Quantitative Multiplex Chromogenic Immunohistochemistry

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Abstract

Immunohistochemistry has been used widely as a diagnostic aid and has gained acceptance over the last decade as a stand-alone method to select patients that may benefit from targeted therapeutics. As researchers gain a better understanding of factors that predict sensitivity to chemotherapeutic agents, the need for evaluating multiple antigens on one slide is becoming apparent. Multiplex immunohistochemical assays are currently being developed using both fluorescent and chromogenic methods. Multiplex chromogenic immunohistochemistry has many benefits over multiplex fluorescence techniques, including: 1) slides can be viewed under a standard light microscope, 2) chromogens do not suffer from photobleaching, 3) slides stained with standard chromogens can be archived for long periods of time and re-evaluated, 4) the FDA has experience evaluating and approving semi-quantitative chromogen-based theranostic IHC assays. By optimizing multiplex IHC assays appropriately and using a multispectral imaging system, individual chromogens can be quantitatively discriminated and analyzed both independently and for inter-analyte relationships. In order to evaluate the linearity of a sample assay, cell lines with defined expression levels for HER2 and EGFR were evaluated by multiplex immunohistochemistry and staining results were compared to protein concentration. Results indicated a linear relationship with an R^2 value of 0.8571 for HER2 and 0.9707 for EGFR. Examples of multiplex assays for diagnostic and theranostic applications are presented.

Introduction

Multiplex immunohistochemical assays have always been limited by the inability to accurately resolve overlapping stains, but this limitation can be solved using a technology called multispectral imaging. A spectral imaging camera records the intensity of transmitted light in defined increments across the light spectrum of interest. This image stack is then compared to defined chromogen spectra, and the individual chromogens are separated out to produce quantitative grayscale images for each analyte. Using image analysis algorithms, the staining characteristics of the cells can be evaluated.

Care must be taken in developing immunohistochemical procedures to ensure adequate sensitivity for each analyte, lack of cross-reactivity and minimal interference. This poster presents data on the linearity of HER2 and EGFR in a multiplex assay and presents a number of other examples of multiplex immunohistochemical assays.

Spectral Imaging

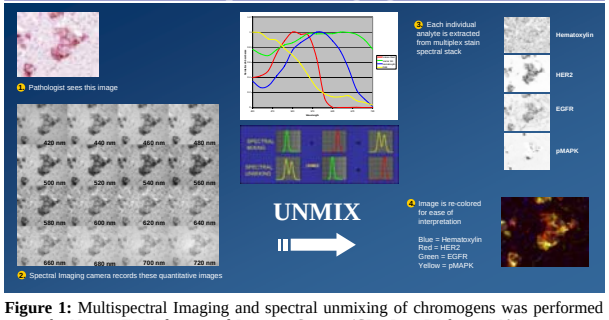


Figure 1: Multispectral Imaging and spectral unmixing of chromogens was performed using the Nuance™ Multispectral Imaging System (CRi, Inc., Woburn, MA).

Results

Cell Line	Herceptin Sensitivity	Iressa Sensitivity	Lapatinib IC50*	RGB	HER2	EGFR	pAKT	Hematoxylin	Pseudocolored
SKOV-3	Minimal	Sensitive	ND						
SK-BR-3	Sensitive	Sensitive	0.037 μM						
MDA-MB-453	Sensitive	ND	3.9 μM						
MCF-7	Resistant	Sensitive	7.7 μM						
BT-20	ND	ND	9.8 μM						
A-431	ND	Sensitive	ND						

*Koncny et al., Cancer Res, 2006. Other sensitivity data was collected from a survey of the literature including Anderson et al., Int J Cancer, 2001; Camarind et al., Breast Cancer Res, 2005; Pegram et al., JNCI, 2004.
ND = not determined

Figure 2: Multiplex IHC of Cell Line Controls

Cell lines with known HER2 and EGFR expression levels were stained using multiplex IHC followed by multispectral imaging and spectral unmixing. Staining for each individual analyte is represented by a quantitative grayscale image of optical density. Composite pseudocolored images at right include HER2 re-colored Red, EGFR re-colored yellow and pAKT re-colored blue.

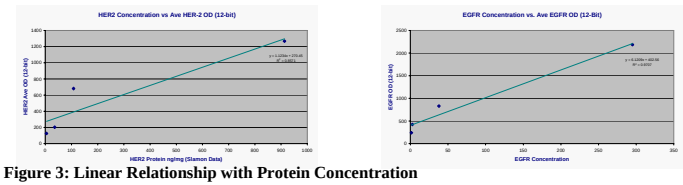


Figure 3: Linear Relationship with Protein Concentration

HER2 and EGFR staining was unmixed from a HER2 / EGFR / pAKT / Hematoxylin multiplex and was compare to reported protein expression levels as determined by ELISA. The average cellular staining intensity demonstrated a linear relationship with the reported EGFR concentration (R^2 = 0.9707) and HER2 concentration (R^2 = 0.8571). HER2 staining intensity was compared to HER2 protein concentration in the SK-BR-3, MDA-MB-453, BT-20 and MCF-7 cell lines. EGFR staining intensity was compared to EGFR protein concentration in SK-BR-3, MDA-MB-453, BT-20, MCF-7 and A-431 cell lines.

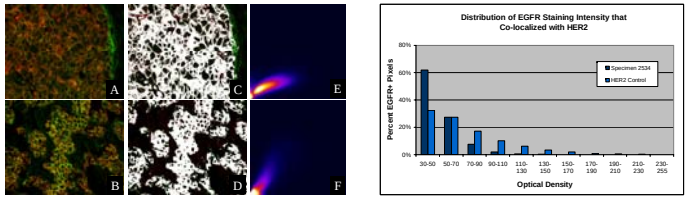
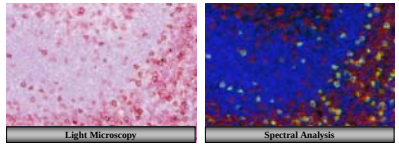
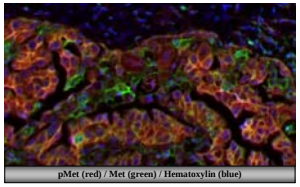
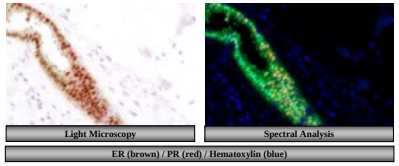


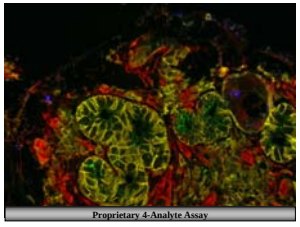
Figure 4: HER2 and EGFR Co-localization Analyses

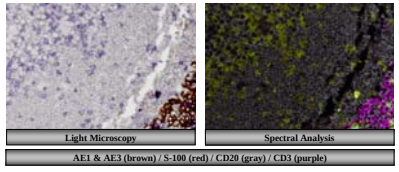
HER2 and EGFR co-localization analyses were performed on spectrally unmixed HER2 and EGFR images from HER2/EGFR/pAKT/hematoxylin multiplex assays. HER2 was pseudocolored red and EGFR was pseudocolored green.

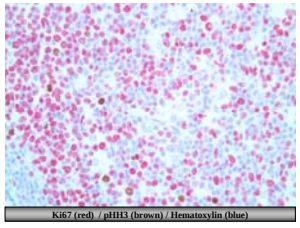


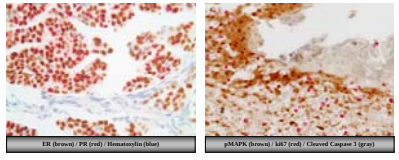


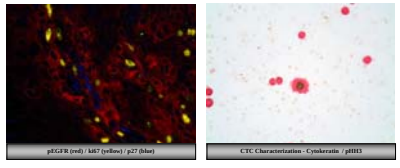


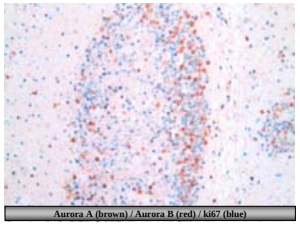












Conclusions

- Spectral analysis and spectral unmixing of multi-analyte chromogenic immunohistochemistry accurately segregated overlapping chromogens, but care must be taken in evaluating acceptable color combinations
- Multiplex chromogenic IHC with multispectral imaging can be quantitative with a strong linear correlation to protein concentration if assays are designed correctly
- Multiplex IHC assays enable "flow cytometry on a slide" co-localization type analyses
- The ability to combine assays for ErbB-family members, signal transduction molecules and other proteins will play an important role in future theranostic assays