



Determination of Fluorescence Excitation and Emission Peaks for Qdot[®] Nanocrystals Using the Synergy[™] 4 Multi-Mode Microplate Reader

Using Monochromators for Fluorescence

Paul Held Ph.D., Senior Scientist, Applications Dept., BioTek Instruments, Inc.

Qdot[®] Nanocrystals have become an important tool in the biological sciences. The ability to coat the crystals with a polymer enables the attachment of specific biomolecules allowing these crystals to serve as solid surfaces for biological reactions. Fundamentally Qdot Nanocrystals are fluorophores, roughly the size of proteins, whose emission wavelengths vary based on the size of the nanocrystal. Here we describe the use of the Synergy[™] 4 Multi-Mode Microplate Reader to perform fluorescent excitation and emission spectrum analysis on Qdot nanocrystals.

Introduction

Quantum dots (Qdot[®]), also known as nanocrystals, are made up of materials commonly referred to as semiconductors. They are very small (10-20 nm), roughly the size of proteins. Qdot nanocrystals are a specialized form of nanocrystal composed of a core, usually made up of a few thousand atoms of a semiconductor material such as cadmium mixed with selenium (CdSe). A semiconductor shell made of zinc sulfide (ZnS) surrounds the core and serves to both improve the optical and physical characteristics of the nanocrystal. The core and shell are further covered by an amphiphilic polymer, which provides water miscibility, as well as the ability to be conjugated with biomolecules. The amphiphilic coating has been covalently modified with a functionalized polyethylene glycol (PEG) outer coating, which has been shown to reduce nonspecific binding (Figure 1).

The optical properties of nanocrystals such as Qdots are unique. These particles do not fluoresce as a result of the π to π^* electron transitions found with traditional fluorophores. Fluorescence by nanocrystals is the result of the formation of Coulomb correlated electron-hole pairs, often referred to as excitons. While excitons can be thought of as excited state electrons, they have much longer lifetimes. The physical properties of these materials also result in several unique optical characteristics. The emission profiles of the dyes are dependent on the size of the particle. This phenomenon, referred to as tuneability, can be exploited with multicolor assays. The emission wavelengths are generally both narrow and symmetric and because the particles are made from the same material they all have the same excitation wavelength. Unlike traditional fluorescence compounds, nanocrystals achieve fluorescence without a conjugated

double-bond molecular structure, resulting in much better photostability.

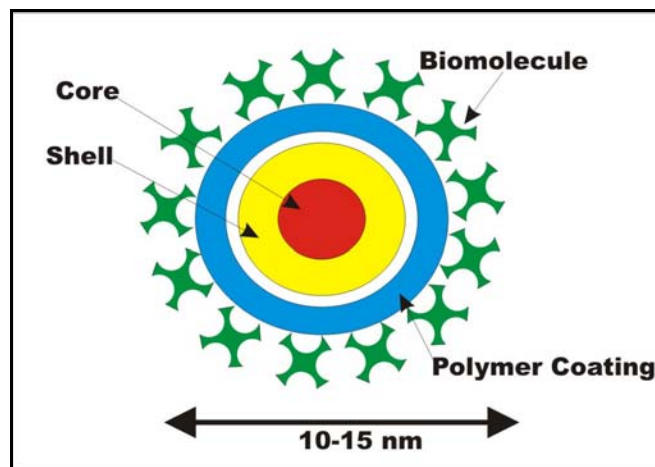


Figure 1. Schematic of the Structure of a Qdot Bioconjugate.

In order for the Qdots to be useful in any sort of biological assay, they require that biologically active molecules, such as proteins, oligonucleotides, and small molecules be attached to the exterior surface of the particles. The large surface area of the nanocrystal provides the ability to conjugate many biomolecules to its surface.

The Synergy[™] 4 is a new type of reader that provides research laboratories with performance and technologies usually found on high-end HTS instrumentation, while at the

same time delivering flexibility and efficient cost-control to screening laboratories.

The Synergy 4 utilizes multiple sets of optics to provide optimal performance regardless of the detection technology. Absorbance measurements use a xenon-flash lamp with a monochromator for wavelength selection, allowing the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm. Fluorescence measurements are made using either a continuous tungsten-halogen lamp or a xenon-flash lamp. Wavelength selection can be made using either monochromators or bandpass filters with or without dichroic mirrors and PMT for detection. Fluorescence polarization is accomplished with the use of polarizing filters in conjunction with label-specific dichroic mirrors for wavelength specificity. For time-resolved fluorescence measurements, the Synergy 4 integrates a high-energy xenon-flash lamp with excitation and emission filters and PMT detector. Luminescence measurements are made using a liquid-filled optical fiber to capture light along with a low noise PMT. The Synergy 4 is capable of reading plate formats up to 1536 wells, robotic compatible, provides temperature control, with shaking as standard features.

Materials and Methods

A Qdot Streptavidin Sampler kit (catalogue # Q10151MP) was purchased from Invitrogen (Carlsbad, CA). Phosphate Buffered Saline (PBS) was reconstituted from tablets (catalogue # P4417) purchased from Sigma-Aldrich (St. Louis, MO). Solid black microplates (catalogue # 3915) were obtained from Corning (Corning, NY).

Fluorescent spectral scans were performed using a Synergy 4 Multi-Mode Microplate Reader from BioTek Instruments (Winooski, VT). For emission scans, the excitation wavelength was set to 330 nm and the emission wavelengths were scanned from 400 nm to 750 nm in 1 nm increments. The xenon flash lamp was used as the light source, with the lamp energy set to high. For each data point 10 measurements were taken with the PMT sensitivity set at 125. The vertical offset was set at 4.00 and the column offset was set at 0.00. In all cases a final concentration of 1 nM of each Qdot examined was used.

For endpoint determinations where filters were used for wavelength specificity, working Qdot-stock solutions were made by diluting the commercially available 1 μ M solutions to 1 nM using PBS as the diluent. Further serial dilutions (1:2) were then made using PBS as the diluent. For each dilution 200 μ l aliquots were pipetted in replicates of 8 into the microplate and the fluorescence determined.

Reading parameters: Initial studies investigated the use of several available excitation filters and emission filter combinations for each of the Qdot nanocrystals contained in the sampler kit. In all cases, a 400 nm cut-off dichroic mirror (catalogue # 7138400) was used in conjunction with appropriate filters. The use of a 340/30 excitation filter was found to provide the best signal-to-noise ratio for subsequent experiments. All measurements were made from the top using the tungsten-halogen light source, with a PMT sensitivity setting of 125.

Results

Using an excitation wavelength of 340 nm, a spectral emission scan of Qdot 655 was performed with the Synergy 4. As demonstrated in Figure 2, the nanocrystals have a peak emission wavelength of 655 nm.

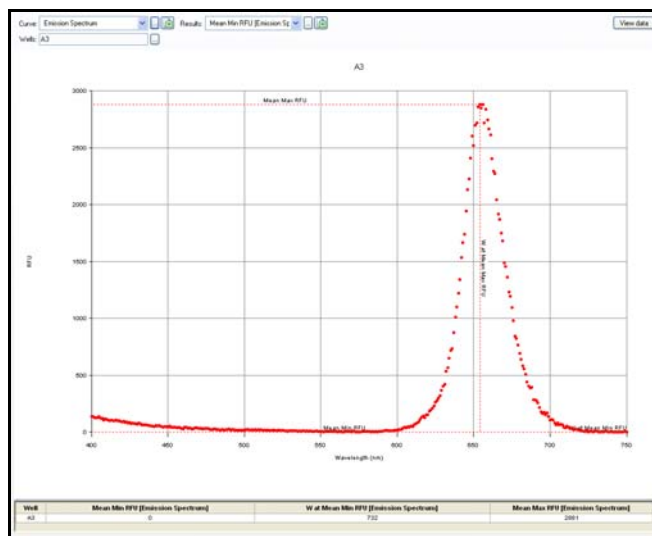


Figure 2. Emission Scan of Qdot 655 nanocrystals. Using an excitation wavelength of 340 nm, the emission from 400 nm to 750 nm spectra in 1 nm increments was obtained using a Synergy 4 Multi-Detection Microplate Reader.

The peak wavelength was then used to monitor fluorescence generated from an excitation wavelength scan. The excitation wavelength was varied from 250 nm to 610 nm in 1 nm increments. As shown in Figure 3, Qdots can be excited by light over a broad range of wavelengths, particularly in the UV range. In fact, emission intensity is directly related to the energy of the excitatory photons.

The peak wavelengths obtained using the Synergy 4 Multi-Mode Microplate Reader compare very well with the emission peaks reported by the manufacturer. As shown in Figure 4, when normalized fluorescence values for Qdot 655 obtained from the Synergy 4 and those from the manufacturer are plotted, the curves superimpose on one another very well. Not only are the determined peak wavelengths the same, but also the shapes of the emission curves are virtually identical. The emission and excitation scans depicted in Figures 2 and 3 respectively were used to determine appropriate filters for end-point fluorescence determinations.

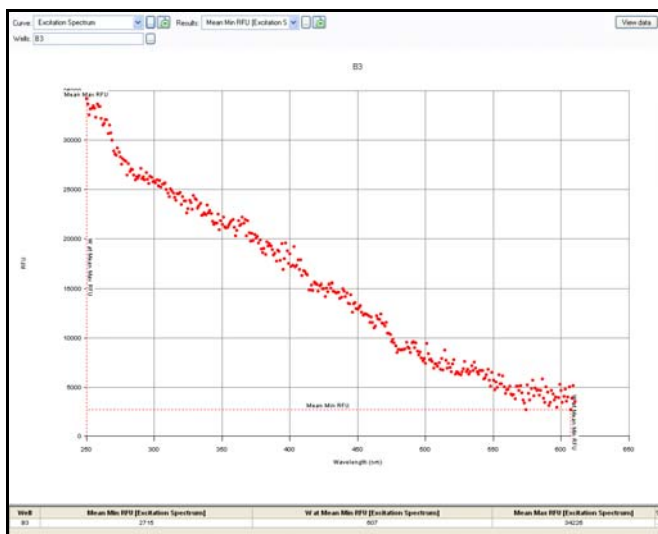


Figure 3. Excitation Scan of Qdot Nanocrystals. The emission at 655 nm was recorded for a spectral scan of excitation wavelengths from 250 nm to 610 nm in 1 nm increments.

Fluorescence spectral scans can also be used to discern different Qdot species from within a mixture. As demonstrated in Figure 5, when the fluorescence of 5 different species of Qdot nanocrystals are scanned several discrete peaks are observed. Interestingly, the fluorescence intensity of different Qdots is markedly dissimilar despite having equimolar concentrations. This difference is due to differences in the extinction coefficients of the nanocrystals, as the response of the photomultiplier tube in the Synergy 4 is constant over these wavelength ranges.

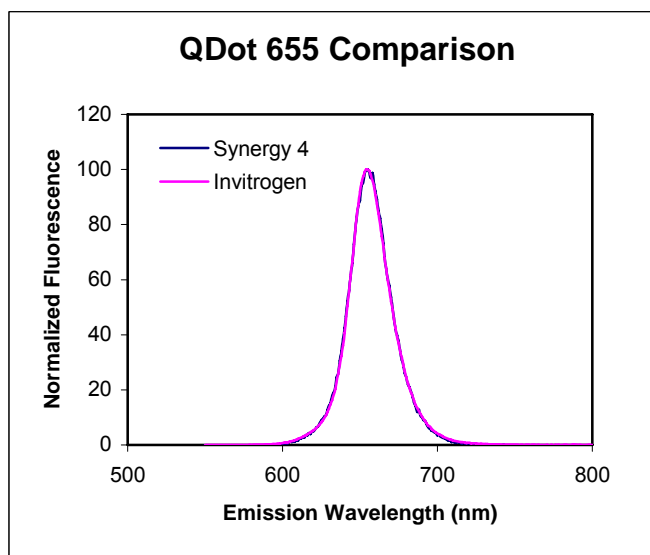


Figure 4. Comparison of the Synergy 4 emission spectrum with reported spectrum. Normalized fluorescence obtained using the Synergy 4 was compared to that provided by Invitrogen.

When the normalized values for both excitation and emission scans are plotted on the same graph, one can very easily superimpose the transmission bandwidth of potential filters on the same graph. As depicted in Figure 6, using a 340/30-excitation filter will utilize a region of the excitation with high efficiency. Likewise, using a 645/40-emission filter will capture a large portion of the emission energy generated by Qdot 655 nanocrystals.

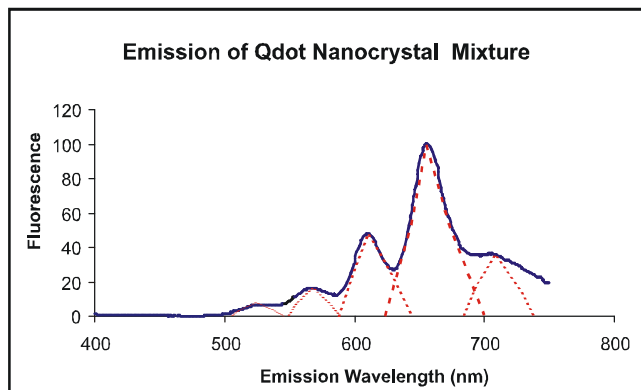


Figure 5. Fluorescence emission scan of a Qdot mixture. Equimolar (1 nM) amounts of Qdot 525, 565, 605, 655, and 705 were excited at 340 nm and the fluorescent emission measured from 400 nm to 750 nm in 1 nm increments. Solid blue line indicates the actual fluorescence, while dashed red lines indicate estimated signal of each Qdot nanocrystal.

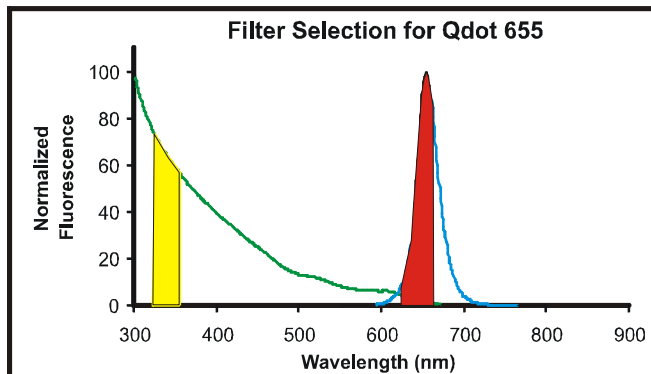


Figure 6. Filter Selection Based on Fluorescent Scans. Fluorescent excitation (—) and emission (—) scans from Qdot 655 were performed using a Synergy 4. The bandpass of the selected excitation (■) and emission (■) filters were then superimposed on the plots.

Using filter-based wavelength selection, the fluorescence of a series of dilutions was measured. As shown in Figure 7 when dilutions of Qdot 655 are in the range of 0 to 1000 pM the signal increases in a hyperbolic nature. Note that the signal, at very low concentrations, is quite linear (Figure 7 inset). Concentrations as low as 4 pM are significantly different than the buffer-only control.

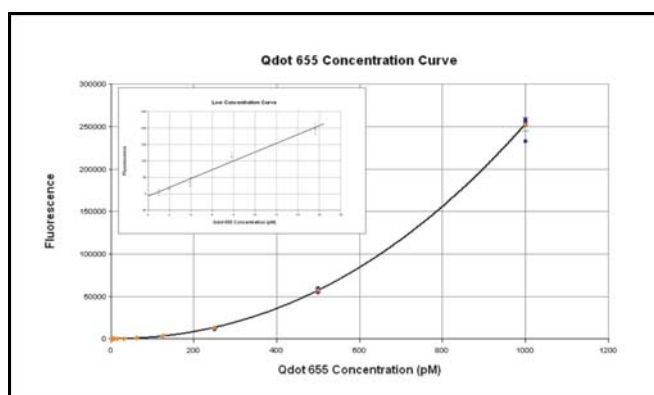


Figure 7. Qdot 655 Concentration Curve. The fluorescence of a series of dilutions ranging from 0 to 1000 pM of Qdot 655 was determined using a Synergy 4 reader with a 340/40 excitation and a 645/40 emission-filter. The reader was controlled and the data collected and plotted using Gen5™ Data Analysis Software (BioTek Instruments).

Discussion

The Synergy™ 4 Multi-Detection Microplate Reader and Qdots have many compatible features. The Synergy 4 uses a very powerful xenon flash lamp that provides a large amount of output in the UV wavelength range. Qdot nanocrystals, as mentioned previously, exhibit a strong absorption profile in this region of the spectrum. Mixtures of Qdots can be discerned using the monochromators to scan the emission of the mixture. As seen in Figure 5, where several Qdot species are present, different peaks can be identified that correspond to various Qdot species. The ability to use monochromators allows the user to select wavelengths that meet their needs in terms of both excitation and emission wavelengths. For example, the measurement of fluorescence in a mixture of Qdot species may require very specific emission wavelengths to minimize the signal overlap between Qdot species. In terms of excitation wavelength, fluorescent scans of a sample and a blank allow the determination of the best compromise between signal and background generation, as measured by a signal-to-noise ratio.

For maximum sensitivity, the Synergy 4 Multi-Detection Microplate Reader also offers filter-directed wavelength selection for fluorescence. The use of filters provides more excitation light, as well as the recovery of greater amount of the fluorescence emission. The use of filters rather than diffraction grating monochromators generally provides greater sensitivity in terms of detection limits of known fluorescent compounds. Deeper blocking at the edges of the bandpass allows for samples with small Stoke's shifts to be measured.

Qdot crystals have several features that make them good candidates for biological assays. Qdots have quantum yields approaching 60% even after attachment to biomolecules, where as many conventional fluorophores may have very low efficiency after conjugation. Because there is no requirement for conjugated double bonds, covalently linking molecules to the nanocrystal does not affect the fluorescence. Qdots have variable emission wavelengths despite having a common excitation profile, which allows the potential use of multiplex reactions.

Qdot® is a registered trademark of Invitrogen.