Cancer InCytes

Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity

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Abstract

Immunohistochemistry is a routine procedure for detecting the expression of biological markers in formalin-fixed and paraffin-embedded tissues. Chromogens, which can appear as different colors (brown, blue, red) under bright field microscopy, are localized in fixed tissues to antigens of interest via an antibody-antigen detection system. The advantage of a chromogen system is that the stained tissue section is permanently fixed, and the staining quality is maintained for many years. The shortcoming, however, is quantifying the intensity of such stains. Unlike immunofluorescent protocols in which the brightness of a region is directly proportional to the amount of localized antigen, chromogen stains appear darker in regions with more antigen. This dark staining is visible to human eye under white light, but darker spots have lower intensity values; something that is counterintuitive and cumbersome for the purposes of quantitation. We report that this limitation can be overcome by measuring the "reciprocal intensity" of the chromogen stain. A typical red-green-blue image resulting from bright field microscopy has its maximum intensity value found in the white, non-stained area. Areas that contain any coloration, due to the chromogen or a counterstain, have an intensity of less than the maximum. By subtracting the intensity of the stained area of interest from the maximum, the staining in these areas can be represented as a quantity that is positively correlated with increasing darkness. This is a more intuitive means of assessing the intensity of a chromogen stain, and allows for more sensitivity in quantifying gradients of coloration between treatment groups. This approach has the potential to stratify nuanced protein expression in previously published human specimen data sets into cohorts with clearer clinical outcomes.

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Immunohistochemistry (IHC) is an effective method for localizing the expression of a specific protein in tissues. It is a routine procedure in biomedical laboratories, first reported by Coons et al. in 1942 [1]. Pathologists influence which treatment a patient should receive based on IHC results of a clinically relevant marker, such as the estrogen receptor-α (ER) in breast cancer. A common current approach to quantifying variations of expression between samples and treatment groups is to use a semi-quantitative method, e.g., the "three plus" scale: + indicating low expression, ++ indicating moderate expression, and +++ indicating high expression. Alternatively, a semi-quantitative cut-off (e.g., 10%) can be used to define whether a sample is positive or negative for a marker. These semi-quantitative approaches have been useful for defining cohorts with differential prognoses or responses to therapy. However, there are cases of discordance in which patient samples exhibit a certain score, yet the patient outcomes are better or worse than would be predicted by a direct correlation of outcome with low, moderate, or high expression of the marker. In such cases, a quantitative method that has a more objective and more highly graduated dynamic range would allow researchers and pathologists to define sub-groups based on nuanced expression of the marker.

Antigens of interest in formalin-fixed paraffin-embedded tissues are detected by an antigen-antibody detection system. Diaminobenzidine (DAB), first introduced by Graham and Karnovsky in 1966 [2], is a common chromogen for detecting the location of an antigen. DAB is a substrate for the enzyme horse radish peroxidase (HRP), which turns DAB it into a brown precipitate that appears

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Figure 1

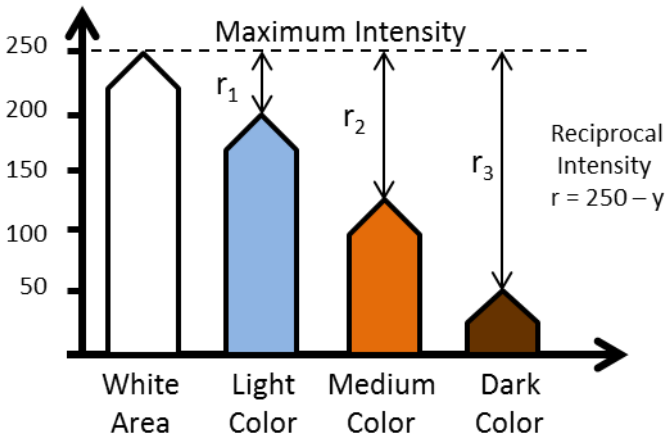

Figure 1. Schematic of method for deriving reciprocal intensity from chromogen intensity. Standard red-green-blue images from bright field microscopy have a maximum pixel intensity of 250 in white, unstained areas. Counter stains (i.e. hematoxylin) and specific stains (i.e. chromogens) result in varying degrees of coloration, which exhibit an intensity of less than 250, inversely correlating with the intensity of the stain. Subtracting the intensity of the region of interest from 250 will yield the reciprocal intensity, which is positively correlated with the intensity of the stain.

darker as more precipitate builds up in one place. DAB has been widely used as a chromogen in IHC for many decades.

Here we report a method that is able to numerically quantify the intensity of a chromogen stain in IHC under bright field microscopy. Standard red-green-blue (RGB) color images acquired from bright field microscopy have a maximum intensity of value 250 (represented by white, unstained areas) as measured by the standard intensity function in the open source Fiji software (ImageJ) (http://fiji.sc/Fiji). Stained areas, as marked by either a nuclear counterstain such as hematoxylin or the chromogen, appear to the human eye as varying degrees of coloration; the more antigen-chromogen present, the darker the area appears. However, darker areas have lower intensity values, resulting in an inverse correlation between the amount of antigen and its numerical value. This is counterintuitive and cumbersome for the purposes of data analysis. One way around this dilemma is to measure the "reciprocal intensity" of the stained area. Since the maximum intensity value of an RGB image analyzed in ImageJ is 250, we can subtract the intensity of a stained region of interest (ROI) from 250, thereby deriving a reciprocal intensity that is directly proportional to the amount of chromogen present (Figure 1). The maximum intensity value of 250 is somewhat arbitrary, since it serves as a reference from which reciprocal intensities are derived.

Figure 2

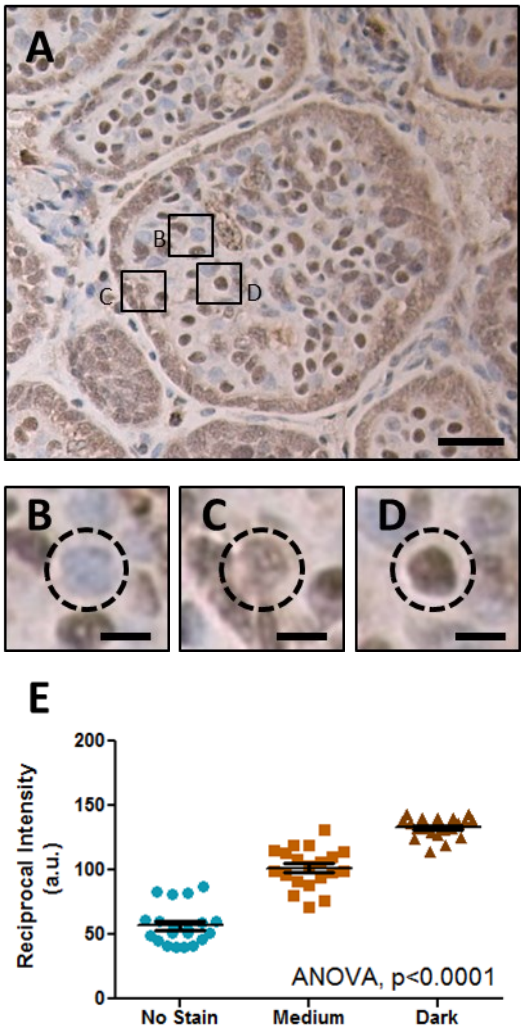

Figure 2. Reciprocal intensity of nuclear estrogen receptor-α in breast cancer. (A) *Trp53 null* mouse mammary tumor stained with an antibody that detects the estrogen receptor-α (ER) protein with a brown DAB precipitate; nuclei are counter-stained with hematoxylin (blue) (bar, 50 μm). (B, C, D) Insets from A; bar, 10 μm; dotted line, region of interest (ROI). (B) Nucleus that is negative for ER but appears blue because of hematoxylin. (C) Nucleus that exhibits moderate staining for ER. (D) Nucleus that exhibits dark staining for ER. (E) Individual mean intensity within ROI covering 20 independent nuclei were quantified for each visually identified category of ER staining: no stain, medium, or dark. Reciprocal intensity positively correlated with increasing ER staining (ANOVA, $p<0.0001$; a. u., arbitrary units).

Therefore this maximum point should be reasonably selected and systematically applied to all measurements in a data set. This is relevant to images that were not white balanced prior to image capture or were edited for contrast afterwards.

The estrogen receptor-α (ER) is one of the most important clinical markers in breast cancer. Its level of expression often determines the aggressiveness of the tumor as well as application of therapies that specifically target the ER pathway [3-5]. We stained for ER in a *Trp53 null* BALB/c mouse mammary tumor; a mouse model that has many features in common with human breast cancer [6-9]. The tumor was formalin-fixed and paraffin-embedded before being sectioned into 5 um slices. We then used the Vectastain ABC Kit (Catalog # PK-4001) and ImmPACT DAB substrate (Catalog # SK-4105) from Vector Labs (Burlingame, CA) to detect the estrogen receptor-α (C1355) antibody from Millipore (Billerica, MA) (rabbit anti-mouse, Catalog # 06-935). De-paraffinization, rehydration, antigen retrieval, and antibody incubation conditions (primary antibody, overnight at 4 degrees Celsius) were done as previously reported [10, 11]. Nuclei were counterstained with a 30 second pulse of hematoxylin (Vector Labs, Catalog # H-3404), to avoid unnecessarily dark blue nuclei, which would have increased the reciprocal intensity of non-positive nuclei. The *Trp53 null* BALB/c model produces tumors that express ER to varying degrees in each cell. As proof of principle, we first quantified the reciprocal intensity of nuclear-localized ER in this tumor model (Figure 2A) using the open source Fiji (ImageJ) software (http://fiji.sc/Fiji). A uniformly sized region of interest was placed over each nuclei using the draw tool (Figure 2B-D), and the mean intensity was measured using the "Measure" function under the "Analyze" menu of Fiji [Note: desired outputs of the measure function (i.e. mean intensity, area, etc.) can be specified in the "Set Measurements..." option under the "Analyze" menu.]. ER was expressed to varying degrees in the nuclei, ranging from no staining (Figure 2B), to moderate staining (Figure 2C), to dark staining (Figure 2D). Reciprocal intensities directly correlated with increasing staining intensity (Figure 2E) (ANOVA, $p<0.0001$).

The reciprocal intensity method can also be used to re-analyze previously published data sets. However, some of those data sets may have been over-exposed; resulting in saturated densities in regions that otherwise would show higher differentials. The data within the images may still be salvageable, contingent upon how much the sample is over-exposed during incubation with DAB. Depending on whether the antibody has non-specific binding and/or whether the antibody has specific binding outside of areas of interest, a background subtraction threshold can be determined. Some regions only show slight staining at pre-saturation incubation times, but become dark when the sample is over-exposed. The goal is to normalize the values of the over-exposed ROIs by the derived background intensity in a way that will amplify the dynamic range that has been narrowed due to over-exposure. We suggest one approach here. To determine the background intensity, certain biological structures or regions should be identified as representing only background staining due to over-exposure. These selected regions should generally not be as dark as the regions that contain the staining of interest. The reciprocal intensity of these ROIs should be determined for an appropriate number of independent regions. This will be referred to as the ad hoc background intensity (AHB). Next, the reciprocal intensity of the stained regions of interest (nuclear, cytoplasmic, etc.) should be determined as previously described. Once this is done, background subtraction can be done by dividing the reciprocal intensities of the regions of interest by the AHB. Since over-exposed regions of non-interest will also be dark, dividing the reciprocal intensities by the AHB will result in values that are fractions ranging from 0 to 2. The fractions from this hypothetical scenario can then be transformed through an exponential function such as $y=e^{(1.6358*x)}$, in order to increase the dynamic range of the data to be between 1 and 25. These data manipulations are far from ideal and should generally be employed only if over-exposed data sets cannot be re-stained. For such retrospective analyses, the ultimate validation of these numerical manipulations is whether they result in patient cohorts that exhibit clinical outcomes that were unidentified by previous quantitation methods. Combined with a rational, systematic selection of the 10-15 fields of view that are reasonably placed throughout each specimen based on contextualized knowledge of what is of interest, this reciprocal intensity approach has tremendous potential for stratifying sub-groups of patients.

To our knowledge, this is the first quantification of chromogen intensity via a reciprocal intensity approach. This method has the potential to clarify previous enigmas in data sets in which protein expression phenotypes did not correlate with clinical outcomes in certain patient cohorts. Published data sets can be retrospectively analyzed and the results will dictate future prospective studies.

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Conflict of Interests

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References

1. Coons AA, Creech HJ, Jones RN, Berliner E: The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *Journal of Immunology* 1942, 45:159-170.
2. Graham RC, Jr., Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 1966, 14:291-302.
3. Harvey JM, Clark GM, Osborne CK, Allred DC: Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* 1999, 17:1474-1481.
4. Hackshaw A, Roughton M, Forsyth S, Monson K, Reczko K, Sainsbury R, Baum M: Long-Term Benefits of 5 Years of Tamoxifen: 10-Year Follow-Up of a Large Randomized Trial in Women at Least 50 Years of Age With Early Breast Cancer. *J Clin Oncol* 2011.
5. John-Baptiste AA, Wu W, Rochon P, Anderson GM, Bell CM: A Systematic Review and Methodological Evaluation of Published Cost-Effectiveness Analyses of Aromatase Inhibitors versus Tamoxifen in Early Stage Breast Cancer. *PLoS One* 2013, 8:e62614.
6. Nguyen DH, Fredlund E, Zhao W, Perou CM, Balmain A, Mao JH, Barcellos-Hoff MH: Murine microenvironment metaprofiles associate with human cancer etiology and intrinsic subtypes. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2013:1-10.
7. Herschkowitz JI, Zhao W, Zhang M, Usary J, Murrow G, Edwards D, Knezevic J, Greene SB, Darr D, Troester MA, et al: Breast Cancer Special Feature: Comparative oncogenomics identifies breast tumors enriched in functional tumor-initiating cells. *Proc Natl Acad Sci U S A* 2011.
8. Jerry DJ, Kittrell FS, Kuperwasser C, Laucirica R, Dickinson ES, Bonilla PJ, Butel JS, Medina D: A mammary-specific model demonstrates the role of the p53 tumor suppressor gene in tumor development. *Oncogene* 2000, 19:1052-1058.
9. Medina D: Biological and molecular characteristics of the premalignant mouse mammary gland. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2002, 1603:1-9.
10. Nguyen DH, Oketch-Rabah HA, Illa-Bochaca I, Geyer FC, Reis-Filho JS, Mao JH, Ravani SA, Zavadil J, Borowsky AD, Jerry DJ, et al: Radiation Acts on the Microenvironment to Affect Breast Carcinogenesis by Distinct Mechanisms that Decrease Cancer Latency and Affect Tumor Type. *Cancer Cell* 2011, 19:640-651.
11. Nguyen DH, Zhou T, Shu J, Hua H, Mao JH: Two applications of a fluorescent chromogen in immunohisto-chemistry of formalin-fixed paraffin-embedded tissue sections to overcome common limitations of detection. *Cancer InCytes* 2012, 1(1):e.