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Validating the Use of ImageJ for the Quantitative Analysis of Immunofluorescence

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Abstract

We sought to validate the use of the free software ImageJ to calculate weighted luminescence (WL) as an outcome measure for immunofluorescence. The noise signal seen in direct immunofluorescence (DIF) demonstrates an inverse relationship with the concentration of added antibody. Thus, the drop in this antigen-independent signal which accounts for the majority of each image allows for mathematical modelling of signal intensity. DIF was performed on specimens from 10 bullous pemphigoid (BP) patients and 6 pemphigus vulgaris (PV) patients. DIF was prepared using dilutions of tagged antibodies for IgG and C3 at a dilution ranging from 1:100 to 1:800. Images were processed using ImageJ, with green luminescence histograms generated for each dilution. To determine the relationship of mean WL as the dilution of antibody increased, we generated histograms with logarithmic regression curves for anti-IgG and anti-C3 antibodies. To account for the role of antigen-dependent signal (i.e. the immunoreactive portion of the DIF picture), an additional regression curve limited to dilutions ranging from 1:100 to 1:400 was performed to reduce the risk of the Fc receptor or C3 becoming unsaturated. Logarithmic regression for BP IgG and C3 dilutions demonstrated a significant and moderate to strong correlation, (R^2=0.51, P<0.001) and (R^2=0.50, P<0.001) respectively with a mild drop in relationship strength, but still statistically significant when corrected for antigen-dependent signal. A weaker relationship was seen in PV, but this also was statistically significant regardless of correction. WL is potentially a valuable measure for quantitative comparison of fluorescent signaling in the experimental setting. Further uses of comparative immunofluorescence using ImageJ such as comparing antibody binding or antigen expression require validation of WL which we herein demonstrate.

Keywords: Quantitative immunofluorescence; Comparative immunofluorescence; Immunology methods

Introduction

Quantitative immunohistochemistry (IHC) using open-source image software has allowed for digital comparison of protein expression [1-6]. This can aid in experimental comparison of protein expression as well as clinical classification of certain tumors' receptor status. While the use of luminescence as an outcome measure in immunohistochemistry has been validated [6], its use in immunofluorescence has yet to be convincingly demonstrated.

The use of image software analysis in immunofluorescence has several theoretical advantages over its use in quantitative IHC. Assuming the use of a green chromophore, no additional color filtration is needed to isolate the desired chromophore. The use of weighted luminescence takes into account protein density. As the selection of color thresholds demonstrates significant inter-user variability, this is desirable [7]. Additionally, the cellular location of the target antigen which may obfuscate isolation of the chromophore in IHC [8] need not be accounted for in immunofluorescence.

Since only a single primary color need be assessed, a focused histogram can be generated for analysis. In order to take into account that more intense colors represent an increased concentration fluorescein isothiocyanate (FITC)-tagged antibody, weighted luminescence can be used as an outcome measure. Black pixels (those devoid of chromophore) are weighed at zero, while those fully saturated with chromophore are weighed highest.

Comparative immunofluorescence has several applications including comparing the binding of different antibodies to a fixed tissue or comparing the antigen expression of different tissue sources using a fixed antibody or probe such as in fluorescence *in situ* hybridization [9-12]. In order to use ImageJ for any of these applications, we sought to assess the validity of weighted luminescence (WL) as an outcome measure for quantifying immunofluorescence signal. The use of logarithmically increasing concentrations of FITC-tagged antibodies in DIF allows the opportunity to mathematically model a logarithmic drop in overall signal intensity, as the rate of signal noise which accounts for the majority of signal is inversely related to the concentration of tagged antibody and is not dependent on the concentration of target antigen. The antigen-dependent section of the DIF (i.e the immunoreactive portion) represents a minority of total signal except at more diluted concentrations of FITC-tagged antibodies. While measurement of fluorescence signal in DIF itself is not of clinical significance, it allows a controlled manner of validating calculation of luminescence as the tissue and primary antibody remains unchanged in each specimen, while only the secondary antibody is diluted. We thus analyzed the overall weighted luminescence for DIF taken from patients with bullous pemphigoid and pemphigus vulgaris. While the quantification of DIF signal is not in itself a clinically useful measure due to saturation of the tissue antigen, the use of quantitative immunofluorescence has been evaluated in several applications to quantify and compare the amount of antigen expression between different tissues [13]. Thus, the experiment provides a validation for the use of WL as a measure for quantitative immunofluorescence which can be used in different experimental applications.

Methods

DIF was performed on histopathology sections of perilesional skin biopsies taken from patients previously diagnosed with bullous pemphigoid (n=10) and pemphigus vulgaris (n=6). DIF methods have been previously described in full detail [14]. In brief, tissue sections from each sample were incubated with dilutions of FITC-tagged antibodies targeting IgG (BioRad, Hamburg, Germany) and C3 (Biologo, Kiel, Germany). Each slide was immediately imaged using the BIOREVO (Keyence, Neu-Isenburg, Germany) system. Settings were kept the same throughout. Three photomicrographs at a magnification of 40 were taken, with a full pixel count of 1360 × 1024 for each dilution.

Images were subsequently processed using the ImageJ (version 1.48, National Institutes of Health, Bethesda, Maryland USA). Each image was inserted into the ImageJ software and a histogram specifically limited to green the green spectrum was generated. Data for each image were exported into the SPSS 20 software. Extracted data points from each histogram represent the number of pixels on the spectrum of green luminescence from 0 (black) to 255 (saturated green). We performed a data transformation to calculate the weighted luminescence. This was calculated by the summation of each color from 0 to 255 by *n/255* multiplied by the number of pixels at each color. Thus, black pixels would be filtered out, and the brightest level of luminescence would be weighed highest.

To determine the relationship of mean weighted luminescence as the dilution of each specimen increased, we generated histograms with logarithmic regression curves of weighted luminescence and anti-IgG and anti-C3 dilutions for bullous pemphigoid and pemphigus vulgaris patients. As signal noise is not dependent on the presence of antigen, but instead the immunoreactive portion of the DIF is antigen-dependent, two separate logarithmic regression analyses were performed. A logarithmic regression curve comparing weighted luminescence with dilutions of anti-IgG and anti-C3 ranging from 1:100 to 1:800 was performed which does not take into account loss of Fc receptor or C3 saturation. An additional logarithmic regression was performed on anti-IgG and anti-C3 dilutions ranging from 1:100 to 1:400, thus increasing the chance that the Fc receptor of C3 remained saturated. An ANOVA was performed to confirm the homogeneity of WL for multiple images taken from the same patient at various FITC-titers. A value of $P>0.05$ was considered a confirmation of homogeneity.

Results

Representative images of anti IgG and C3 in BP and PV are shown in figures 1 and 2 respectively. Logarithmic regression assessing the relationship between weighted luminescence for anti-IgG and anti-C3 dilutions demonstrated a significant and moderate to strong correlation, ($R^2=0.51$, $P<0.001$) and ($R^2=0.50$, $P<0.001$). Correction for antigen dependent immunofluorescence (measuring only at dilutions of 1:400 rather than 1:800) led to a decrease in the strength of the logarithmic relationship between weighted luminescence and anti-IgG and anti-C3, but the relationship remained statistically significant. Regression curves in pemphigus vulgaris demonstrated a less strong relationship between weighted immunofluorescence and anti-IgG and anti-C3 dilution as compared to those in bullous pemphigoid, but these still remained statistically significant. Like in bullous pemphigoid, the strength of the relationship between weighted luminescence and anti-IgG and anti-C3 decreased when accounting for antigen dependent immunofluorescence. These results are provided in table 1. The logarithmic regression curves for antigen-independent calculations (dilutions ranging from 1:100 to 1:800) are provided in figure 3. All ANOVA for comparing multiple specimens from the same patient were not significant ($P>0.05$), indicating homogeneity.

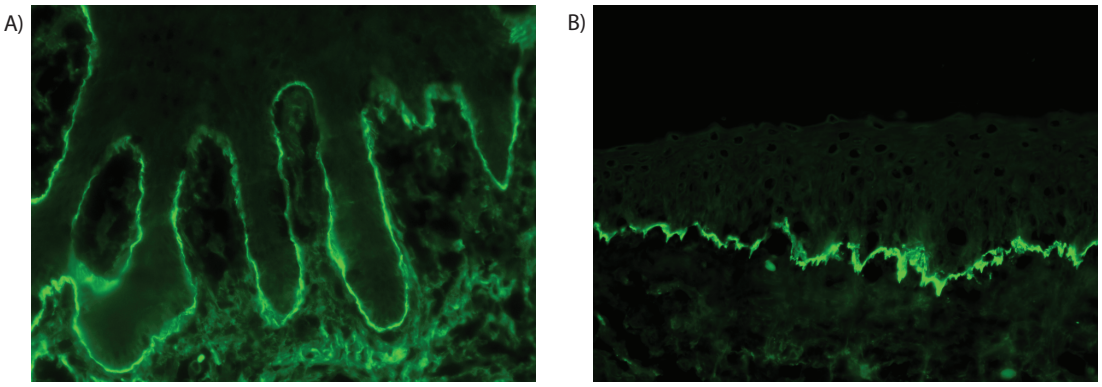

Figure 1: Direct immunofluorescence in bullous pemphigoid targeting **A)** IgG at a titer of 1:200 and **B)** C3at a titer of 1:200

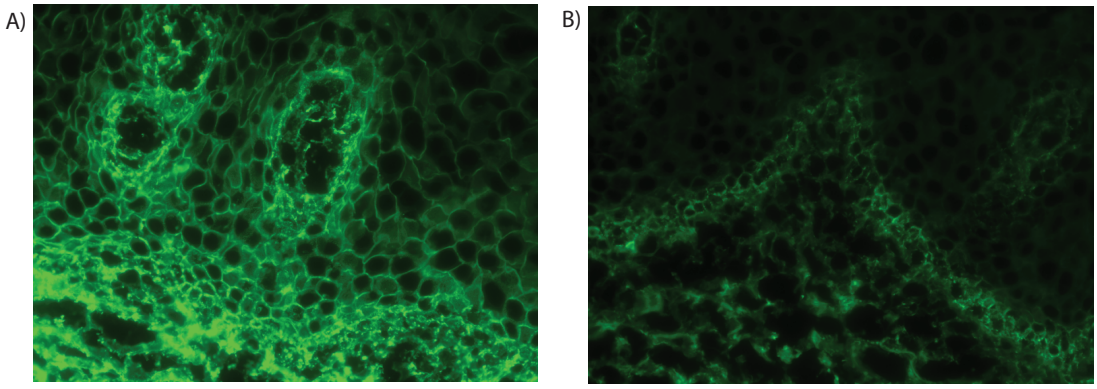

Figure 2: Direct immunofluorescence in pemphigus vulgaris targeting **A)** IgG at a titer of 1:200 and **B)** C3at a titer of 1:200

	Raw analysis Dilution of 1:100–1:800				Correction for antigen-dependent signal 1:100–1:400		
Bullous pemphigoid	Dilution	WL	R^2	P	Bullous pemphigoid	R^2	P
IgG	1:100	1029	0.51	<0.001	IgG	0.41	<0.001
	1:200	522					
	1:400	337					
	1:800	117					
C3	1:100	572	0.50	<0.001	C3	0.24	<0.001
	1:200	496					
	1:400	299					
	1:800	100					
Pemphigus vulgaris			R^2	P	Pemphigus vulgaris	R^2	P
IgG	1:100	1235	0.43	<0.001	IgG	0.20	0.043
	1:200	1030					
	1:400	667					
	1:800	284					
C3	1:100	699	0.32	<0.001	C3	0.25	0.005
	1:200	358					
	1:400	274					
	1:800	161					

Table 1: Logarithmic regression coefficients and P values for the relationship between weighted luminescence and the dilution of added anti-IgG and anti-C3 antibodies in direct immunofluorescence studies of patients with bullous pemphigoid and pemphigus vulgaris. Corrected analysis to account antigen-dependent signaling provided. (WL) weighted luminescence

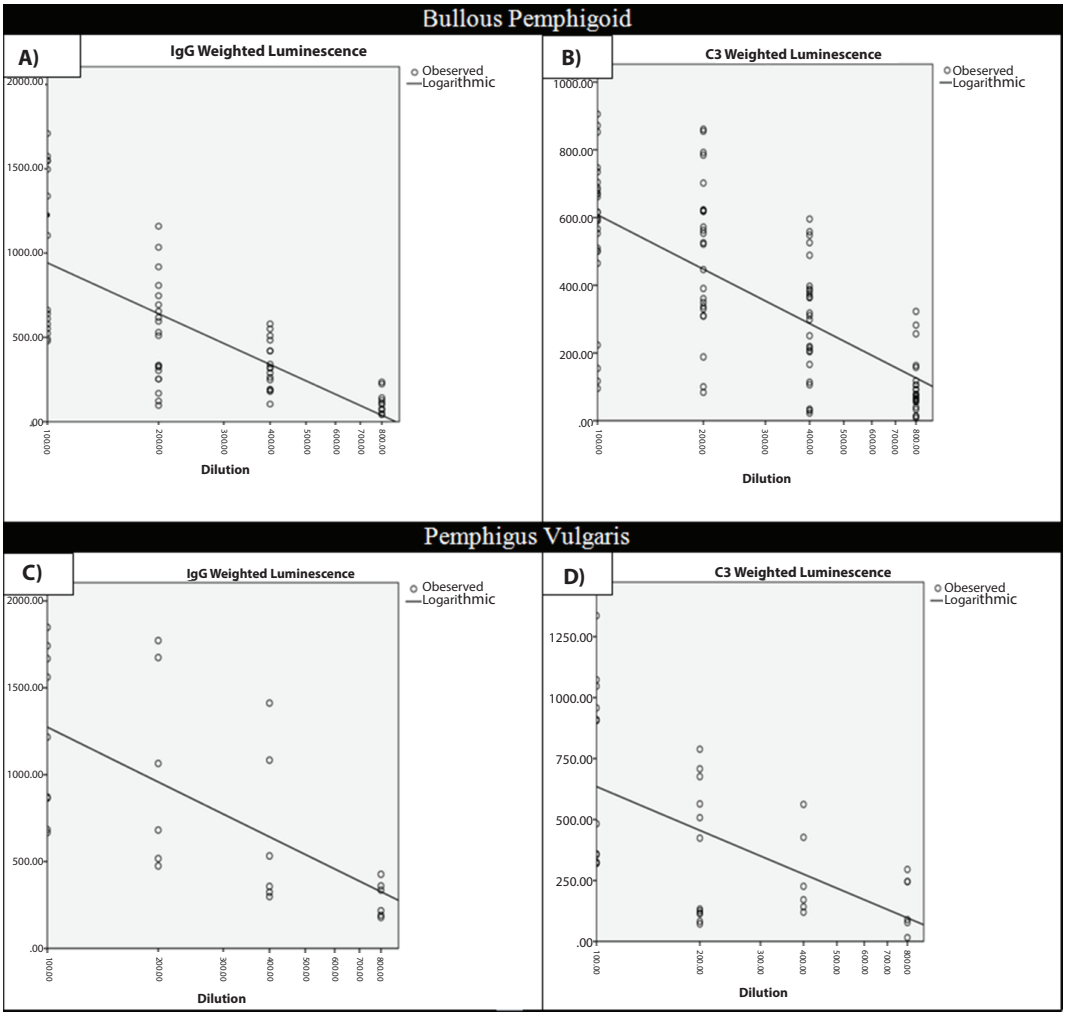

Figure 3: Weighted luminescence* with logarithmic regression line shown for direct immunofluorescent samples from patients with bullous pemphigoid (a,b) and pemphigus vulgaris (c,d) respectively with logarithmic transformation of horizontal axis.
**Weighted luminescence defined as Σ[pixel count x (n/255)], where n is the green color value on a scale of 0 to 255.*

Discussion

Weighted luminescence, calculated using the ImageJ software can serve as a helpful means of quantifying immunofluorescent signal from controlled photographs without necessitating expensive image analysis software or user set thresholds which may affect inter-observer reliability. Its accuracy requires the use of standardized camera settings and antibody preparations. Thus comparison can be made should the settings and antibody preparations be the same throughout. The use of DIF for creating a logarithmic regression model provides some advantages and disadvantages as compared to indirect immunofluorescence. As the majority of signal is antigen-independent due to signal noise, the logarithmic increase in dilutions predictably results in a logarithmic drop in signal noise. This is at the expense of containing an immunoreactive region of the image which is antigen dependent. This is best demonstrated by the decreased strength of relationship between weighed luminescence and antibody dilution seen in pemphigus compared to bullous pemphigoid. As IgG is deposited intercellularly in pemphigus vulgaris, there is relatively more antigen-dependent signal than in BP which is limited to the basement membrane zone. Thus, the assumption of a logarithmic drop in weighted luminescence with increasing dilutions would be dependent on the saturation of the target antigen (Fc receptor or C3) which wouldn't necessarily drop in a predictable manner as would the antigen-independent signal noise. The use of indirect immunofluorescence would limit this analysis as logarithmic decreases in signal would only begin following loss of saturation of the Fc receptor at a certain serum dilution. Thus, while DIF does not allow a pure analysis of antigen-independent signal changes at various dilutions of anti-IgG and anti-C3 antibodies, it provides an estimate. While further confirmatory analysis could be performed to the non immunoreactive areas of the DIF, this was avoided to prevent any changes in settings or selected areas which often plague the use of digital imaging software [7]. Thus, the performed study provides a conservative estimate of the relationship between weighted luminescence and the logarithmic drop in signal intensity.

Unfortunately DIF cannot be used to predict antibody titers, as it is related to the amount of antigen in the patient's skin in addition the quantity of anti-desmosomal IgG. However, by demonstrating that ImageJ can reliably measure fluorescence signal in a controlled setting (serial dilutions of the same specimens in this study), this technology can be applied to other applications where fluorescent signal is of clinical utility.

The use of imaging software in the analysis of immunofluorescence offers several promising features. Immediate photography of immunofluorescence images can allow for long-term cataloging, thus reducing the risk of loss of reactivity due to changing conditions and light which may affect luminescence at weak signals [15]. Likewise, should all other settings and laboratory conditions be controlled, quantitative and comparative immunofluorescence could be performed using weighted luminescence as a value for statistical comparison offering a free method of performing quantitative immunofluorescence for different experimental settings.

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Conflict of Interest

The authors have no potential conflicts of interest to disclose. The study did not receive funding.

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