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Quality Control of Highly Multiplexed Proteomic Immunostaining with Quantum Dots: Correcting for Crosstalk

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***Abstract*—The process of developing molecular assays for disease diagnosis and prognosis requires cross-disciplinary research which monitors quality and reproducibility at all levels. This paper discusses challenges in the quality control of highly multiplexed Quantum Dot (QD) staining and provides a method for improving accuracy of QD quantification in two phases. Phase one is the estimation of unintended crosstalk between multiplexed QD-antibody reporters, and phase two is digital correction of this crosstalk. Results show that crosstalk varies among tissues and reagents, and in some cases it can be on the same order of magnitude as the original intended signal. In cases where target protein expression is assumed to be independent, crosstalk can be empirically estimated from imaging data and corrected for. This work is expected to improve the overall reproducibility and quantification of multiplexed QD staining.**

I. Introduction

THE promise of personalized medicine and molecular diagnosis is inherently limited by the quality and reproducibility of intermediate technologies which lead from basic discovery to clinical practice. Figure 1 shows an overview of the process of developing a clinical assay, beginning with high-throughput discovery technology and concluding with a panel of biomarkers fit for an assay. This process is called *Translational Bioinformatics* and includes many interfaces between computational technologies and traditional clinical experimentation. The pipeline can be broken into three sections with three corresponding areas of quality research: (1) high-throughput data collection and quality control, (2) biomarker selection and knowledge integration, and (3) clinical testing and quality control.

The pipeline begins with raw input from a high-throughput discovery technology. In this case, gene expression microarrays are shown, but the pipeline can be easily modified to accommodate other similar technologies such as single nucleotide polymorphism chips, gene copy number chips, or next-generation sequencing technologies. Existing work on microarray quality control includes methods for robust probe design and robust gene expression calculation [1-4], as well as the United States Food and Drug Administration (FDA) MicoArray Quality Control (MAQC) project [5].

The FDA has already published results of the phase-I MAQC study which supports the use of gene expression microarrays for biomarker discovery (area one of the pipeline), and recently, the FDA has concluded work on phase II of the MAQC project which investigates the use of microarrays for clinical decision making (area 2 of the pipeline). Most reputable biomarker discovery work now includes some form of targeted validation, either through quantitative Polymerase Chain Reaction (PCR) or independent microarray experimentation. In addition to quality assurance through validation, methods also exist to proactively increase quality by combining heterogeneous datasets or incorporating existing knowledge such as known biomarkers or gene ontology information [6, 7].

Xing et al. have presented a detailed protocol which allows for up to 10-plex staining of tissue samples using Quantum Dots (QDs) and primary antibodies form only two

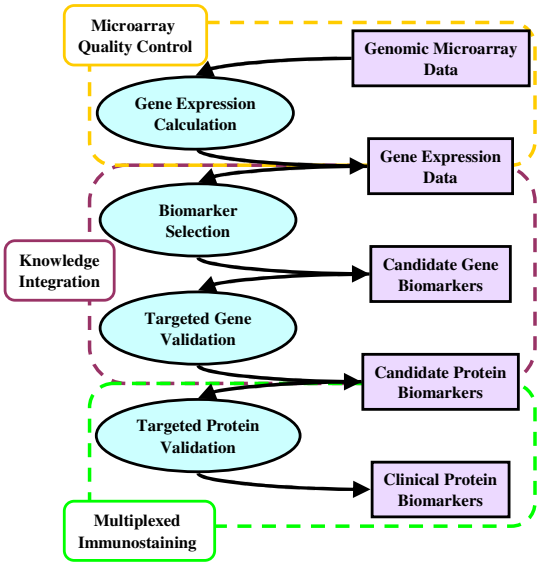

Fig. 1. Translational Bioinformatics Pipeline for Clinical Biomarker Development. Data concepts are shown as rectangular boxes on the right while process concepts are ovals on the left. Three key areas of current research are highlighted with dashed borders.

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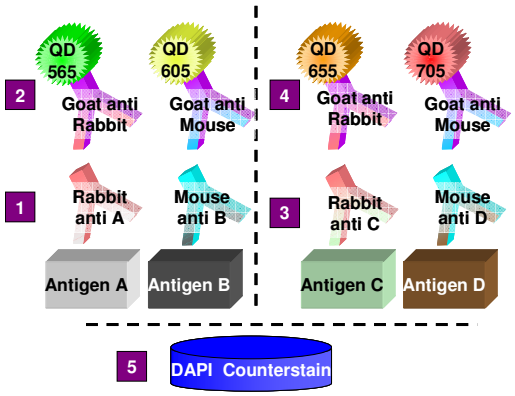

Fig. 2. Multiplexed Immunostaining Workflow. Step 1 and 3 are addition of primary antibodies, Steps 2 and 4 are the addition of QD conjugated secondary antibodies and step 5 is nuclear counterstain with DAPI. A 4-plex procedure is depicted, but Steps 3 and 4 may be repeated with different targets and QDs to increase the multiplicity of staining.

animal sources (Figure 2) [8]. While this protocol is extremely effective in reducing cost and increasing public accessibility of multiplexed staining, it opens up the possibility of unintended yet chemically specific staining of antigen by QD. We refer to this phenomenon as *crosstalk* between QDs. The hypothesized method of QD crosstalk is depicted in figure 3, which shows how QD-secondary antibody conjugates may bind to unintended primary antibody sites, and thus generate QD signal at the site of the incorrect antigen. Identification and amelioration of such crosstalk is the subject of this paper.

II. Methods

A. *Multiplexed Staining Protocol*

Multiplexed QD staining was carried out according to previously published protocols [8]. Briefly, Formalin-fixed paraffin-embedded (FFPE) human specimen tissue sections (5µm thickness) were pre-heated, deparaffinized, hydrated, and rinsed. Antigen retrieval was performed by using a decloaking chamber and standard decloaking buffers and slides were stored in PBS-Tween buffer solution until staining.

Multiplexed QD staining was carried out by the Nemesis 7200 robotic system, (Biocare Medical). Staining was preceded with blocking the slide surfaces by 2% BSA, 5% goat serum, and 1x PBS for 30 minutes at room temperature. The blocked tissue slides were incubated with a mixture of two primary antibodies at room temperature for one hour (step 1 in fig 2). These two polyclonal primary antibodies were from mouse and rabbit respectively, and recognize two different tissue protein markers (Biocare Medical). After washing with 1x PBS-Tween twice, a mixture of two secondary antibody QD conjugates (goat anti-rabbit QD and goat anti-mouse QD, Invitrogen) was applied to the slides and were incubated for 2 hours at room temperature (step 2 in figure 2). After washing with PBS-Tween three times the

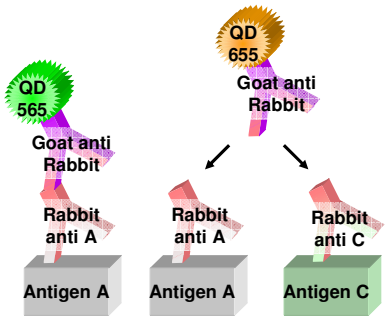

Fig. 3. Undesirable Signal Crosstalk. This cartoon illustrates a scenario where QD655 signal will be present at antigen A as well as antigen C. This occurs if there are unsaturated or unblocked primary anti-A antibody sites left over from the previous round of staining.

same staining protocols were used for more tissue antigens coupled with more QD colors (steps 3 and 4 in figure 2). The overall staining procedure was finished by DAPI counterstaining (step 5 in figure 2), followed by dehydration and mounting on glass slides for fluorescence imaging.

B. *Imaging and Spectral Unmixing*

Wavelength-resolved fluorescence imaging was achieved by using a multispectral imaging system (Nuance, CRI, Woburn, MA) attached to an inverted fluorescence microscope (Olympus 1X71). With near-UV lamp excitation at 350-360nm and a long-pass dielectric filter (cut-on wavelength 500nm), a wavelength-resolved stack of fluorescence images (called an *image cube*) was acquired over the spectral range of 500nm to 800nm at 10nm increments, yielding a total of 31 images. A library of pure QD and tissue autofluorescence was previously acquired from pure antibody-QD solutions spotted on glass slides and from unstained tissue sections, respectively. Library spectral components were then used as a basis for spectral unmixing of each pixel in the image cube using a previously defined positively-constrained least squared error approach [9].

C. *Quantification of crosstalk*

Before quantification, each image cube was segmented to include only regions of interest according to the original intended purpose of the image. For example, the images which were stained for progesterone receptor were segmented to only include glandular regions of breast tissue where the protein is expected. Following this segmentation process, spectral unmixing was performed. This produced one intensity image for each QD and each auto fluorescence component used during unmixing. Each component image was normalized by dividing by the maximum observed intensity in the image followed by multiplication by 255 and rounding to conform to standard 8-bit image formats. 2D histograms were constructed from the paired intensities of corresponding pixels for the first and second QD which share the same secondary antibody target. Crosstalk was not assessed for staining protocols with more than 4QD.

For each histogram column with at least 20 entries, (chosen to be large enough to robustly assess quantiles), the

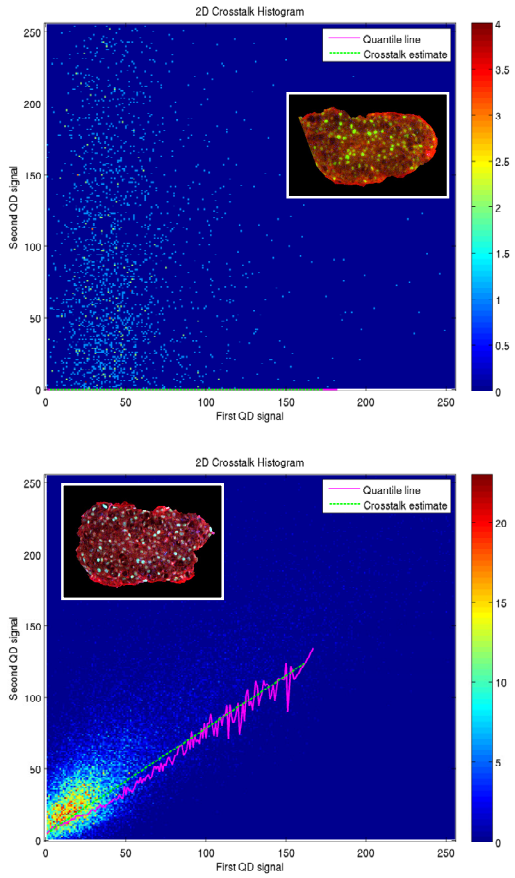

Fig. 4. Observed Signal Crosstalk in Singularly Stained Tissues. Figures show a 2-D histogram of normalized and unmixed QD signals. Lines show the estimated cross-talk among probes used. Inset is a pseudocolored portion of the original normalized (0-255) image used for each histogram. In each inset, the first QD is colored green, the second is blue, and tissue autofluorescence is red. Primary antibody staining is for a single biomarker known to be present exclusively in the nucleus. (top) only the first QD is used, and no crosstalk is detected. Any signal from the second QD is due to noise in the imaging and unmixing processes. (bottom) The first QD was applied before application of the second QD, but with no addition of any additional primary antibody in between. Signal of the second QD is expected to be due entirely to crosstalk.

bottom 20th percentile was estimated. From the paired list (first QD intensity, second QD lower quintile), the centroids of the brightest and darkest three pairs are calculated. The line which connects these two centroids is called the *crosstalk estimate line*, and serves as a linear model of interaction between presence of primary antibody (as assessed by the first QD signal) and improper presence of secondary QD due to crosstalk.

D. Correction for Crosstalk

The slope and intercept of the *crosstalk estimate line* are calculated such that the line may be represented by the following equation:

$$QD_{2\_crosstalk} = b + m*QD_1$$

The observed signal for the second QD is assumed to be a combination of true signal and crosstalk according to the following model:

$$QD_{2\_observed} = QD_{2\_crosstalk} + QD_{2\_true}$$

Thus, a better estimate of the true signal of the second QD can be constructed by subtracting the expected value of the crosstalk-component (calculated with the first QD signal) from the observed second QD signal.

$$QD_{2\_true} = QD_{2\_observed} - (b + m*QD_1)$$

Using this correction equation, the intensity image of the second QD can be modified on a pixel-by-pixel basis using information from the first QD intensity image. The result is an approximation of how the true image should appear without crosstalk. Corrected images may then be quantified using standard procedures.

III. Results

A. Control Experiments

To observe the minimum and maximum possible crosstalk, control slides were stained with only one primary antibody. These slides were stained with the first QD-conjugated secondary antibody and then by either the second QD-conjugated secondary antibody or plain buffer solution. In either case, libraries for both QDs were used during the unmixing process.

Figure 4 (top) shows a representative result of the case where no second QD is added. In this case the regression line was reproducibly and consistently estimated to have slope and intercept of zero indicating no crosstalk.

Figure 4 (bottom) shows a representative result of the case where the second QD is added with no additional primary antibody. In this case, the slope and intercept were reproducibly estimated across different locations on the same slide as well as for serial sections stained simultaneously (10% slide to slide error, data not shown). The magnitude of the estimated slope suggested a nearly one-to one relationship between the first and second QD intensities.

B. Crosstalk in Molecular Profiling Samples

Crosstalk was also observed in actual molecular profiling images. The original intended purpose of these images was for diagnosis and grading of prostate tissue, or assessment of tumor subtype in Renal Cell Carcinoma (RCC). Because of this, these images can be considered a good example of crosstalk which might be observed under normal circumstances

The first staining protocol, which had well-known and biologically independent protein targets, crosstalk was on the order of 10% of the first QD signal. Figure 5 (top) shows a variety of pixels with either no stain or only green stain or only red stain. A transition region between only red or only green is due to the pixels which lie on the border between nucleus and membrane. Still, the cluster of red-only stained cells is clearly greener than the cluster representing un-stained cells, indicating crosstalk.

In the second staining protocol. which had novel and biologically uncharacterized protein targets (Figure 5

bottom), nonspecific crosstalk was also estimated to be on the order of 10% of the first QD signal. This crosstalk was considered biological because of the fact that the only two QDs used were conjugated to different and independent secondary antibodies (only steps 1,2 in Figure 2).

IV. Discussion

Negative controls with only one QD demonstrated good reliability of spectral unmixing results, and rule out unmixing procedure as a source of crosstalk. The possibility that observed crosstalk is due to antibody nonspecificity is ruled out by a similar comparison of any autofluorescence signature to a QD signature. These controls detect little to no crosstalk between autofluorescence and biomarker. Even in this case, however, the estimated crosstalk does not go to zero due to the fact that biomarker only stains tissue, and thus some correlation is expected.

One shortcoming of this methodology is clear in the result derived from uncharacterized biomarkers. When the association of biomarkers is unknown, simple 2-QD assays should be performed first to ensure no biological co-expression.

The magnitude of the effect of crosstalk can vary between tissue types and antibody pairs and, although it can be estimated empirically, it remains unpredictable from a theoretical standpoint. Future study will focus on characterizing the effects that different blocking methods and incubation times have on crosstalk. It has also been hypothesized that free antibody binding sites are generated by a significant amount of disassociation between primary and secondary antibodies which occurs during the multiple washing and incubation steps of a multiplexing protocol. Investigations into this phenomenon are ongoing.

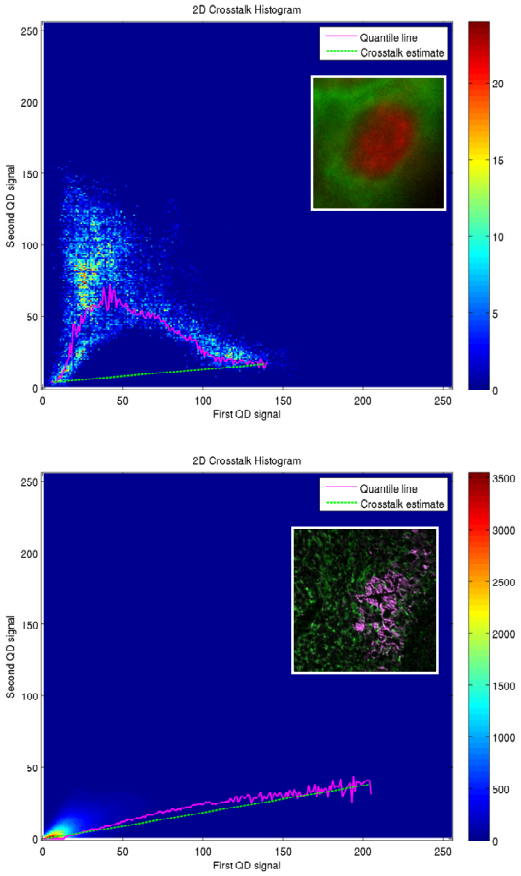

Fig. 5. Observed Signal Crosstalk in Differentially Stained Tissues. Figures show a 2-D histogram of normalized and unmixed QD signals. Lines show the estimated cross-talk among probes used. Inset is a pseudocolored portion of the original normalized (0-255) image used for each histogram. (top) Staining is for two biomarkers known to be present exclusively in the nucleus, which is pseudocolored red, or membrane, which is pseudocolored green. QDs use the same secondary antibody, and the red color was stained first. Any linear relationship of QD signals is expected to be from crosstalk. (bottom) Staining is for two experimental biomarkers with undefined subcellular locations, which are pseudocolored magenta and green. QDs use different secondary antibodies. Both primary antibodies were applied simultaneously. Perceived crosstalk is expected to be due to biological co-occurrence of the biomarkers.

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