Quantitative immunohistochemistry using an antibody-fused bioluminescent protein

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ABSTRACT

We established a quantitative detection method for immunohistochemistry based on a reference standard light-emitting diode, protein microarray and antibody-fused bioluminescent protein. In this procedure, we calibrated the bioluminescence imaging system and prepared the calibration curve between antigen and antibody-fused bioluminescent protein using a protein microarray. Then we converted the detecting light signal to antigen count via absolute photon number in the bioluminescent images; there was a resulting threefold difference in the target antigen number between normal and cancerous tissues. Our technique can easily compare immunohistological images and evaluate tumor progression in quantitative pathological diagnosis.

METHOD SUMMARY

Here we present a quantitative detection method for immunohistochemistry using a reference standard light-emitting diode and a protein microarray. In this method, the light signal of antibody-fused bioluminescent protein converts to the number of target antigens via the absolute photon number by a calibrated imaging system.

KEYWORDS

antibody imaging • antigen detection • bioluminescence • immunohistochemistry • luciferase

Developed in 1941, immunohistochemistry (IHC) has been an important technique underlying sensitive detection systems including colorogenic substrates, fluorescent probes and bioluminescence (BL) probes [1–5]. Fundamentally, IHC detects a target molecule using antigen-specific antibodies in tissue sections. However, quantitative visualizations of IHC images are not yet possible, despite the ability to quantify immune assay signals. We currently cannot quantify IHC images because they are not based on an antigen-versus-signal calibration curve. However, the ability to count the number of target antigens in tumor tissue would allow us to categorize tumor stages. Here we developed a novel quantitative detection method using IHC based on combining a reference standard light-emitting diode (LED), protein microarray and antibody-fused bioluminescent (BL) protein.

Previously, we had established a rapid direct detection system for IHC using an antibody-fused BL probe, demonstrating the potential to detect tumor antigens with high sensitivity in routine immune histological examinations [5]. Furthermore, we established a reference LED light source that was characteristic of total flux and light distribution, then calibrated the bioluminescence imaging (BLI) system as an absolute light signal [6]. Using this LED, time-averaged emission intensity could be controlled linearly to cover a wide dynamic range of about four orders of magnitude for several years. This calibrated BLI system revealed that luciferase had an average light signal at one attowatt, corresponding to a few thousand photons per second per cell [7]. Based on luciferase quantum yield, we could estimate the amount of luciferase protein in a living cell.

Carcinoembryonic antigen (CEA) is usually present at very low levels in the blood of healthy adults. However, some tumor types have serum CEA levels that are elevated by approximately 10 ng/ml, suggesting that the antigen can be used as a tumor marker and its antibody can be used as a tumor probe [8,9]. In this study, we therefore used existing methods [5] to prepare biotin–CLuc (recombinant *Cypridina noctiluca* luciferases) and finally, 50 µg/ml of anti-CEA-antibody-fused bioluminescent (anti-CEA-BL) probe from CLuc. Diluted anti-CEA-BL and luminescence activity exhibited a strong linear relationship across a wide range (Figure 1A). Before antigen detection, we followed an existing method to calibrate the tissue BLI system for IHC, based on a reference LED light source [7]. We used a cooled

Figure 1. Background of quantitative immunohistochemistry. (A) Relationships between diluted probe and luminescence activity. Light intensity was measured with a tube-type luminometer after adding 1 mM cypridinid luciferin for 10 s. (B) Calibration schematic for the bioluminescence imaging system based on the reference LED signal. At 474 nm, the Radiant-flux reference LED standard contained 2.36×10^8 photons/s, corresponding to 4.98×10^6 RLU on the CCD camera. Supplementary Table 1 summarizes the conversion process. (C) Bioluminescence imaging of microarray. The microarray contained 0.2–1.0 mg/ml control CEA protein per spot (size ~ 0.014 mm²) (GENE SILICON chip, Toyo Kohan, Osaka, Japan) and analyzed in triplicate using a Protein Microarrayer Robot (Kaken Geneqs Inc., Matsudo, Japan) [10]. Spotted slides were incubated for 1 h with a blocking buffer and incubated with diluted anti-CEA-BL (50-fold) probe for 5 min. After adding 10 μ M Cypridinid luciferin for 5 min, followed by two washes in phosphate-buffered saline, bioluminescence imaging was taken using a MultiVersa WSL-1300 (ATTO, Tokyo, Japan) with a 1 $\times$ Telecentric Lens (NA 0.093). (D) Calibration curve between antigen count and photon numbers. Light signal was measured in CS Analyzer (ATTO). CCD: Charge-coupled device; CEA: Carcinoembryonic antigen; LED: Light-emitting diode; RLU: Relative intensity.

charge-coupled device (CCD) camera with a 1 $\times$ telecentric lens (NA 0.093) and a reference LED light source set at an aperture of 164 pW. The camera was a frame transfer type iKon (Andor, Belfast, UK), with a pixel size of 13 μ m and pixel number of 1024 $\times$ 1024. Pre-amplifier gain was set at 4 and binning at 2 $\times$ 2. We followed a specific calibration schematic for the BLI system (Figure 1B) and a conversion process (Supplementary Table 1) to obtain the absolute light signal value from the reference LED light source. As the light source distribution is $I(\theta) = \cos^n \theta$, the reference distribution is $\cos^{1.33} \theta$. The light collection efficiency of the objective lens $\eta(\theta)$ is calculated with the following formula:

$$\eta(\theta) = 1 - \cos^{n+1} \theta = 1 - \cos^{2.33} \theta$$

Total photon number (Tp) during exposure time t (s) is determined based on accumulated light in the CCD camera and photon energy at 474 nm (E_{474}), and total flux of light source (Rf) is 164 pW using the following equation:

$$Tp = Rf \times \eta(\theta) \times t/E_{474}$$

To determine absolute sensitivity, we measured the reference LED light source five-times at exposures of 0.5 and 1.0 s. Relative luminescence activity (RLU) was measured with the imaging software CS Analyzer (ATTO). Average RLU values were 7.13×10^6 and

Figure 2. Quantitative immunohistochemistry of surgical pathological specimens (serial cancer tissue sections). (A) Hematoxylin and eosin (H&E) staining of sections. (B) Immunohistochemical staining of sections using indirect peroxidase labeling. Immunohistochemical staining was performed using monoclonal mouse anti-human CEA (30 min, 1:1000; Dako Corporation, Glostrup, Denmark). Dako EnVision Labeled Polymer Peroxidase was the secondary antibody (30 min; Dako Corporation, Tokyo, Japan). The chromogen was diaminobenzidine. (C) Bioluminescence imaging of sections. Each slide was incubated with diluted anti-CEA-BL (50-fold) probe for 5 min. After adding 10 μ M luciferin solution in phosphate-buffered saline, sections were visualized using a cooled charge-coupled device camera system (MultiVersa WSL-1300, ATTO, Tokyo, Japan) with a 5-min exposure time. Light signal was measured in CS Analyzer (ATTO). (D) CEA distribution of five randomly selected spots in the colon cancer (shown in yellow) and normal epithelial tissues (shown in blue) from Figure 2C. The histogram of light signal in the pixel per each box was measured in NIH Image J. The frequencies of appearance were plotted against the detecting photon number in the histogram.

1.21×10^7 at 0.5 and 1.0 s, respectively. We then determined the photon conversion coefficient as 0.398 photons/RLU, and the absolute photon number as 2.36×10^3 photons/ mm^2 at 474 nm, based on the average RLU value at an exposure time of 0.5 s. After calibrating the BLI system, we confirmed that recalibration was not required for several weeks (data not shown). The BLI system must, however, be calibrated when changing an antibody-fused BL probe and/or a detecting apparatus.

Next, we prepared the protein microarray, containing control CEA protein for 0.2–1.0 mg/ml per spot ($\sim 0.014 \text{ mm}^2$). The analyses were performed in triplicate using a Protein Microarrayer Robot [10]. Spotted slides were incubated for 1 h with a blocking buffer. In microarray experiments, we added diluted anti-CEA-BL (50-fold) probe on the spotted slide and incubated for 5 min. We then washed the slide twice with phosphate-buffered saline before adding 1 mM Cypridinid luciferin to detect the light signal for 5 min. The image taken of protein microarray revealed approximately 480–1000 photons/ s/mm^2 (Figure 1C). The calibration curve between antigen and photon numbers indicates a linear relationship ranging between 1.0 and 4.0 ng/ mm^2 (Figure 1D). Based on spot area, 1 ng/ mm^2 CEA corresponds to 580 photons/ s/mm^2 ; however, the linear relationship is less obvious at 1.0 ng/ mm^2 due to a weak signal. We could not confirm linearity above 4.0 ng/ mm^2 because the microarray does not have the capacity to absorb proteins beyond that point. We can consider that the linearity between antigen and photon numbers could be more widely based on the linearity of immunoassay using the antibody-fused CLuc extended from 7.8 to 500 pg/ml IFN- α [11]. Despite this, we demonstrated the usefulness of the 1.0–4.0 ng/ mm^2 range on the protein microarray. Overall, these results indicate that light signal can be converted to amount of antigen via absolute photon numbers.

We used frozen surgical pathology sections of colon cancer tissue specimens to verify the effectiveness of quantitative IHC. Each section comprised colon cancer tissue, normal epithelial tissue and muscle layer tissue. Total average CEA in one section was approximately 460 ng/tissue (Supplementary Figure 1). Previous research using radioimmunoassay has suggested that tumor tissues contain

around 145–17,850 ng/mg CEA [7]. A bicinchoninic acid assay of another frozen section found about 0.03 mg total protein, indicating around 15,000 ng/mg CEA. Thus surgical specimens could represent a higher tumor stage.

Hematoxylin and eosin (H&E) staining (Figure 2A) and peroxidase-based IHC staining (Figure 2B) revealed that CEA was mainly distributed in colon cancer tissue, partially in normal epithelial tissue, and not in muscle layer tissue. Our findings do not contradict previous work that faintly detected CEA in normal epithelial tissue [12]. However, because CEA signals are an indirect measure dependent on substrate exposure time and enzymatic reactions, they are not ideal for determination, which results in insufficient judgment of tumor progression. Instead, directly counting tumor antigens in IHC would yield a more accurate understanding. We therefore incubated diluted anti-CEA-BL (50-fold) probe for 5 min with the colon cancer tissue specimens used in protein microarrays (Figure 1C) to detect a light signal. The procedure (including washes) was completed in approximately 15 min. The BLI of IHC was nearly identical to IHC staining of CEA using anti-CEA antibody (Figure 2C), showing stronger and fainter CEA signals in colon cancer tissue (encircling yellow line) and faint signal in normal epithelial tissue (encircling blue line), respectively. The total number of detected photons in the colon cancer area ($\sim 35 \text{ mm}^2$) was 4.87×10^4 photons/s, corresponding to an estimated 222 ng CEA based on the calibration curve; this value was less than that obtained using ELISA. Our IHC using anti-CEA-BL might underestimate the target antigen because an antibody can only detect a limited amount of surface antigens; therefore we set the detection limit at 1.0 ng/mm^2 .

To compare CEA distribution in colon cancer and normal epithelial tissues, we randomly selected five spots of each area and counted photons. Each spot contained 1000–2800 photons/s/ mm^2 in colon cancer (shown in yellow in Figure 2D) and 50–1,000 photons/s/ mm^2 in normal epithelial tissue (shown in blue in Figure 2D). The frequency of appearance against the detecting photon number were plotted as a histogram (Figure 2D). The amount of CEA was 4–15 ng/ mm^2 and the maximum frequency of appearance was approximately 10 ng/ mm^2 in colon cancer. These results indicate a threefold difference between the two tissues. Our quantitative IHC technique is therefore able to categorize and evaluate tumor progression. Additionally, it allows us to construct 3D IHC images using serial sections of pathological tissue samples after normalization (Supplementary Figure 2 & Supplementary movie).

In conclusion, after calibrating the BLI system using the standard LED, we prepared a calibration curve between the antibody-fused bioluminescent protein and the target antigen in the protein microarray, then converted the light signal to antigen count via absolute photon number in the tissue. We counted the target antigen in an immunohistological tissue sample as a proof of concept and found that the amount of CEA was increased threefold in colon cancer tissues compared with normal epithelial tissues. Our technique allows for easy comparisons of multiple immunohistochemical images regardless of when they were taken, the equipment used or the use of different bioluminescent probes.

Future perspective

Validation of the quantitative immunohistochemistry with other antibody-fused bioluminescence probes and target tissues may provide a useful and easy analytical method to evaluate tumor progress in the laboratory and clinic. Furthermore, the method enables categorization of tumor stage in quantitative pathological diagnosis.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0006

Author contributions

Y Ohmiya directed the bioluminescence imaging project, planned the experiments and prepared the manuscript. C Wu produced the anti-CEA-BL probe. K Wang, S Shimajiri, T Enomoto and H Kubota performed all the experiments. H Kubota and H Akiyama calibrated the imaging system.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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