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Feasibility of Multiplex Quantum Dot Stain Using Primary Antibodies from Four Distinct Host Animals

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***Abstract*— We discuss the feasibility of multiplex QD stain for four biomarkers and our progress in finding four suitable biomarkers from four different hosts. There is a demand for using more than three fluorescent probes on a single tissue sample for disease detection to offer a more reliable prediction of disease progression. We developed a protocol for targeting four biomarkers using four primary antibodies from four different animal hosts. We performed primary-secondary antibody binding assays on nitrocellulose paper and stained breast cancer microarray slides with known expression of ER, PR, and HER2. We identified the lack of a standard protocol and the limited supply of primary antibodies from hosts other than rabbit and mouse in the market as key challenges. The results show variable success in both assays, but indicate future potential for this protocol with more development.**

I. Introduction

Breast cancer accounts for 1 in 3 cancers diagnosed in women. In 2011, the American Cancer Society expected approximately 230,480 new cases of invasive breast cancer and 39,520 deaths are expected among US women.[1] A common method for verifying the success of a herceptin treatment for breast cancer is by staining for the HER2 protein through IHC stains [2]. While HER2 is not the only protein associated with breast cancer, 15% to 25% of breast cancer cases involve elevated levels of HER2 [3]. Each patient has a unique biomarker profile and biochemical response to chemotherapy, so knowing the expression level of HER2 is not enough to indicate the best treatment for all patients. It is important to stain for multiple proteins that are linked to breast cancer in order to properly diagnose a patient. In addition, many tumors exhibit heterogeneous protein expression in different regions, so a multiplexed staining strategy is useful to avoid the need to run many IHC protocols on large amounts of tissue sample.

Clinical IHC protocols rely mostly on traditional organic dyes. Staining for multiple biomarkers is possible, but differentiating each biomarker is difficult due to overlaps in stain colors. Quantum dots (QD) are tiny light-emitting particle semiconductors and have a broad absorption spectrum and a narrow excitation bandwidth. The ability for QDs to multiplex makes them favorable to use when studying tumor heterogeneity and mechanisms of cancer development [4-7]. Unlike other fluorescent molecules, QDs are resistant to photobleaching, which make them useful for diagnostic particles because slides can be reviewed many times over many months without significant signal reduction.

QDs are conjugated to secondary antibodies extracted from goats and generally targeting the common binding site of another animal's antibodies. QD conjugates properly bind to their respective primary antibodies as long as species cross-reactivity is minimized. Several protocols suggested using only two different host animals and two separate primary and secondary antibody incubation sets which will only require four antibodies made from two different animals [4, 5]. However, there is concern about nonspecific binding and QD cross talk with this method [8].

We investigate the feasibility of identifying four primary antibodies from different host animals. However, the availability of antibodies derived from different hosts is very limited. We tested 12 antibodies from a range of companies (U.S. Biological, Abcam, DAKO, Abgent) for species cross-reactivity using a standard assay, and evaluated QD multiplexing on paraffin embedded human cancer tissue microarrays.

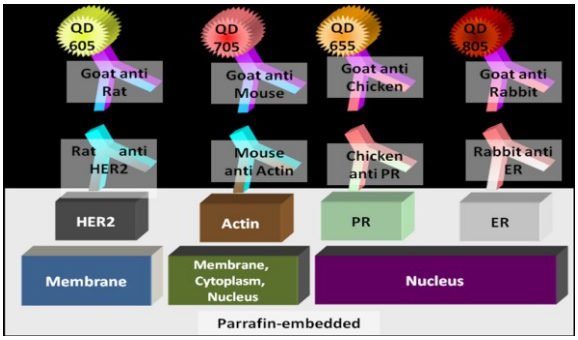

Figure 1: Diagram of multiplex stain experimental concept. Each primary antibody will come from a different host and each will target a different protein which in turn will be marked with a secondary conjugate QD (primary and secondary will not necessarily be pair in the same order as the diagram).

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Code	Company	Catalog #	Antibody	Host	Clonality	Grade	Secondary Binding	Breast TMA
RtH2-1	US Biological	E3451-25N	Anti -ErbB2 (c-erbB2, HER2, neu Oncoprotein)	Rat	Mono	Purified	Pass (mouse reactivity)	Fail**
RtH2-2		C0026-15E	Anti -c-erbB2 (HER-2, neu Receptor, CD340)	Rat	Mono	Highly Purified	Pass	Pass
RtER-1		E3564-89	Anti -Estrogen Receptor (ER)	Rat	Mono	Affinity Purified	Fail	Fail
CkAct-1		A0760-43	Anti -Actin, beta	Chicken	Poly	Affinity Purified	Pass	Pass
CkBRCA-1		B2709-01J	Anti -BRCA1	Chicken	Poly	Affinity Purified	Pass*	Not Tested+
CkPR-1		P9007-01B	Anti -Progesterone Receptor (PgR)	Chicken	Poly	Affinity Purified	Pass	Fail**
MsFox-1		F9045-02A	Anti -FOXA1 (Forkhead Box A1, Hepatocyte Nuclear Factor 3 alpha, HNF3A, MGC33105, TCF3A)	Mouse	Mono	Affinity Purified	Pass (chicken reactivity)	Not Tested+
RbER-1		E3565-15D	Anti -ESR2, ID (Estrogen Receptor beta, ER-beta, Nuclear Receptor Subfamily 3 Group A Member 2)	Rabbit	Poly	Purified	Pass	Fail**
MsPr-1	Abcam	ab2764	Anti-Progesterone Receptor antibody [PR-AT 4.14]	Mouse	Mono	unknown	Fail	Not Tested
RbPr-1		ab16661	Anti-Progesterone Receptor antibody [SP2]	Rabbit	Mono	unknown	Fail	Not Tested
RbH2-1	DAKO	A0485	c-erbB-2 Oncoprotein	Rabbit	Poly	unknown	Pass	Pass

Table 1: Table of all antibodies tested. Two antibodies were chosen from each host animal and tested in order to find a set of antibodies what will give optimal signal for multiplex stains. The goal is find four primary antibodies that will target four different proteins (3 of which will be ER, PR, and HER2) and will each come from a different host. * CkBRCA-1 only tested with rat and chicken secondaries (not with mouse or rabbit). ** These Breast TMA stains were performed without blocking centrifuge tubes during dilution, so the results are indeterminate. + Control tissue microarrays could not be found for FOXA1 and BRCA1 proteins.

II. Methods

A. Antibody Selection

Our multiplex protocol uses four secondary antibody QD conjugates (goat anti-rat QD605, goat anti-chicken QD655, goat anti-mouse QD705, goat anti-rabbit QD805, Invitrogen). Primary antibody selection was made based on whether they could target the human ER, PR, and HER2 proteins and if they could bind to the secondary antibody QD conjugates. Ideally, there will be four primary antibodies that will target four different proteins (3 of which will be ER, PR, and HER2) and will each come from a different host so that the secondary antibody can mark each primary antibody specifically. Table 1 shows the primary antibodies we tested.

B. Staining Protocol

We first test if the primary antibodies are compatible with their secondary antibody QD conjugates using a binding assay on nitrocellulose paper. First, the primary antibody being tested is spotted onto the nitrocellulose paper and allowed to air dry for 30 min. Next, the paper is placed in a blocking solution of 10% milk in an already blocked plastic chamber for 30 min. All four secondary antibody QD conjugates are diluted to their desired concentrations by adding them to the blocking solution used to block the nitrocellulose paper. They are incubated for one hour. After the secondary antibody incubation, the nitrocellulose paper is washed in TBST buffer and mounted without coverslip. Images were acquired with the Zeiss imaging system at 9 wavelengths between 565 and 785nm and a clinical DAPI filter.

Antibodies were tested on human tissue with known levels of protein based on traditional IHC. Formalin-fixed paraffin-embedded trial slides were obtained from US Biomax, Rockville, Maryland. We preheat the slides at 60^{o}C for 15 min before a xylene substitute treatment and hydration in an autostainer. The autostainer uses a series of alcohol solutions at 100%, 95%, 80%, 70%, and finally a pure H2O wash. We perform antigen retrieval by heating the slides in a commercial pressure cooker with Tris-EDTA-Tween20 buffer up to 15 psi. The slides are then allowed to cool and washed with TBST. We draw a wax boundary around the

tissue sample with an ImmEdge pen to keep the incubation solutions in a droplet over the tissue. The blocking step is then performed in 5% goat serum and 2% bovine serum albumin for 30 min. Each washing step uses fresh TBST three times for three minutes each (see Figure 2). The solution containing all four primary antibodies is pipetted within the wax boundary and incubated for one hour. The solution containing all four secondary antibody-conjugated QDs is pipetted onto the slide and incubated overnight in a commercial refrigerator. Some slides were counterstained with 1:4000 DAPI for 5-10 minutes for nuclei detection. Images were acquired with the Zeiss imaging system at 9 wavelengths between 565 and 785nm and a clinical DAPI filter.

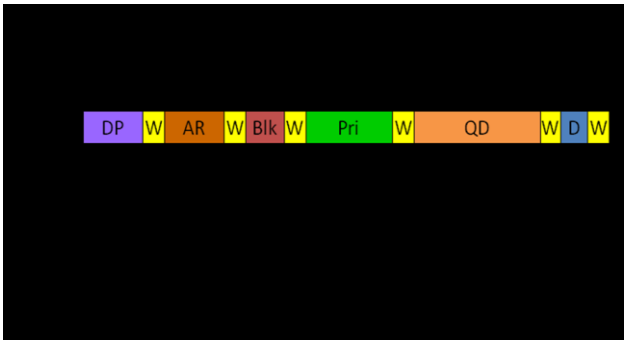

Figure 2: Diagram of multiplex staining protocol on paraffin embedded tissue slides. Primary antibody incubation was done at 4°C for 1 hour. Secondary antibody incubation was done at 4°C overnight.

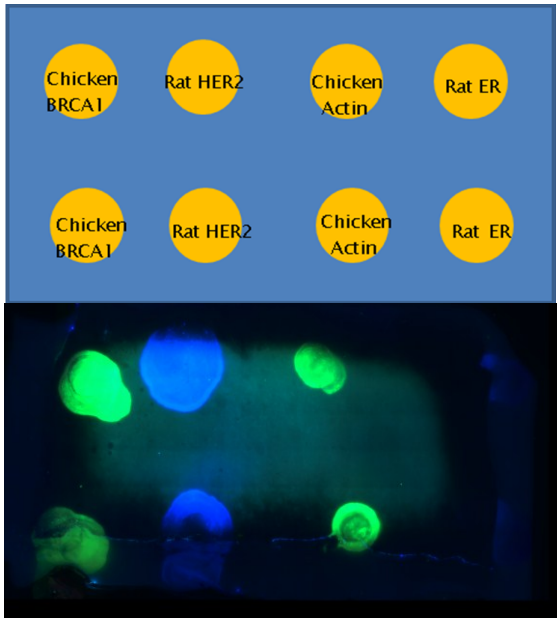

Figure 3: The antibody binding assay was done on nitrocellulose. In the first row from left to right, the antibodies CkBRCA-1, RtH2-2, CkAct-1, and RtER-1. In the second row are the same set of antibodies in the same order. The assay shows strong signal for the CkBRCA-1, RtH2-2 and CkAct-1 spot which means that these antibodies were able to bind to their secondary antibody RtER-1 did not show any signal and does not seem to be compatible with the secondary antibody

III. Results

The antibody binding assay was done first to check if the primary antibodies were able to bind to their secondary antibody. The first assay tested CkBRCA-1, RtH2-2, CkAct-1, and RtER-1 (figure 3). The assay shows strong signal for the CkBRCA-1, RtH2-2 and , CkAct-1 spot which means that these antibodies were able to bind to their secondary antibody. RtER-1 did not show any signal and does not seem to be compatible with the secondary antibody.

A second assay was done to test RbPr-1, MsPr-1, and RbH2-1. In addition, RtH2-2, CkAct-1, and RtER-1 were also tested again to confirm the results from the first assay. A strong signal was observed for RtH2-2 and CkAct-1and moderate signals from RbH2-1. Very little or no signal was observed for RbPr-1, MsPr-1, and RtER-1. From these two assays, CkBRCA-1, RtH2-2, CkAct-1, and RbH2-1 show potential for being suitable antibodies for our multiplex protocol.

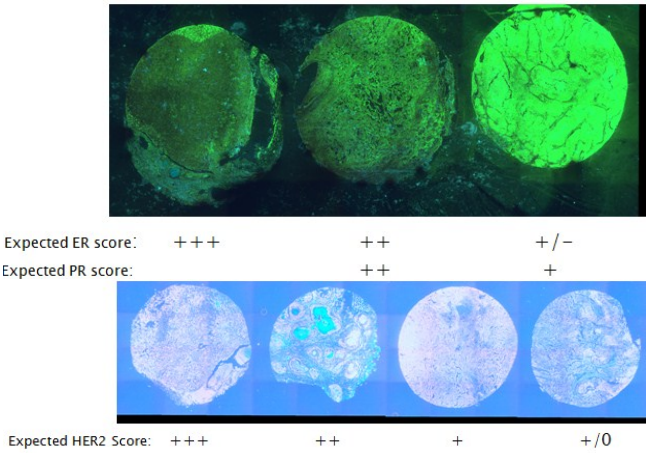

Figure 4: (top) ERC031 slides have three tissue spots with different ER and PR expression scores. This example slide was stained using only CkAct-1 (green) and RtER-1 (blue). (bottom) HRC041 slides have four tissue spots with different scores for HER2. This example slide was stained with CkAct-1 (green), RtH2-2 (blue), and RbH2-1 (red).

Two types of microarray slides were used to test the primary antibodies, ERC031 and HRC041 (US Biomax). ERC031 slides have three tissue spots from different cores with different levels of ER and PR; the first spot is +++ for ER and unknown for PR, the second spot is ++ for ER and PR, and the third spot is +/- for ER and + for PR. HRC041 slides have four tissue spots from different cores; the first spot is +++ for HER2, the second spot is ++ HER2, one that is single positive HER2 and one that was +/0 for HER2.

The primary antibodies tested on the ERC031 are CkAct-1, RbPr-1, and RtER-1. CkAct-1 showed strong signal the third tissue spots and moderate amounts in the first and second. Both RtER-1 and RbPr-1 showed the same amounts of signal in all three spots but the overall signal was barely above background. In addition, RtER-1 and RbPr-1 do not seem to stain cell nuclei as expected.

For the HRC041 slide, CkAct-1, RtH2-2, and RbH2-1 were tested. We test two antibodies that both target HER2 to

compare which binds more competitively. The more competitive antibody will have more antibody bound to the tissue and will produce a stronger signal. The overall signal of all the primary antibodies was very weak. All four spots produced about the same amount of signal despite having different levels of HER2. The RbH2-1 had a better signal compared to the RtH2-2 which may indicate that the RbH2-1 is more competitive; however, neither HER2 primary stained cell membranes as expected. CkAct-1 was seen in all four tissue spots as well but also had a very low signal.

IV. Discussion

Two antibodies of the 12 we tested (MsPr-1 and RbPr-1) were ruled out because of lack binding with the secondary antibody QD conjugates and very low signal when used to test on trial slides. All other primary antibodies show potential for multiplexing since they have been successful in binding to their secondary antibodies. When applied to staining the tissue microarray slides, not many of the antibodies showed strong signal. This may be due to the multiplexed staining protocol. We found several errors with the protocol while staining the tissue microarrays. One of the biggest mistakes was allowing the secondary antibodies to bind to unblocked plastic centrifuge tubes, causing the final concentration applied to the tissue to be very low. We blocked both the pipette tips and the centrifuge tubes before they come into contact with the antibodies for four of the seven stains.

The lack of variety in host animals for the primary antibodies has also been a major obstacle in our antibody selection. The majority of antibody companies only supply antibodies from rabbit and mouse; however for our multiplex protocol to work, it is essential that there are four antibodies each coming from a different host. Antibodies not from these two host are available but in only small varieties. For multiplexing, it will more helpful if antibody suppliers can provide more antibodies made from more animals.

V. Conclusion

Much proteomics and pathology research concentrates on multiplex staining to better understand cancer biology and develop more accurate diagnostics for personalized medicine. Finding reliable targeting strategies (e.g. primary antibodies) is a critical step in this research. There are a few new promising alternatives to antibodies derived from animal immune systems. One of these is synthetically-produced RNA aptamers for targeting breast cancer cells [8]. Synthetically-produced (i.e. "engineered") targeting molecules should be more reliable than host-derived primary antibodies because the production process does not involve the highly complex and incompletely understood immune systems of host animals.

The primary antibodies we identified for multiplex staining had a very low success rate. Critics of this research may point to a complex and variable staining protocol for paraffin-embedded tissue slides as a reason. The simple nitrocellulose binding assay still had a success rate of only 73% with some cross-species reactivity. Several improvements in the protocol have already been made, such as blocking pipette tips and dilution tubes before use. We also plan to retest the primary antibodies on fixed cell slides with known ER and HER2 expression levels. The stains on fixed cell slides will produce a clearer signal which will allow us to make conclusive decisions on which primary antibodies are feasible to use for staining.

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