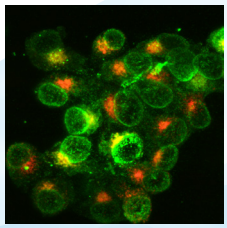
HORIBA Scientific

Fluorescence

# Synchronous Scanning with Duetta



Application Note
Life Sciences

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Figure 1: Duetta 2-in-1 fluorescence and absorbance spectrometer

## Introduction

Synchronous scanning is a spectroscopic method designed to extract information from complex fluorescence spectra that results from multiple fluorescing components. With a traditional spectrofluorometer, both the excitation and the emission monochromators are scanned at the same time, in synchrony, with a fixed offset wavelength value. In such an acquisition, the user selects the starting and ending excitation wavelength for a scan, and the wavelength offset for the emission monochromator. A synchronous scan thus results in no signal at wavelengths where there is no overlap between the fluorescence excitation and emission wavelengths, and a narrow spectrum where there is an overlap between the excitation and emission spectrum. For fluorescing molecules with a large Stokes Shift, this overlap is very small, but for samples with a small Stokes Shift this overlap occurs over a wider wavelength range.

Figure 2 shows examples of individual fluorescence excitation and emission spectra of three fluorochromes in water: quinine sulfate, fluorescein, and rhodamine 101. As you can see from each spectral pair, there is a region of overlap between each excitation and emission spectrum. Stokes Shift (the difference between the excitation and emission spectral peaks) and the overlap between the excitation and emission both play a part in the synchronous scan. The overlap for quinine sulfate is quite small but has a large Stokes Shift, while the overlap between the other two molecules is larger, but the Stokes Shift is smaller.

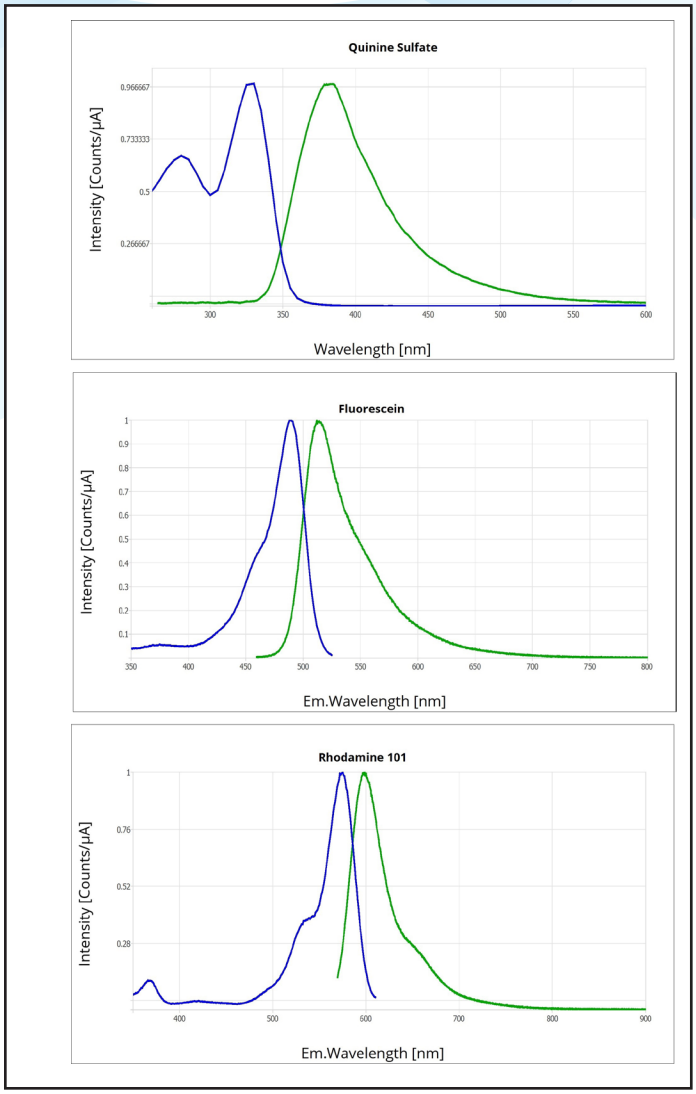


Figure 2: Excitation spectra (blue) and emission spectra (green) of quinine sulfate (top), fluorescein (center) and rhodamine 101 (bottom) in water (neutral pH).

Below are single excitation and emission spectra for the solution mixture of the three dyes, using an emission wavelength of 610 nm to acquire the excitation scan and an excitation wavelength of 340 nm to acquire the emission scan. Both spectra show multiple peaks, giving an indication that the solution is a mixture, but it is unclear from each of these that the mixture has three distinct dyes in it. Excitation and emission spectra for the three dyes do overlap somewhat, so these methods of using fluorescence emission and excitation are not suitable methods for identifying the components in the solution mixture. In fact, single spectra can often be misleading in the case of unknown components if the spectra measured use 2-dimentional methods that are specific to one dye or fluorescing compound.

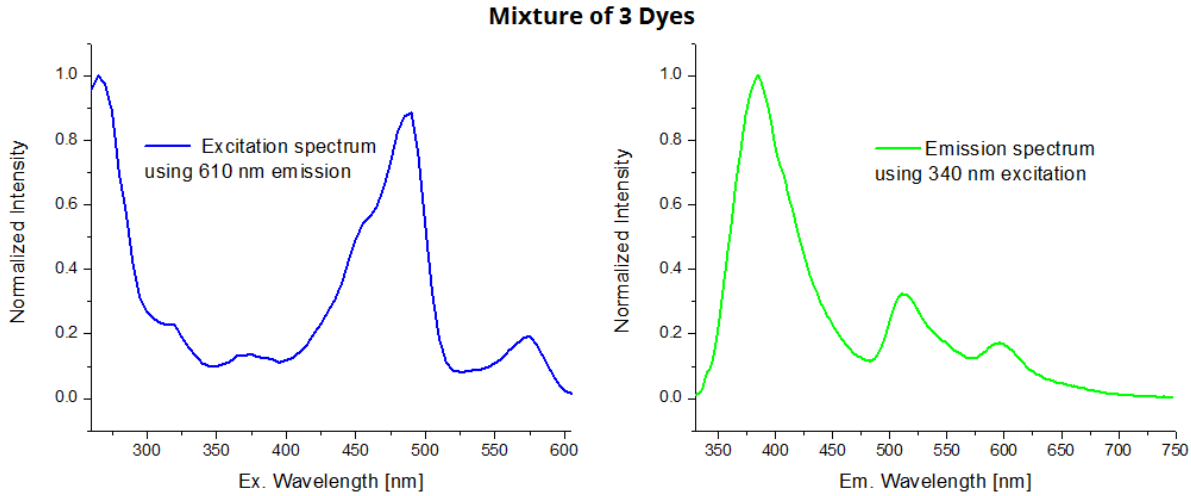


Figure 3: Left: A fluorescence excitation spectrum and Right: A fluorescence emission spectrum of the mixture of quinine sulfate, fluorescein, and rhodamine 101 in water.

By measuring the synchronous scan of each of the dye solutions, a single peak for each of the dyes comes out very clearly. The graph in Figure 4 shows the individual synchronous peaks for a solution of quinine sulfate, a solution of fluorescein, and a solution of rhodamine 101, using a 40-nm offset. This means that for every plotted emission wavelength, the excitation wavelength was 40 nm lower than the emission wavelength. The graph in Figure 4 is normalized for easy viewing, but the peaks will differ in intensity based on concentration of the dye, overlap region, and Stokes Shift as mentioned previously. Using a 40-nm offset between excitation and emission monochromators, quinine sulfate in water has a spectral overlap peak centered at 375 nm, while fluorescein has a peak centered at 525 nm and rhodamine 101 has a peak centered at 610 nm.

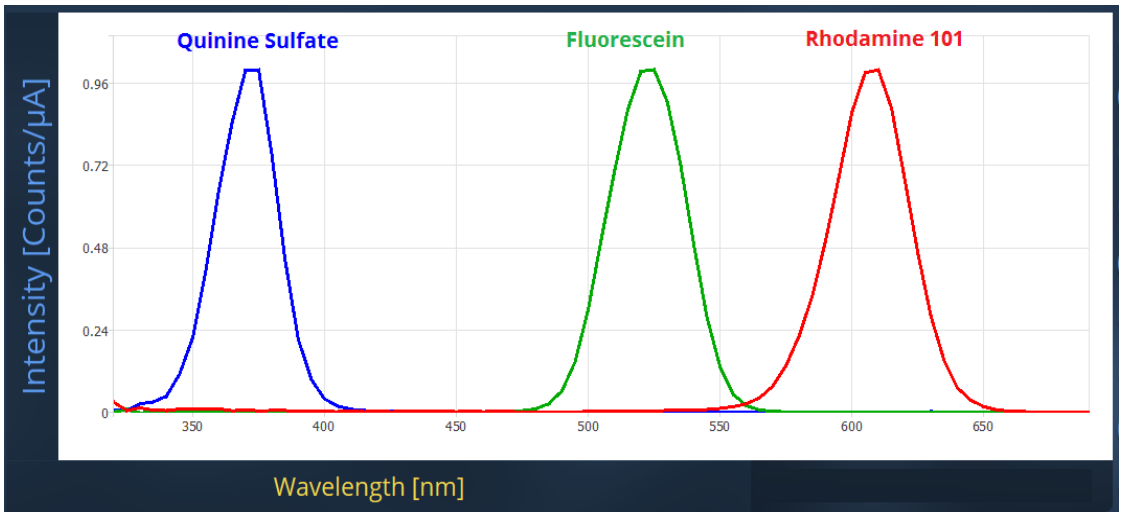


Figure 4: Synchronous scans (normalized) using 40-nm offset for quinine sulfate, fluorescein, and rhodamine 101 in three individual solutions

While the synchronous scan method has been used for decades, a much better method for fluorescence component analysis is to acquire a complete 3-D excitation and emission matrix (EEM). The EEM captures much more information and can be used with chemometrics software for very precise, and complex, component analysis. Duetta can certainly provide synchronous scans, but it can also provide much more, and much faster. Duetta EEMs are required very quickly because Duetta uses a CCD detector with a fixed grating spectrograph for fluorescence emission rather than a scanning monochromator and a PMT.

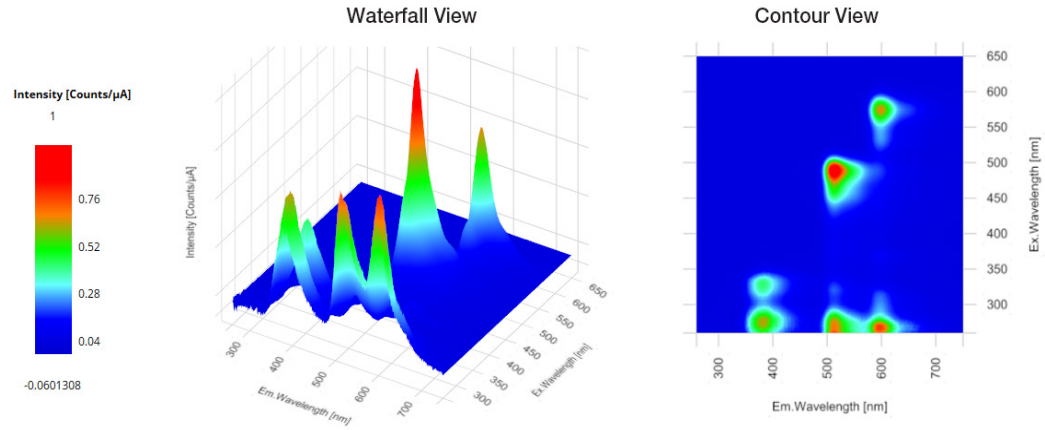


Figure 5: An excitation emission matrix (EEM) of the mixture of quinine sulfate, fluorescein, and rhodamine 101, shown as a waterfall plot (left) and a contour plot (right).

Figure 5 is an EEM acquired and shown as a waterfall plot (left) and contour plot (right). This EEM was acquired from a mixture of three florescent dyes: quinine sulfate, fluorescein, and rhodamine 101. The three dimensional nature of this EEM is provides a wonderful tool for chemometric component analysis. Figure 6 shows EEMs collected for the individual dyes. It is easy to see the fingerprint of each of them contained in the EEM measured for the mixture in Figure 5.

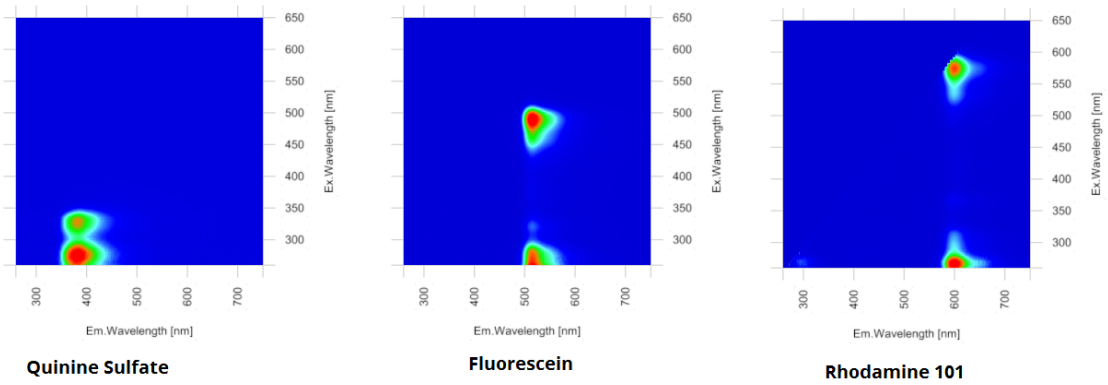


Figure 6: Duetta EEMs for the three individual dyes: quinine sulfate, fluorescein, and rhodamine 101 in water.

In spite of the fact that an EEM provides more information, one can extract a synchronous scan from an EEM collected with Duetta. In fact, Duetta provides much more than a traditional scanning fluorometer. The EzSpec software that powers Duetta has a synchronous scan analysis tool that allows you to extract a synchronous scan from any acquired EEM. Once you have acquired an EEM with Duetta, you can enter wavelength values to simply display a synchronous scan, but a nice benefit is that you can change the synchronous scanning ranges and offsets to quickly extract a different synchronous scan. Another important note is that the Duetta EEM acquisition time is much faster than a traditional scanning fluorometer, so not only is it faster to acquire, but you do not need to run a new acquisition to change the synchronous scan parameters.

Below is the Synchronous Scan extracted from the EEM of the mixture solution with 5 nm excitation step and 40 nm offset between excitation and emission.

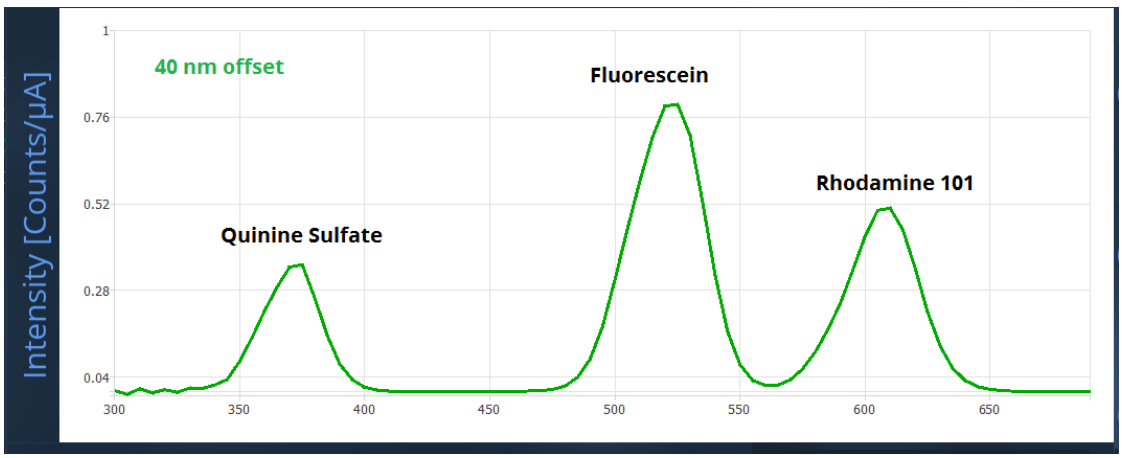


Figure 7: Synchronous scan using a 40-nm offset, extracted from the Duetta EEM in Figure 6 showing distinct peaks for each of the three components.

This trace is effectively the diagonal cross section of the EEM of Figure 5, above, corresponding to a 40 nm offset. The three peaks are easily seen from the extracted synchronous scan and can be used to identify components. However this two-dimensional data set, by definition, has much less information about the sample measurement than the three dimensional EEM.

The synchronous scan peak location and intensity will shift with the excitation/emission offset chosen to measure the synchronous scan. In the image below, the synchronous peaks are changing for each dye when a difference in offset is used. Because the synchronous scan peak intensity for each peak depends on the Stokes Shift for each dye and the relative amount of each dye in the solution, it is somewhat difficult to use synchronous scans for quantitative analysis. As shown in Figure 8 , the quinine sulfate peak increases in intensity when the shift is increased since quinine sulfate has a larger Stokes Shift. The other two dyes have a decreasing synchronous peak as the offset is increased since they have a smaller Stokes Shift.

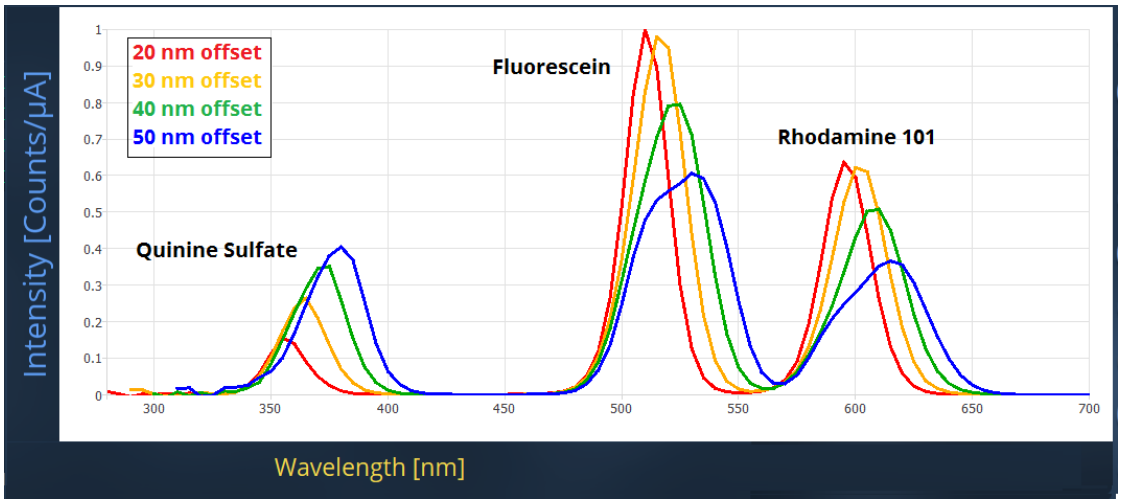


Figure 8: Synchronous scans extracted from the EEM of the mixture using various offsets: 20, 30, 40, and 50-nm showing resulting peak shifts.

While synchronous scans can be, and certainly have been used to measure and analyze mixtures of fluorescing compounds, an EEM gives much more information on the full spectral characteristics of the mixture and lends itself to giving a true molecular fingerprint.

As such, the unique design of Duetta, which quickly and easily measures EEMs and also offers Absorbance-Transmission parameters and correction of the EEM for the inner filter effect (A-TEEM™), is the ideal tool for fluorescence component analysis.