission	Filter: 560(20) nm MCR: 560 nm	Filter: 610(20) nm MCR: 610 nm
Flashes	100	
Integration time	40 µs	
Gain	119 (optimal)	150 (optimal)
Mirror	510 nm dichroic	Wide-band
Z-optimization	Calculated from well	
Settle time	0 ms	
Measurement mode	**Emission scan**	
	Fluorescein	**Alexa Fluor 555**
Excitation	Filter: 460(10) nm MCR: 460 nm	Filter: 510(10) nm MCR: 510 nm
Emission	500 – 600 nm (MCR)	440 - 530 nm (MCR)
Flashes	100	
Integration time	40 µs	
Gain	114 (optimal)	81 (optimal)
Mirror	510 nm dichroic	Wide-band
Z-optimization	Calculated from well	
Settle time	0 ms	
Measurement mode	**Excitation scan**	
	1 nM europium	**0.5 pM europium**
Excitation	300 – 400 nm (MCR)	
Emission	Filter: 612(10) nm MCR: 617 nm	
Flashes	100	
Lag time	100 µs	
Integration time	400 µs	
Gain	119 (optimal)	255 (manual)
Mirror	510 nm dichroic	
Z-optimization	Calculated from well	
Settle time	0 ms	
Measurement mode	**Emission scan**	
	1 nM europium	**0.5 pM europium**
Excitation	Filter: 340(35) nm MCR: 340 nm	
Emission	550 – 650 nm (MCR)	
Flashes	100	
Lag time	100 µs	
Integration time	400 µs	
Gain	119 (optimal)	255 (manual)
Mirror	510 nm dichroic	
Z-optimization	Calculated from well	
Settle time	0 ms	

Table 4: Parameters used for excitation and emission scans of fluorescein, Alexa Fluor 555, and europium.

RESULTS

Both excitation scans and emission scans for fluorescein, Alexa Fluor 555 and europium revealed that sensitivity is increased when using filters for the side with the fixed wavelength and MCRs for scanning. Using the same gain, the peak excitation/emission signal obtained with MCRs on both sides was approximately 20-25 % of the peak signal using a combination of filters and MCRs (Figure 3). For high concentrations of fluorophore, this lower signal intensity can be compensated for by adjusting the gain appropriately. However, when the concentration is low and the gain is set to the maximum, the increased sensitivity of filters can improve the signal-to-noise ratio, thereby enabling the acquisition of scans at lower concentrations than with MCRs alone (Figure 4).

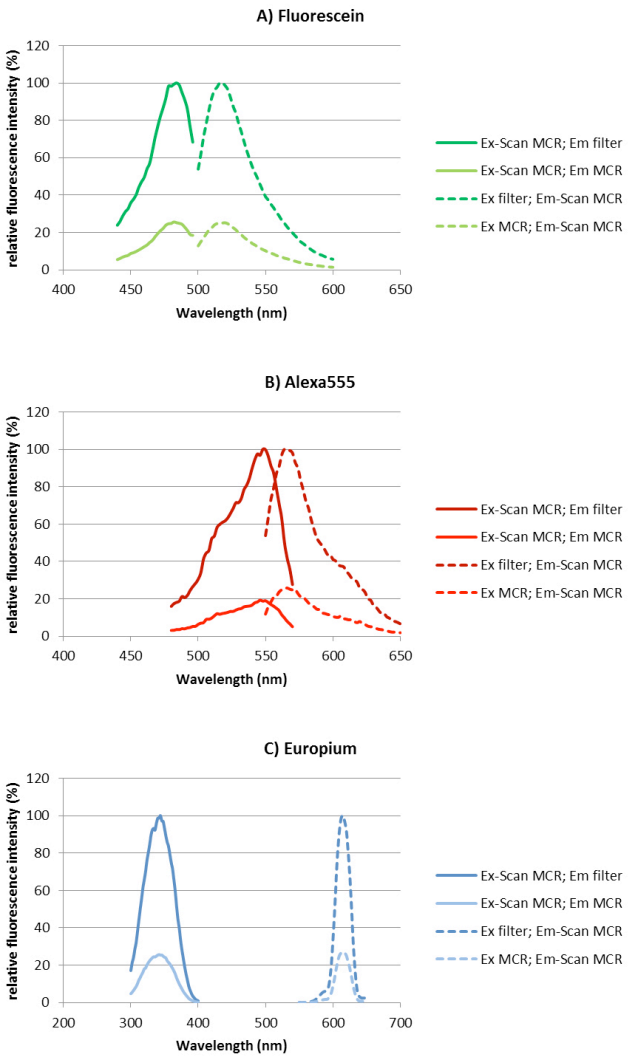

Figure 3: Comparison of excitation and emission scans for fluorescein (A), Alexa Fluor 555 (B) and europium (C) reveal that MCRs only detect ~25 % of the signal compared to using filters on the side with the fixed wavelength.

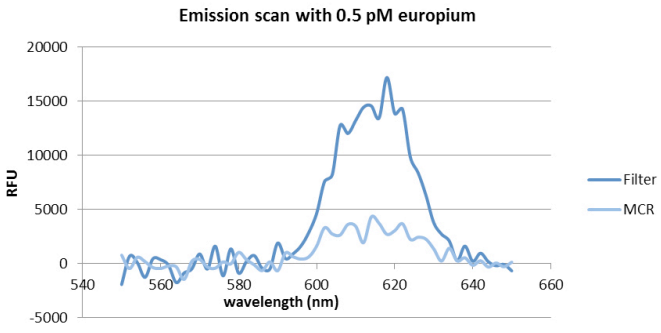

Figure 4: Comparison of emission scans with a very low concentration of europium (0.5 pM) acquired with maximum gain. Using MCRs on both sides results in a poor signal-to-noise ratio, whereas using a combination of filters and MCRs provides a reasonable spectrum.

FUSION OPTICS FOR HTRF

The HTRF technology from Cisbio Bioassays combines the principles of FRET and time-resolved fluorescence. It is based on the energy transfer between two fluorophores, a long-lived europium or terbium cryptate donor and a chemically-modified allophycocyanine (XL665) or a d2 acceptor. HTRF-based applications have become increasingly popular for the analysis of various molecular interactions and binding studies, due to their homogeneity, robustness, sensitivity and potential for miniaturization.

For HTRF measurements, high sensitivity readers which are able to work at low light levels are necessary for accurate detection of the fluorescent signal. At the same time, the emission should be measured with a low bandwidth to reduce background and provide a broad dynamic range. Many multimode-readers using MCRs are not sensitive enough to obtain good results for HTRF, however Tecan's Infinite® M1000 PRO is an exception to this, demonstrating that an MCR-based instrument can be sensitive enough for HTRF. Here, the performance of the Spark equipped with its standard fluorescence module for HTRF measurements was tested.

MATERIAL AND METHODS

- Spark multimode reader with standard fluorescence module (Tecan, Austria)
- 96-well half area microplates, white (Greiner Bio-One, Germany)
- HTRF Reader Control Kit (Cisbio Bioassays, USA)
- Heraeus Labofuge 400e (Thermo Scientific, USA))

The HTRF Reader Control Kit is intended for the calibration of HTRF-compatible instruments and for validation of their ability to perform HTRF measurements.

The Reader Control Kit was used according to the manufacturer's instructions. A 96-well half area microplate was filled to a final volume of 100 µl using the plate layout shown in Figure 5. To remove air bubbles, the plate may be quickly spun down at 500-2,000 rpm (Heraeus Labofuge 400e). The plate was then measured using the parameters shown in Table 6.

	1	2	3	4	5	6	7	8
A	620 nm Control	Standard 0		Low Control	High Control	Buffer Blank	XL665 Blank	Cryp Blank
B								
C								
D								
E								
F						Buffer Blank		
G								
H								

Figure 5: Plate layout for the HTRF assay.

Parameter	Setting
Measurement mode	Fluorescence Intensity Top
Label 1	
Excitation	Filter: 320 (25) nm MCR: 320 nm
Emission	Filter: 620 (10) nm MCR: 620 nm
Lag time	150 µs
Integration time	500 µs
Gain	Optimal
Mirror	510 nm dichroic
Flashes	100
Settle time	0 ms
Z-optimization	Calculated from well
Label 2	
Excitation	Filter: 320 (25) nm MCR: 320 nm
Emission	Filter: 665 (8) nm MCR: 665 nm
Lag time	150 µs
Integration time	500 µs
Gain	Optimal
Mirror	510 nm dichroic
Flashes	100
Settle time	0 ms
Z-optimization	Calculated from well

Table 6: Measurement parameters for the HTRF assay using different combinations of the Fusion Optics.

RESULTS

The measurement results obtained with the HTRF Reader Control Kit are summarized in Table 7. Using filters on the emission side, the Spark exceeds all performance criteria specified by the manufacturer, resulting in an exceptionally good measurement range.

Due to the 20 nm bandwidth, which allows detection of too much background signal, the results using MCRs on the emission side have a decreased dynamic range (evident from the reduced ΔF values) (Table 7).

	S/B	CV std0 (%)	CV low ctrl (%)	CV high ctrl (%)	ΔF high	ΔF low	Z'
MCR/MCR	448,13	3,81	3,61	2,57	419,37	15,15	0,87
MCR/Filter	619,93	2,72	3,08	1,38	916,35	28,09	0,94
Filter/MCR	555,16	1,65	2,29	1,09	419,61	14,02	0,94
Filter/Filter	560,92	1,14	1,47	1,69	922,14	29,93	0,94
CisBio Criteria	*40*	*10*	*10*	*10*	*600*	*15*	*0,7*

Table 7: Measurement results obtained with the reader control kit using different combinations with the Fusion Optics. S/B = signal to blank ratio; CV std0 = Green fields meet the Cisbio performance criteria (yellow), red fields do not.

The results acquired with the HTRF Reader Control Kit demonstrate the suitability of the Spark equipped with its standard fluorescence module for HTRF measurements. Either filters or MCRs can be used on the excitation side, offering complete flexibility in excitation wavelength, while filters on the emission side guarantee optimal sensitivity.

SUMMARY

Tecan's innovative Spark multimode reader provides optimized, tailor-made solutions for customers to take advantage of the unique Fusion Optics.

Quad4 Monochromators implemented in the standard fluorescence module of the Spark ensure excellent sensitivity, offering high quality results for standard FI measurements. In addition, for critical applications with weak fluorescence signals which require very sensitive detection, the signal can be boosted by combining the MCRs with filter-based optics on either the excitation or emission sides. For spectral scans of fluorophores at low concentrations, using filters on the fixed wavelength side can significantly improve the quality of the spectral curve while the spectrum is scanned by MCRs. Using filters on the emission side also allows HTRF-based measurements, with the instrument performance exceeding the manufacturer's criteria for their technology.

For experiments requiring extremely high sensitivity, users benefit from our newly developed Fusion Optics, which enable them to easily define their optimal MCR/filter combination for both excitation and emission without having to compromise between sensitivity and flexibility anymore. This can make the difference between a good result and an excellent result.

REFERENCES

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Dr. Christian Oberdanner is Product Manager at Tecan Austria. He studied molecular biology at the University of Salzburg with a strong focus on cell- and tumor biology during his PhD study. Christian started to work for Tecan Austria as external scientific consultant in 2005 and permanently joined the company in 2006. Since then he held the role of an Application Specialist in the research and development department as well as in the sales and marketing department and since 2015 of a Product Manager. Christian's priority within Tecan is the multimode microplate reader portfolio.

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