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Quantum Dots-Based Multiplexed Staining of FFPE Tissues

Introduction

We developed two ways of staining tissue preparations (that can also be applied to cell line preparations) using quantum dots (QDs): 1) non specific staining using quantum dots (QDs) with carboxyl group on the surface (Catalog # QSH) or 2) specific staining using QDs that are conjugated with specific targeting molecules. In non specific staining, the cells are exposed to QDs with carboxyl groups on the surface without specific targeting moiety. The cells are colored as a result of the non-specific binding of the QDs to the cell surface. On the other hand, specific staining involves attachment of specific targeting molecules to the QD surface to allow attachment to biomarkers found on the cell membrane or inside the cell. One example of specific staining with QDs is the use of specific antibody (Ab) conjugated QDs, QD~Ab, for single stain. This can also be extended into the use of different specific antibodies on different QDs emitting different colors for multiplexed staining. In multiplexed QD-Ab staining, different primary Abs that are specific to different biomarkers on cells are conjugated to different QDs emitting at various wavelengths. Cells in tissues expressing these biomarkers are stained with a cock tail of the QD-Abs. The various colors of the QDs are easily observed under fluorescent microscope with a single UV light source. In combination with a multispectral CRI camera, quantification of each biomarker is possible.

Materials:

Formalin fixed paraffin embedded (FFPE) tissue slide; xylene, 100% EtOH, 90% EtOH,70% EtOH, 30% EtOH, sodium citrate buffer 10 mM, pH 6.0, 10 mM PBS buffer pH 7.2; PBS-0.05% TritonX100.

Use Ocean's QDs such as those with catalog numbers QSH, QMG, QFA (QD with folic acid coating) or QD-Ab conjugates (may be prepared in your own lab using our conjugation kits (Catalog # QCK, or through our customer conjugation service). Our blocking buffer that has been optimized with our nanoparticles, (Catalog # BBB) is used to minimize non specific binding of the QDs.

Procedure:

1. **Dewax and rehydrate the tissue:** Bake the slide at 60 °C for 30 min to melt the wax. Place the slide in xylene for 10 min and repeat 4 times, followed by 100% EtOH for 10 min, 90% EtOH for 5 min, 70% EtOH for 5 min, and 30% EtOH for 5 min. At the end of this process, rinse with ddH_2O for 2 min.
2. **Heat-induced epitope retrieval with microwave:** Immerse the slide in boiling citrate buffer (10 mM, pH 6.0) and keep boiling for 12 min. Cool down the slide at RT for 20-25 min.
3. **Permeabilization:** Immerse the slide in ddH_2O for 2 min followed by PBS 5 min, PBS-0.05% TritonX100 5 min, and finally in PBS 5 min
4. **Serum block:** Block slide by placing 1 ml of 10% bovine calf serum (in PBS) at RT for 10 min.
5. **Ab incubation:** Place 75 uL of QD-Ab cocktail (for multiplexed staining) that has been diluted to the desired concentrations or ratios with 6% BSA/PBS solution on the slide with tissue. Incubate at RT for 1 h.
6. **Washing:** Wash the slide with BBB twice and PBS once for 5 min each time.
7. **DAPI staining:** Place one drop of 10 ug/mL DAPI solution on the slide and incubate for 5 min. Wash off the DAPI solution with DI water.
8. **Mounting:** Place a new cover slip with 90% glycerol on the section of the slide containing the cells/tissue. Seal the cover slip with nail polish and allow to dry at RT.
9. **Observation:** Mount the slide on a microscope and observe for fluorescence under a UV light source.

Results and Discussion

We used head & neck cancer tissues as the model tissue to demonstrate multiplexed staining using QD-Abs. As shown in **Figure 1**, FFPE tissue was stained with QD520-anti-EF1α (green), QD620-anti-survivin (red) and nuclear specific dye DAPI (blue). Images were captured using multispectral imaging system (CRI) to generate intensity dependent images from each of the three color channels that were identified through the program. The autofluorescence from FFPE tissue was removed by assigning autofluorescence channel as black. The house keeping protein EF1α (elongation factor 1 α) was present universally throughout the slide while the cancer tissue specific biomarker survivin was concentrated in the cancer tissue (indicated by the arrows). As shown through DAPI staining, the cancer cells had larger nucleus and less cytoplasm due to frequent cell division.

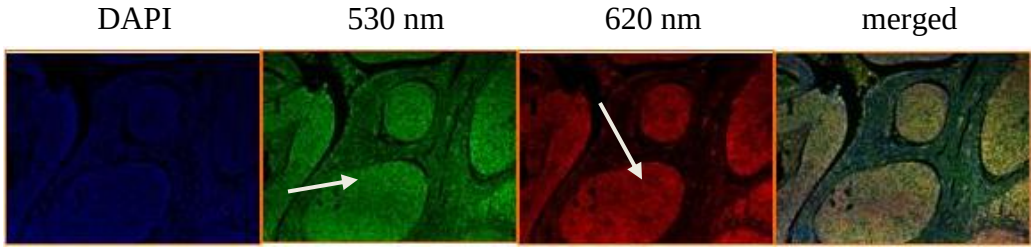

Figure 1. Intensity-dependent images from different channels of human head & neck cancer tissue slice stained with QD-Ab cocktail. FFPE tissue was multiplexed stained with QD520-anti-EF1α, QD620-anti-survivin and nuclear specific dye DAPI. Images were captured using multispectral imaging system (CRI) to generate intensity dependent images from each of the three color channels. The autofluorescence from FFPE tissue was removed. The house keeping protein EF1α (elongation factor 1 α) was present universally in all cells throughout the slide while the cancer biomarker survivin was concentrated in the cancer tissue (indicated by the arrows). Cancer cells tend to have larger nucleus and less cytoplasm due to frequent cell division. The merged images were composite images of each color. The pseudo color assignments for merged images are as follows: DAPI, blue; QD530, green; QD620, magenta.

We quantified the signals from the different channels and different tissue types on the same slide. The software that was integrated with the camera took the exposure time into account and gave the readings as counts/s. The survivin expression level was much higher than that of EF1 α in the cancer tissue while the expression levels for these two proteins were not much different in the cancer surrounding tissue (Table 1).

Table 1. Quantification of different channels of human head & neck cancer tissue slice stained with QD-Ab cocktail. FFPE tissue was multiplexed stained with QD520-anti-EF1α, QD620-anti-survivin and nuclear specific dye DAPI. Images were captured using multispectral imaging system (CRI) to generate intensity dependent images from each of the three color channels and autofluorescence channel. Signals from QD emitting at 620nm, 530 nm, and autofluorescence were quantified with the software integrated with CRI camera.

Tissue types	Detection channels	Total signal (counts/s)
Cancer tissue	620 nm	3163.08
	530 nm	1153.56
	Autofluorescence	381.87
Normal tissue	620 nm	43.67
	530 nm	34.91
	Autofluorescence	20.74

Figure 2 demonstrated the resolution of this QD-based multiplexed staining system. The same multiplexed stained tissue slide was reanalyzed with QD620 and QD635 as fluorescent stains. After the image was deconvoluted and the signals were quantified, the signal from QDs emitting at 620 nm was more than 1000 times stronger than those from QD emitting at 635 nm. This indicated that the QDs had very narrow emission bandwidth and the imaging system was able to identify the difference from the two QDs whose emission maximum wavelengths were only 15 nm apart. This novel method provides a number of choices for the emission color of the QDs that can be used for multiplex staining in the visible light range because QDs are size tunable to emit different colors.

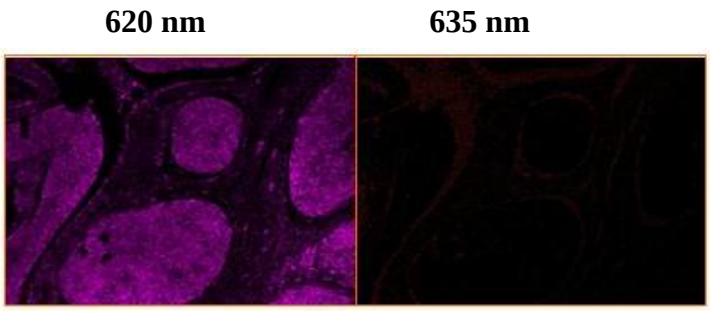

Figure 2. Intensity-dependent images from different human head & neck cancer tissue slice stained with QD-Ab. FFPE tissue was stained with QD 620-anti-survivin. Images were captured using multispectral imaging system (CRI) to generate intensity dependent images from QD emitting at 635nm and 620 nm for comparison. The signal from QD emitting at 620 nm was more than 1000 times stronger than that from the QD emitting at 635 nm, indicating the narrow bandwidth of QDs.

Unlike the current popular method of conjugating the QDs to secondary antibodies that leads to non-specific staining, we conjugated the primary Ab directly to the QDs. This was very risky because the primary Abs was very expensive but we have developed a conjugation kit for preparing QD-Abs that works very well. **Figure 3** shows the comparison between tissue samples stained with our method (right panels) and the currently popular method (left panels). One tissue slide was stained using QD620 (Ocean's)-anti survivin and the other slide was stained first with anti-survivin and then QD655-secondary Ab (Invitrogen), which took much longer time because of one extra antibody incubation step. In addition to shorter experimental time, tissue stained with our method also showed brighter color in cancer cells with less background color in the surrounding cells that are not expected to express large amount of cancer cell specific biomarker, in this case, survivin. The optical stability of Ocean's QDs was much higher than the QDs from the other company. Two weeks after staining, Ocean's QDs still showed bright signals on the tissue stained with QD-Ab but the signal from the other commercial source of QD almost faded away completely (lower panels). Our method offers the advantage of direct staining of the analyte with the QD~Ab. It also avoids the inconvenience of matching the primary Ab with secondary Ab-QD which becomes cumbersome for rare antibodies that are difficult to match with commercially available

secondary Ab-QD for multiplexed staining. This protocol also shortens the time frame of the entire staining process.

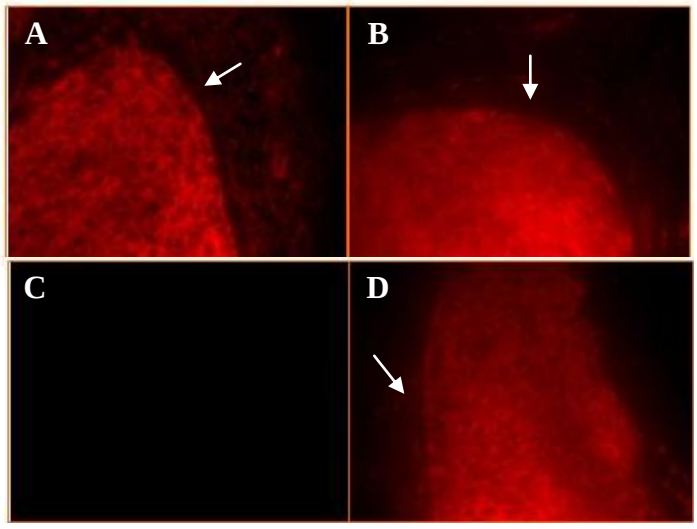

Figure 3. Intensity-dependent images from human head & neck cancer tissue slice stained with QD-Ab from different sources. FFPE tissues were stained with anti-survivin/QD655-secondary Ab from another QD supplier company (A, C) or QD620-anti-survivin from Ocean Nanotech (B, D). Images were captured using multispectral imaging system (CRI) to generate intensity dependent images from 655nm (A, C) or 620 nm (B, D) immediately (A, B) or two weeks (C, D) after staining for comparison. Survivin was specifically over expressed in cancer tissue, indicated by the arrows. Brighter and longer lasting signal and less non specific binding in the surrounding tissue were observed in B and D when compared to A and C.