35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

Unmixing of Spectrally Similar Quantum Dots Using Filter Selection

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***Abstract*— This paper explores the possibilities for quantitative analysis of multiplexed Quantum Dot Immunohistochemical (QDIHC) staining using a 10-slot fluorescence microscope filter wheel. QDs are an ideal fluorophore for staining biomarkers due to their unique properties, including greater photostability and relatively narrower emission bandwidths compared to organic dyes. We imaged a slide containing 5 pure QD spots and 6 QD mixtures with a customized scanning fluorescence microscope. The QD mixtures contained either two or three QDs in equal amounts. Ten filter cubes were used to gather emission signal and then fast non-negative least squares regression (FNNLS) performed the unmixing process by assigning components of the 10-channel raw data to one of the five QDs used. the average error in the unmixing process was measured to be 7.60% when all filters were used and 7.80% when only 6 filters were used.**

I. Introduction

The use of fluorescence imaging to distinguish proteins, organelles, or cells in a sample has grown among researchers due to its high specificity in targeting [1]. Although the use a single fluorophore for targeting has proven to be a reliable method in biological imaging applications, the use of multiple fluorophores would be useful in applications that may benefit from identifying multiple biomarkers. For example, ER and PR, markers for breast cancer can be both be present, one be present, or not present at all. Fluorescence imaging has come to be the preferred method for distinguishing multiple biomarkers due to the flexibility of multi-spectral imaging using different filters of the microscope [2]. Testing for multiple markers at once can increase the chance of detection while decreasing time and cost for testing each marker separately.

Staining for multiple markers does present challenges that otherwise would not be a problem. One major issue is that the emission spectra from the multiple fluorophores are likely to overlap, skewing what biomarkers might be present and in what amount. The fluorophores used in this study are Quantum Dots (QDs), which have greater photostability and large absorption coefficients compared to organic dyes. These particles are covalently linked to corresponding biorecognition molecules such as antibodies for specific targeting [3]. The QDs used in this paper are in similar spectral ranges that have overlapping emission signals, and therefore can be difficult to distinguish for specific targeting applications.

Due to the unique properties of QDs, fluorescent signal may quantified to determine cell and tissue composition. Although QDs are an excellent candidate as a quantitative tool, there are still some issues that must be accounted for when using multiple QDs as a detection tool. One issue is the slight change in QD profile with each sample, which can be affected by the type of tissue, background, and can even vary depending on different batches of the QD being used. Another issue that occurs when using multiple fluorophores is the issue of spectral overlap. Some of the emission signal overlaps between different QDs, and the closer the QDs are in spectra, the greater amount of emission signal that gets overlapped. This is a major problem when determining where the signal belongs.

Crosstalk between bioconjugated QDs when quantifying signal would normally have to be considered, quantified, and reduced in order to produce reliable results [4]. The QDs in this study are not bioconjugated to antibodies for tissue staining in order to eliminate this issue to more accurately evaluate the unmixing process.

This paper evaluates the spectral overlapping using multiple QDs in a given sample using varying amounts of

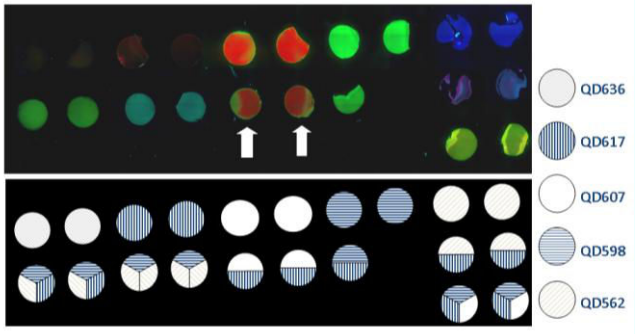

Figure 1. A sample psuedo-colored image of the quantum dot microarray layout. In this example, the red channel is QD607, green is QD598, and blue is QD562. The top row contains "pure" duplicate QDs while the second row contains duplicate mixtures of 2-3 QDs. Some error in the unmixing is detectable by green regions in the QD607 spots labeled with the white arrows, due to variation in emission distribution. We are still investigating the cause of this signal variation. The pure QD636 and QD617 spots should be black, but some error is expected in the very bright edges.

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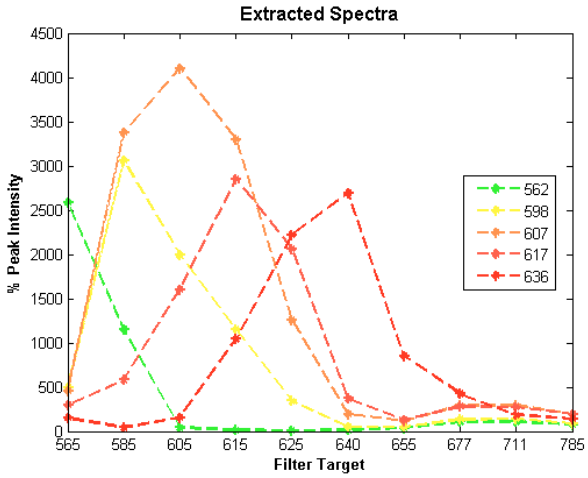

Figure 2. Templates derived from the 5 "pure QD" spots. QD598 (yellow) and QD607 (orange) have very similar emission profiles using this filter set. The 785/62 filter does not provide useful information for unmixing (all signals are near zero). Baseline signal has been subtracted.

microscope filters. A spectral unmixing algorithm that uses imaging data from multiple wavelength channels from a fluorescence microscope has been developed in order to accurately distinguish multiple QD fluorophore emissions as well as autofluorescence in an assay. The successful unmixing of multiple signals is useful in research and clinical settings as this allows for the tagging of multiple biomarkers in the same tissue, which may be necessary for conclusive results.

II. Materials and Methods

A. Slide Setup and Fluorescence Imaging

We evaluate the effects of different filter sets used in fluorescence imaging for unmixing by imaging an entire microscope slide containing 5 emission-overlapping Quantum Dots in various mixtures using our multi-spectral microscope with 10 filters tuned for quantum dot imaging. The slide contains 5 spots in duplicate with pure QD sample and 6 spots with a mixture of 2-3 QDs (Fig. 1). This slide was prepared in the lab of Prof. Shuming Nie by mixing pure quantum dots with a collagen matrix and processing that gel normally as formalin fixed paraffin embedded (FFPE) tissue. We acquire microscope images at 5x magnification covering the entire slide using an automated motorized stage and filter turret provided with our Zeiss AxioImager Z2. Looking ahead to detecting multiplexed QDs for identification of biomarkers in clinical settings, the Zeiss AxioImager Z2 microscope contains standard equipment used for histological evaluation that is available in pathology labs. While other microscopes, such as the Ziess LSM 780, also have the ability to unmix fluorescent dyes and fluorophores, at about 8 times the cost and 12 times longer acquisition rate for a standard slide than the AxioImager Z2, microscopes like the LSM 780 are not likely to be present in pathology labs. We used the following bandpass filters (from Semrock, Inc., Rochester, NY): FF01-565/24-25, FF01-585/29, QD605-A-ZHE-ZERO, FF01-615/20-25, QD625-A-ZHE-ZERO, FF01-640/14-25, QD655-A-ZHE-ZERO, FF01-677/20-25, FF01-711/25-25, and FF01-785/62-25. Catalog number includes center lambda and band width separated by a forward slash ("/"). All filter cubes use the same excitation filter: FF01-435/40-25 and a dichroic mirror to remove excitation from emission before entering the camera.

B. Unmixing Process

The spots containing pure QDs defined templates for our unmixing process (Fig. 2). The templates were acquired by averaging the signal from 1000 randomly selected pixels after trimming the pixel distributions to remove background and noise from dust. We perform FNNLS unmixing as provided by [5] to transform 10 channels of raw data to 6 channels of signal. Algorithms for emission spectra analysis typically constrain the variables to be non-negative and must solve for a model with large amounts of observation inputs, such as the 10 channels of fluorescence emission at each pixel in the slide used in this paper. FNNLS unmixing reduces the computational burden of unmixing when compared to other algorithms [5]. We then register and stitch each 1388x712x6

TABLE I. Unmixing Error of Quantum Dot Mixture Samples

		QD	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5	Mixture 6
	Sample Preparation	562	0.00	0.00	0.50	0.00	0.33	0.00
		598	0.00	0.50	0.00	0.33	0.33	0.33
		607	0.50	0.00	0.00	0.00	0.00	0.33
		617	0.50	0.50	0.50	0.33	0.00	0.33
		636	0.00	0.00	0.00	0.33	0.33	0.00
10 filters	Measured Mixture Ratio	562	0.0050	0.0065	0.3671	0.0159	0.3513	0.0013
		598	0.1549	0.5254	0.2366	0.3389	0.4117	0.4540
		607	0.3201	0.0002	0.0004	0.0055	0.0000	0.1262
		617	0.4863	0.4238	0.3490	0.3265	0.0000	0.3899
		636	0.0336	0.0441	0.0469	0.3133	0.2370	0.0287
	RMSE		0.1074	0.0411	0.1405	0.0124	0.0561	0.1109
6 filters	Measured Mixture Ratio	562	0.0051	0.0065	0.3669	0.0159	0.3513	0.0013
		598	0.1546	0.5253	0.2369	0.3390	0.4116	0.4539
		607	0.3208	0.0002	0.0004	0.0055	0.0001	0.1263
		617	0.4858	0.4238	0.3490	0.3264	0.0000	0.3899
		636	0.0337	0.0441	0.0469	0.3133	0.2370	0.0287
	RMSE		0.1071	0.0411	0.1406	0.0124	0.0561	0.1108

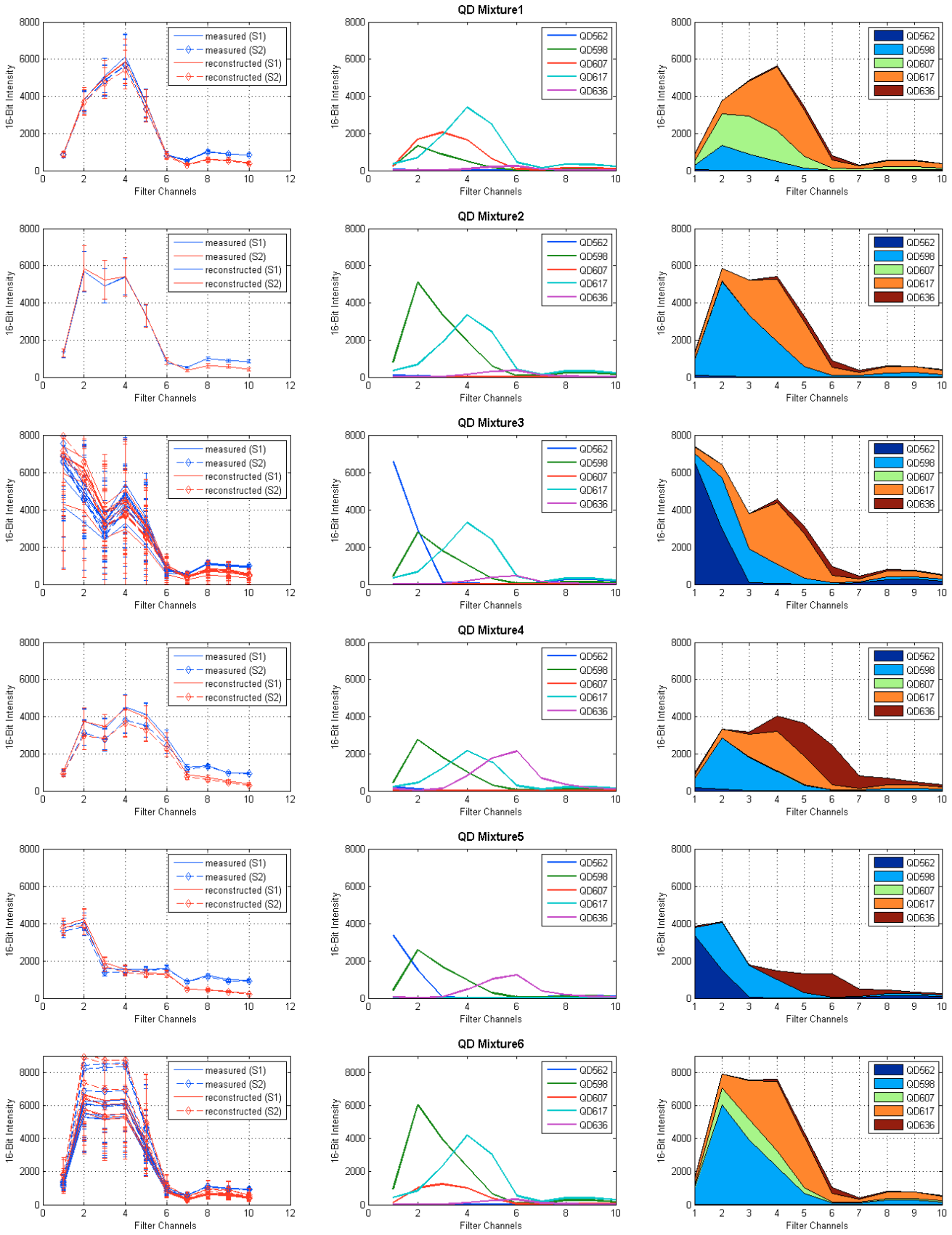

Figure 3. Resulting spectra after running the unmixing process. The first column compares the predicted spectra (reconstructed) based on the extracted spectra to the measured spectra after unmixing. The middle column represents the indiviual spectral components. The last column shows the spectral components that make up the reconstructed spectra.

channels into pseudo-RGB images for visualization [6-7]. Error, calculated by root mean square error, is measured by comparing the predicted fluorescence signal based on the ratio of QDs used in a given spot to the fluorescence signal assigned to each spot through FNNLS unmixing.

III. Results

After determining the extracted spectra from the pure QD spots and then performing the unmixing process by applying an algorithm based on NNLS regression, the fluorescence signal from the mixed QD spots containing 2 to 3 QDs were compared to the predicted fluorescence signal based on the known present QDs. Table 1 contains error of the fluorescence signal from each QD as assigned by the unmixing process. This root mean square error was calculated by using the QD ratio stained in each spot to the QD ratio found through the unmixing process using fluorescence signal. It is worth to note while that while the QDs were stained in each spot in the ratio specified, due to pipetting error this may not be the exact ratio.

In order to view where the unmixing process assigned the fluorescence signal from each spot, components of the reconstructed signal was plotted for each mixture (Fig. 3). Sampling for QD signal from the whole slide image was done individually for each spot as to avoid background. Fifty thousand pixels were randomly selected from a bounded area that only contained the QD signal from each spot. While trying to account for the non-uniformity of some of the spot shape, some mixture spots also appeared to be unevenly stained with QDs. This non-uniformity can be seen especially in Mixture 3 and Mixture 6, which is reflected in the error and the breakdown of spectral components (Fig. 3).

IV. Discussion

Results show an overall success in the unmixing process by identifying the correct QD emissions, with room for improvement. The unmixing process mislabeled some signal as coming from different QDs that were known not to be present in the QD mixture. This mislabeling of signal was only a small percentage of the overall signal, making it fairly obvious that the QD that the fluorescent signal was assigned to was either there in a small amount or not present at all.

Some of the mislabeling of the signal in two of the spots was due to the unintentional staining pattern in the 1st mixture containing QD 607 and QD 617 (Fig 1). One possible reason for the staining seen in the mixed spot could be due to the way the QDs were spotted, allowing the QDs to "float" to the edge of the spot drawn by the surface tension of the droplet. We are still investigating the cause of this staining pattern. Mixtures 3 and 6 also contained irregular staining patterns, introducing greater variation in fluorescence signal when pixel sampling to compare the measured and reconstructed spectra.

We attempted to reduce unmixing error by modifying the trimming parameters we used when defining the templates. Based on our results, we recommend replacing filters that do not measure differentiated signals in QD emissions (e.g. filters centered at 711nm and 785nm) with bandpass filters that detect signal near the borders of confused QD spectra (e.g. 575nm or 595nm).

V. Conclusion

Achieving multiplexed QD stains for three or more biomarkers simultaneously for medical applications will require accurate and reliable filter designs, template calibration, and unmixing procedures. In traditional IHC staining for HER2, the assay must have 5% or less of sample disagree with the overall data [8]. If QD staining can outperform traditional IHC staining for locating biomarkers in tissue, it should provide enhanced diagnostic abilities with potential to detect many biomarkers with less sample. This study shows promise in unmixing fluorescence signal from 2 or 3 QDs for use in multiplexed staining using a commercially-available microscope.

Acknowledgment

The authors would like to thank Aqua Asberry for help in the histology laboratory and Richard Moffitt for help in selection of the microscope filters.

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