

Use of Spectral Scanning Fluorometer and Spectral Analysis to Optimize Assays with Multiple Fluorometric Labels

Jorma Lampinen, Hanna Granö, Vuokko Kytöniemi and Arja Lamberg
Thermo Electron Oy, Vantaa, Finland

Introduction

When fluorometric multianalyte assays are developed it is extremely important to optimize excitation and emission wavelengths for each label to achieve the best possible assay resolution. This paper shows the principle how wavelength selection optimization can be easily performed with the spectral scanning fluorometer and analysis of the fluorescence spectra.

During the optimization procedure the fluorescence excitation and emission spectra of each label is measured separately and in combination with all labels within the same well. Spectra are analyzed by calculating the ratios between the emission signal of each label and the summarized signal of interfering labels. These signal / interference signal ratios were calculated separately for each label and for both excitation and emission spectra. The maxima of the ratio curves are used for measurement of the label in the label mixture.

The optimization of multilabel assay parameters was performed with four common fluorescent labels: Methylumbelliferone (MeU), Alexa Fluor 488, 6-Carboxy-X-Rhodamine (6-ROX) and Alexa Fluor 680. Optimization is performed with the Varioskan spectral scanning fluorometer connected to SkanIt Software and with 96-well black microtiter strip plates (Thermo Microtiter, prod. no. 95029450, Thermo Electron Oy, Vantaa, Finland). The results show that using this optimization principle it is possible to design new multianalyte assays quite easily with optimal label resolution. The results will also show that the optimal wavelengths for multianalyte assays differ from commonly used wavelengths as well as from the spectral maxima of these labels.

Methods

Four common commercial labels were used to optimize multilabel assay parameters: MeU, Alexa Fluor 488, 6-ROX and Alexa Fluor 680. These labels were selected because their fluorescence spectra are divided into a large wavelength area and therefore they are potential candidates as labels to be used in combination in multiplexing assays. All labels were purchased from Molecular Probes Inc. (Eugene, OR).

The fluorochromes were dissolved in 50 mM PBS buffer, pH 9.0, and diluted to a working concentration with the same buffer. The following concentrations of each fluorochrome were used for assay optimization: MeU, 1000; Alexa Fluor 488, 500; 6-ROX, 500 and Alexa Fluor 680, 1000 (pmol/well). 50 µl of each fluorochrome was transferred to the black 96-well microtiter plate according to the layout shown in Picture 1. An aliquot of 50 µl of each fluorochrome was added to each well after which all wells were filled to 200 µl with the PBS buffer.

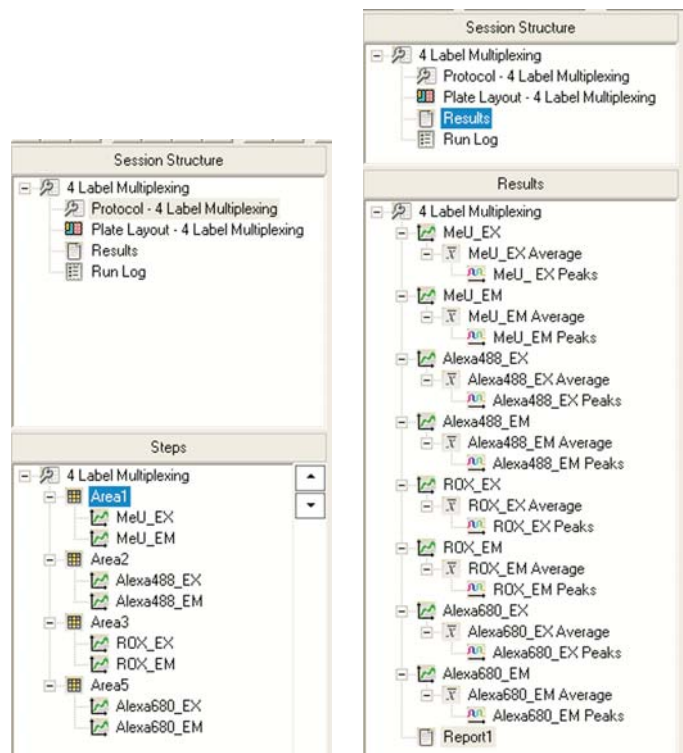
The fluorescence excitation and emission spectra of all wells were measured with the Varioskan spectral scanning microplate fluorometer (Thermo Electron Oy, Vantaa, Finland). The measurement settings were adjusted so that the blanks, a fluorochrome alone, a combination of three other fluorochromes and a combination of four fluorochromes were measured together using the same measurement settings. The measurement settings were as follows:

- MeU: Emission at 448nm, excitation scanning 300 - 430nm
Excitation at 360nm, emission scanning 380 - 520nm
- Alexa 488: Emission at 540nm, excitation scanning 410 - 520nm
Excitation at 480nm, emission scanning 498 - 620nm
- 6-ROX: Emission at 630nm, excitation scanning 480 - 610nm
Excitation at 550nm, emission scanning 570 - 680nm
- Alexa 680: Emission at 722nm, excitation scanning 561 - 703nm
Excitation at 660nm, emission scanning 680 - 760nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS buffer blank	PBS buffer blank	PBS buffer blank	PBS buffer blank	PBS buffer blank	PBS buffer blank	Alexa 488, 6-ROX, Alexa 680	Alexa 488, 6-ROX, Alexa 680	Alexa 488, 6-ROX, Alexa 680	Alexa 488, 6-ROX, Alexa 680	Alexa 488, 6-ROX, Alexa 680	Alexa 488, 6-ROX, Alexa 680
B	MeU standard, 1000 pmol/well	MeU standard, 1000 pmol/well	MeU standard, 1000 pmol/well	MeU standard, 1000 pmol/well	MeU standard, 1000 pmol/well	MeU standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680	MeU, Alexa 488, 6-ROX, Alexa 680	MeU, Alexa 488, 6-ROX, Alexa 680	MeU, Alexa 488, 6-ROX, Alexa 680	MeU, Alexa 488, 6-ROX, Alexa 680	MeU, Alexa 488, 6-ROX, Alexa 680
C	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well
D	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well
E	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well	6-ROX, Alexa 680 standard, 500 pmol/well
F	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 500 pmol/well
G	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well
H	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well	MeU, Alexa 488, 6-ROX, Alexa 680 standard, 1000 pmol/well

Picture 1. 96-well microtiter plate layout used to optimize a multilabel assay with four common fluorochromes.

All measurements were performed using a 5 nm excitation and 12 nm emission slit, 100 ms/well integration time (=10 flashes) and a 2 nm scanning wavelength step size. The assay plate was divided into four different measurement areas to be able to target the scanings to the correct wells. The measurement protocol defined in SkanIt Software was according to Picture 2.



Picture 2. Varioskan measurement protocol structure for the multilabel assay optimization. The assay plate was divided into four different well groups for the spectral scanning measurements. Automated result calculation was used to calculate the average spectra of all scanings and to search for the peaks in each spectra.

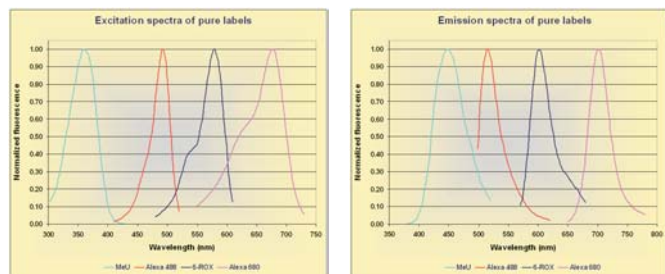
Results

Fluorescence excitation and emission spectra of all four labels were drawn from the spectral scanning result data. The spectra are shown in Picture 3.

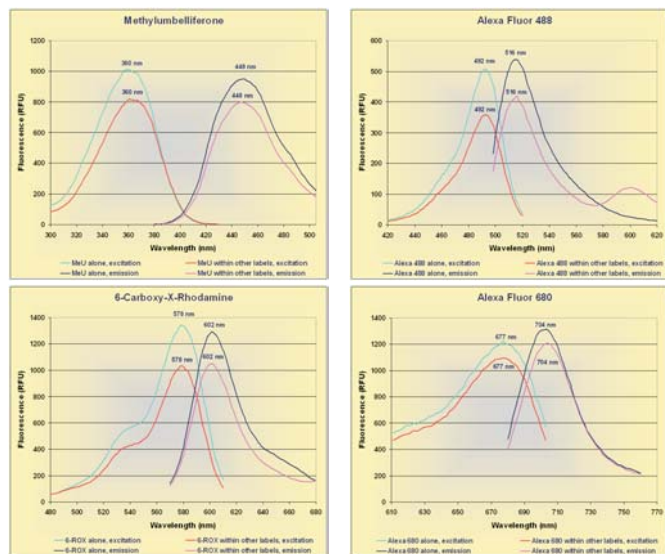
One important feature of suitable fluorochromes for multiplexing assays is that the fluorochromes may not have an effect on the excitation or emission of the other fluorochromes. Therefore, excitation and emission spectra of a pure fluorochrome was compared to the spectra measured from the mixture including all four fluorochromes. The comparison spectra are shown in Picture 4.

As a following step, ratio curves between the fluorochrome spectra and the spectra of the other three fluorochromes was calculated for all excitation and emission scanings. This ratio shows the resolution of the fluorochrome signal from the interfering signal originating from the other fluorochromes and the resulting spectra are shown in Picture 5.

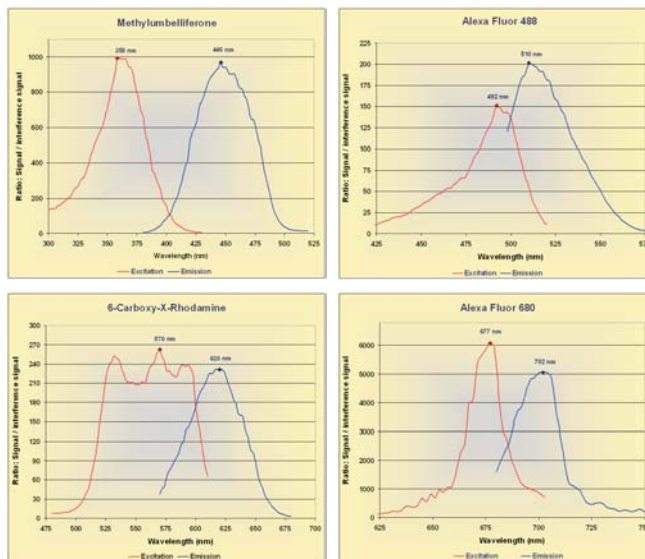
Data from the spectra shown in Pictures 4. and 5. was summarized in Picture 6. The reference data of the excitation and emission maxima from the fluorochrome manufacturer's Web site was additionally collected to the picture together with our common recommendations about the fluorescence filter selections for the measurement of these fluorochromes with filter fluorimeters.



Picture 3. Excitation and emission spectra of the four fluorochromes used in multiplexing assay optimization.



Picture 4. Comparison spectra of the fluorochromes. Fluorochrome excitation and emission spectra were measured both with the fluorochrome alone (pure label) and in the combined mixture with the other fluorochromes.



Picture 5. Fluorescence signal to interfering signal ratio curves of the fluorochromes used in multiplexing optimization. The increased ratio shows a better resolution of the measured fluorochrome over the other fluorochromes present in the same assay mixture.

Fluorometric label	Literature reference peak wavelengths (www.probes.com)		Recommendations for the measurement with filter based fluorimeters	
	Excitation (nm)	Emission (nm)	Excitation (nm)	Emission (nm)
Methylnbelliferone (MeU)	358	450	355	460
Alexa Fluor 488	493	517	485	518
6-Carboxy-X-Rhodamine (6-ROX)	578	604	578	604
Alexa Fluor 680	679	702	678	700

Fluorometric label	Optimal measurement wavelengths for Varioskan when used as single label		Optimal measurement wavelengths for Varioskan when used in combination with others	
	Excitation (nm)	Emission (nm)	Excitation (nm)	Emission (nm)
Methylnbelliferone (MeU)	360	448	358	446
Alexa Fluor 488	492	516	492	510
6-Carboxy-X-Rhodamine (6-ROX)	578	602	570	620
Alexa Fluor 680	677	704	677	702

Picture 6. Summary data from the multiplexing assay optimization.



Conclusions

Both excitation and emission spectra of these four different fluorochromes are well spread over the visible wavelength area and they are sufficiently separated for multiplexing assays. It is possible to measure all four separately from the same well. Mixing these four labels together into the same well has a slight effect on the fluorescence intensity of these fluorochromes but there is no effects on the shape of the excitation of emission spectra. Generally, fluorescence from the mixture has about 20% lower intensity than from the pure label solution. This is probably due to the absorption of the fluorescence emission by other labels because the emission spectra of one fluorochrome always overlaps with the excitation spectra of another fluorochrome, MeU emission spectra overlaps with Alexa Fluor 488 excitation, its emission overlaps with 6-ROX excitation, etc. However, the decrease is acceptable for multiplexing assays.

Detected excitation and emission maxima as well as the shape of the spectra from both the pure label solutions and the label mixture are identical to the maxima reported by the fluorochrome manufacturer showing that the spectral scanning measurement was both reliable and precise.

Optimal measurement wavelengths for multiplexing assays with these fluorochromes are quite similar to the optimal wavelengths for single label assays with all other fluorochromes except 6-ROX where a remarkable difference was noticed.

With 6-ROX, about a 20% higher signal to background ratio was observed both with 570 nm excitation and 620 nm emission compared to the 578 nm excitation and 602 nm emission maxima of the single label assay.

The small decrease in excitation wavelength is useful to decrease excitation of the Alexa Fluor 680 simultaneously with 6-ROX and the increase in emission wavelength is most probably due to the small overlapping of the Alexa Fluor 488 and 6-ROX emission spectra.

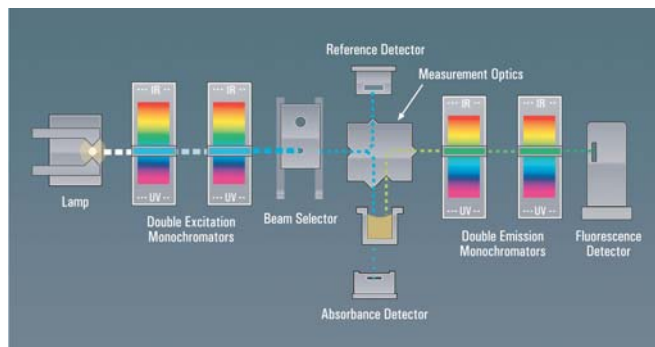
Signal to interfering signal curve of 6-ROX excitation clearly shows three peaks offering three possible excitation wavelengths. These peaks can be explained by the interference of Alexa Fluor 688 excitation that is partially overlapping with 6-ROX excitation. The absolute excitation efficiency is anyhow clearly lower with these other peaks and therefore the middle peak of 570 nm is the best choice for the measurement (about 35% efficiency with the 530 nm peak, about 90% with the 570 nm peak and about 65% with the 594 nm peak).

Similar multiplexing assay optimization was also tested with the fifth fluorometric label, Tetramethyl Rhodamine-isothiocyanate (TRITC), but it has so much overlapping in both excitation and emission spectra with 6-ROX that the separation of those two fluorochromes reliably was proven to be impossible (detailed data not shown).

Based on these experiments, it is essential to optimize measurement wavelengths when one is developing assays with multiple fluorometric labels. Multiple label environment can remarkably affect on the optimal measurement parameters.

The most efficient way to develop new multilabel assays is to analyze the fluorescence spectra of both the pure fluorochrome and different fluorochrome mixtures. The optimal parameters for each label combination can be revealed from these spectra.

A spectral scanning microplate fluorometer is an extremely efficient tool for multiplexing assay development and for optimizing assay parameters.



Operational principle of Varioskan optics.



For more information contact:

Thermo Electron Oy

P.O. Box 100, FI-01621 Vantaa, Finland

Email: info.microplateinstruments@thermo.com

Web: <http://www.thermo.com>

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