A New Approach For Co-localization Of Proteins Using HRP And AP Enzyme Detection In Paraffin Embedded Tissues

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Abstract Co-localization of two proteins is a powerful tool in *immunofluorescence*, however the stability of the fluorescence limits the ability to screen large sample sets. Double and triple staining with the ability to visualize co-localization of proteins using HRP and AP enzymes in paraffin embedded tissue may prove to be a useful tool for discovery and functional analysis of gene and protein expression. Here we present a novel chromogen mix by GBI Labs Inc. of Emerald and Red that yields a third color (Dark Blue/Purple) when proteins are co-localized using HRP and AP enzymes for detection. Initial tests evaluated combinations of ER, Her2, CEA, Keratin, PR, p53, PCNA, and Ki67 in 10 breast and 10 colon cancer cases to screen the percentage of co-localized proteins in these cases. In addition, a 70 case breast cancer tissue array containing duplicate samples and known diagnosis of ER, PR, and Her2 for each case were screened with ER, PR, Her2, p53, PCNA, or Ki67 and keratin antibodies. The core tumor sections showed co-localization of PCNA, Ki67 and p53 proteins. These new double and triple staining protocols in combination with tissue array, will allow researchers to rapidly determine the association between expression of two or more proteins in the tissue and if proteins co-localize within the cell.

Introduction Accurate determination of multiple proteins expressed in cancer significantly contributed to the understanding of disease progression and its clinical implications for patient care. To perform these studies, serial tissue sections are often stained for proteins expressed in a disease subtype in research or clinical setting i.e., ER, PR, and Her2 status in breast cancer. However score interpretation of these screens is semi-quantitative and can be influenced by interpreters bias when assessing multiple proteins where less than 40% of the cell population expresses any or all proteins assessed. Currently, permanent double and triple staining are effective tools if the proteins exist in different cells or cellular locations. However, effective screening of proteins co-localized in the same cellular location can only be accurately assessed by a multispectral imaging system, which can be expensive for small studies or *immuno fluorescence* which can limit the size of small studies further.

Immunohistochemistry using horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzyme antibody conjugates are globally accepted for screening protein expression in tissues. Traditional single stained protocols of these enzymes has been adapted to screen many different antibody combinations in ready to use double and triple staining kits (*GBI Labs Inc.*). Here we present the use of a novel Emerald chromogen (*GBI Labs Inc.*) for HRP with the use AP-Red+ (*GBI Labs Inc.*) for AP enzymes that can produce a third color in the blue/purple range for the assessment of two proteins co-localized to the same cellular location. The expression patterns between two expressed proteins show a greater variability of protein co-localization in the tissue as illustrated in figures 2-6 of the differently colored reaction products. Additionally, we show that blue color is easily distinguished whether the two proteins are co-expressed in the nucleus, cytoplasm, or cell membrane. Finally, images show that semi-quantitative assessment can be made based on which protein level is expressed at higher levels which results in how blue (more emerald antigen) or how purple (more red AP Red + antigen) the color visualized within double stain.

Staining Pattern of Proteins with AP-Red+ & Emerald Chromogen

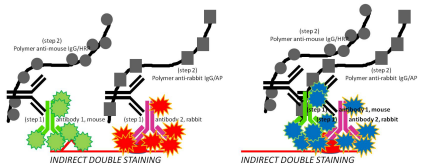

Fig. 1 Cartoon of staining pattern of proteins expressed separately versus co-localized in the cell.

Methods

Colon and breast cancer tissues specimens were obtained from Dr. Shi, Bejing, China and the 70 case breast cancer tissue array was obtained from BioChain Cat# Z7020004. Representative cores were used to generate two tissue arrays, one of breast cancer and the second of colon cancer. Screens were done as follows. Five-µm sections were cut and mounted on coated slides and dried overnight at 37C. Slides were de-waxed in xylene and hydrated using graded alcohols then rinsed in tap water. Endogenous peroxidase was blocked with 3% H2O2 for 10 minutes and washed in several changes of water. If Heat Induced Epitope Retrieval was required for primary antibody this step was done with 10mM Citrate buffer pH6.0 for 15 min at 98C with a cool-down to 45C. The double staining procedure is highly dependent on the primary antibody combination with respect to animal species. Mouse –Rabbit and Mouse –Goat primary antibody combinations were incubated on the tissue together. Mouse- Mouse and Rabbit-Rabbit primary antibody combinations were stained sequentially following protocols used in the double and triple stain kits listed below. Most primary antibody combinations were incubated for 30 minutes at room temperature unless otherwise indicated by primary antibody source. AP-Red+ chromogen requires incubation prior Emerald chromogen as detailed in the multi-staining protocol. Tissues were scored using Olympus BX40 Light microscope. Scoring was assessed on total percent positive cells and intensity of stain. Co-localization was assessed on all levels of positive cells.

Antibody Pairs	Source	Tissue	Double & Triple Staining Kits GBI Labs
Ms anti-p53 & Rb anti-ER	GBI Labs M16-IDB Spring E1644	Breast Cancer	DS201C, DS202C, DS233C
Rb anti-ER & Ms anti-PCNA	Spring E1644 GBI Labs M16-ID1	Breast Cancer	DS203C, DS212C
Ms anti-ER & Rb anti-PR Rb anti-Her2	GBI Labs ER-C Epitomics 1438-1 Epitomics 4201-1	Breast Cancer	TS302A, TS309A
Ms anti-p53 & Ms anti-PCNA & Rb anti-CEA	GBI Labs M16-IDB GBI Labs M16-ID1 GBI Labs P3012	Colon cancer	TS301A, TS308A
Gt anti-EGFR Ms anti-CEA	Santa Cruz SC03-G Gift from Dr. Shi ZC23	Colon Cancer	DS207C,
Gt anti-EGFR Ms anti-CK8/18	Santa Cruz SC03-G GBI Labs M308i	Breast Cancer	DS207C
Ms anti Actin Rb anti-Desmin	Sigma A2547-5ml GBI Labs P4005	Colon Cancer	DS201C, DS202C, DS233C

Results

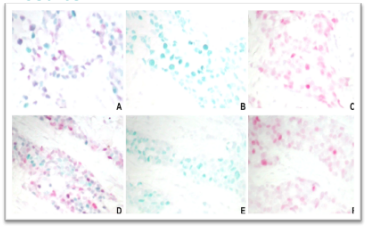

Fig. 2 Expression pattern of Rb anti-ER with Emerald and Ms anti-p53 with AP-Red+ (Panel A&D) on two breast cancers; Rb anti-ER detection with emerald chromogen (Panel B&E); and Ms anti p53 with AP-Red+ chromogen (Panel C&F). Co-Localization expressed in blue/purple color. Although the single stain for these two tumors show similar expression, the amount of co-localization is very different as seen in panel A with >70% of tumor cells expressing both proteins and panel D with 25% of the tumor cells expressing both proteins as indicated by blue/purple color.

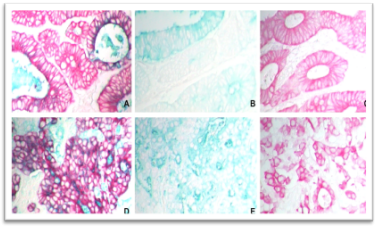

Fig. 3 Two Colon Cancer double stained with Rb anti-CEA with emerald and Ms anti-CK8/18 with AP-Red+ (Panel A&D); Rb anti-CEA only detection with emerald chromogen (Panel B&E); and Ms anti-CK8/18 only with AP-Red+ chromogen. Co-Localization represented in blue/purple color. Panel A shows that when tumor is negative, there is no leakage or overlap; only when the two proteins are co expressed there is a color change

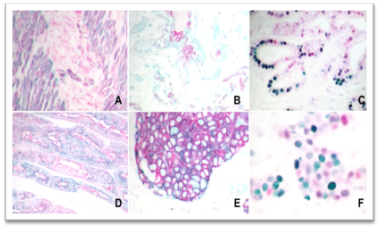

Fig. 4 Panel (A) colon cancer double stained with Rb anti-Desmin /Emerald and Ms anti-Smooth Muscle Actin/AP-Red+. Panel (B, C, E, & F) are breast cancer cases. Panel (B & E) are stained with Ms anti-CK8/18 with AP-Red+ and Gt anti-EGFR with emerald chromogen. Panel (C & F) are stained with Ms anti-ER with AP-Red+ and Rb anti-PR with emerald chromogen. Panel (D) colon cancer stained with Gt anti-EGFR emerald & Ms anti-CEA/AP-Red+.

Tables Data of percent of tumor cells expressing protein are presented to match the pattern of the tissue array layout. Tumor cells are considered positive even in the intensity of the stain is 1 out of scale of 1 to 4. A score of 1 is represents weak staining For example much of the EGFR staining in breast cancer was scored 1 to 2 while much of the score of CK8/18 was scored a 3 to 4 Overlap equals the percent of total tumor cells expressing both proteins.. (BL –No tissue; Neg –Negative; NA- Not Applicable)

Breast Cancer	Ms anti-P53			Rb anti-ER			Ms anti-P53 & Rb anti-ER		
1	90%	10%	Neg	Neg	-	1%	NA	NA	NA
2	Neg	10%	1%	90%	20%	20%	NA	1%	0
3	40%	30%	Neg	50%	70%	Neg	24%	12%	NA
4	1%	5%	BL	10%	1%	BL	NA	1%	BL
5	Neg	Neg	95%	Neg	Neg	Neg	NA	NA	NA

Breast Cancer	Rb anti-ER			Ms anti-PCNA			Rb anti-ER & Ms anti-PCNA		
1	Neg	100%	Neg	50%	70%	10%	NA	63%	NA
2	90%	10%	20%	60%	100%	30%	54%	90%	20%
3	60%	70%	Neg	100%	80%	90%	49%	35%	NA
4	10%	5%	BL	70%	95%	BL	10%	5%	BL
5	Neg	1%	Neg	100%	75%	100%	NA	1%	NA

Breast Cancer	Gt anti-EGFR			Ms anti-CK8/18			Gt anti-EGFR & Ms anti-CK 8/18		
1	100%	50%	70%	80%	70%	60%	72%	25%	30%
2	70%	80%	70%	100%	70%	70%	49%	70%	21%
3	70%	80%	90%	20%	90%	1%	20%	56%	1%
4	90%	Neg	BL	90%	100%	BL	63%	NA	BL
5	70%	90%	90%	70%	80%	50%	35%	72%	50%

Colon Caner	Ms anti-PCNA			Ms anti-p53			Ms anti-PCNA & Ms anti-p53		
1	60%	80%	Bl	Neg	Neg	Bl	NA	NA	NA
2	90%	60%	90%	100%	100%	90%	9%	6%	54%
3	70%	50%	Neg	70%	60%	Neg	21%	45%	NA
4	90%	100%	100%	40%	Neg	1%	28%	NA	NA
5	90%	BL	Neg	Neg	BL	Neg	NA	NA	NA

Colon Cancer	Gt anti-EGFR			Ms anti-CEA			Gt anti-EGFR & Ms anti-CEA		
1	10%	10%	Bl	50%	50%	Bl	2%	2%	NA
2	50%	20%	75%	90%	90%	90%	25%	18%	53%
3	60%	75%	NA	90%	90%	90%	54%	68%	NA
4	25%	60%	100%	90%	100%	100%	6%	42%	90%
5	60%	BL	Neg	90%	BL	Neg	54%	BL	NA

Colon Cancer	Ms anti-Actin			Rb anti-Desmin			Ms anti-Actin & Rb anti-Desmin		
1	90%	80%	Bl	80%	30%	Bl	60%	15%	BL
2	80%	90%	90%	40%	40%	10%	20%	15%	5%
3	90%	90%	90%	10%	40%	10%	5%	20%	5%
4	90%	90%	90%	10%	NEG	5%	5%	NA	2%
5	70%	BL	90%	30%	BL	70%	15%	BL	50%

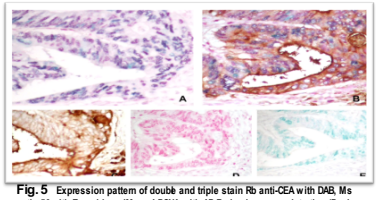

Fig. 5 Expression pattern of double and triple stain Rb anti-CEA with DAB, Ms anti-p53 with Emerald, and Ms anti-PCNA with AP-Red+ chromogen detection (Panel A&B) on colon cancer. Single stain are shown in panel C, D, & E.

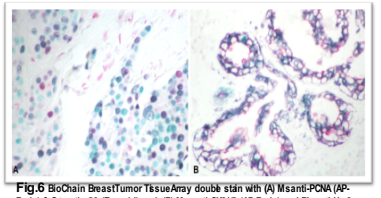

Fig.6 BioChain Breast Tumor Tissue Array double stain with (A) Ms anti-PCNA (AP-Red+) & Gt anti-p53 (Emerald) and (B) Ms anti-CK8/18 (AP-Red+) and Rb anti-Her2 (Emerald).

Tables BioChain Breast Tumor Tissue Array (BCTA) double stain with Ms anti-PCNA & Gt anti-p53 and Ms anti CK8/18 and Rb anti-Her2

BCTA	Ms anti-PCNA & Gt anti-p53													
1	NA	NA	3%	20%	10%	NA	1%	NA	NA	NA	NA	NA	5%	NA
2	NA	NA	1%	20%	90%	NA	NA	NA	NA	NA	10%	NA	5%	NA
3	NA	NA	NA	NA	NA	NA	30%	10%	NA	NA	NA	30%	54%	10%
4	NA	1%	NA	NA	NA	NA	40%	10%	10%	NA	NA	1%	64%	42% 10%
5	NA	0.5%	NA	NA	NA	NA	50%	NA	NA	5%	NA	50%	20%	NA NA
6	NA	NA	NA	NA	NA	40%	45%	1%	NA	2%	NA	50%	16%	NA NA
7	NA	NA	NA	NA	50%	NA	70%	NA	NA	NA	50%	NA	30%	NA 10%
8	50%	NA	NA	45%	20%	36%	NA	NA	NA	NA	10%	NA	10%	1% NA
9	5%	NA	NA	NA	NA	36%	NA	NA	60%	NA	NA	NA	NA	NA NA
10	NA	NA	NA	NA	NA	NA	NA	NA	50%	NA	NA	NA	NA	NA NA

BCTA	Rb anti-HER2 & Ms anti-CK8/18													
1	NA	NA	NA	NA	5%	NA	NA	NA	NA	20%	80%	NA	54%	NA NA
2	NA	NA	NA	NA	30%	NA	NA	NA	NA	10%	64%	NA	63%	NA NA
3	NA	NA	NA	NA	NA	80%	70%	90%	80%	NA	NA	20%	NA	50% 50%
4	NA	90%	NA	NA	NA	80%	70%	90%	90%	NA	NA	NA	10%	NA 50% 50%
5	NA	10%	NA	NA	NA	NA	48%	NA	NA	NA	NA	NA	20%	NA NA
6	10%	10%	NA	90%	NA	NA	40%	NA	NA	NA	NA	70%	20%	NA NA
7	NA	NA	40%	NA	NA	70%	NA	NA	NA	NA	NA	NA	NA	NA NA
8	NA	NA	40%	NA	NA	70%	NA	NA	NA	NA	NA	NA	NA	NA NA
9	NA	NA	70%	NA	NA	NA	80%	NA	35%	NA	NA	NA	NA	NA NA
10	25%	NA	100%	NA	NA	NA	42%	NA	25%	NA	NA	NA	NA	NA NA

Conclusion: Here we presented multiple double staining showing that emerald chromogen in the presence of the AP-Red+ chromogen produces a blue/purple color when two proteins are co-expressed in the nucleus, cytoplasm, or cell membrane. We predict that semi-quantitative assessment can be made based on which protein level is expressed at higher levels which results in how blue (more emerald antigen) or how purple (more red AP Red + antigen) the stain presents. The emerald chromogen will enable the researcher to take a closer look at the co-expression of proteins in larger study sets with the use of a light microscope.