An Automatic Image Based Single Dilution Method for End Point Titre Quantitation of Antinuclear Antibodies Tests using HEp-2 Cells

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***Abstract*—Indirect Immunofluorescence (IIF) on Human epithelial (HEp-2) cells test has been the golden standard for identifying the presence of Anti-Nuclear Antibodies (ANA) due to its high sensitivity and the large range of antigens that can be detected. Furthermore, IIF ANA test allows the positive sample strength (sample end point titre) to be reported. Despite its advantages, the IIF ANA test needs to be performed manually, and therefore it is perceived as an expensive and laborious process. This also applies to determining the strength of positive samples (end point titre) which traditionally is done by serially diluting the specimen using expensive reagent. In this paper, we present an image-based method which is able to automatically determine the end point titre of positive samples based only on a single screening dilution. This can be done by simulating the manual titration process using a mathematical model of the exposure-density curve. Technically, a new Image Titration Endpoint (ITE) unit based on the model is introduced. Each specimen image is then measured in terms of this unit. Finally, the end point titre for the specimen is determined through a standard curve which specifies the end point titre given an ITE unit. This process is fully automated which would give an advantage over the current digital titration methods. The overall endpoint titre agreement between the proposed approach and the manual serial dilution method in the evaluation of 134 positive samples was 100%. This high agreement demonstrates that the proposed approach is suitable for routine ANA IIF testing in the clinical settings.**

I. Introduction

The identification of Antinuclear Antibodies (ANA) in a human serum sample is very important in clinical medicine and immunology [2]. The presence of ANA is often associated to the existence of Connective Tissue Diseases (CTD) such as Systemic Lupus Erythematosus (SLE), Sjogren's syndrome, and Rheumatoid Arthritis. Although there are many methods can be used to identify ANA, the Indirect Immunofluorescence (IIF) on HEp-2 cells test has been the hallmark method for the detection of antinuclear antibodies (ANA) [11]. This is due to its high sensitivity and the large range of antigens can be detected by this method. Figure 1 depicts some patterns exhibiting different detected antigens. Another advantage of using this test is that, the positive sample titres, which indicates the sample strength, can be reported. This is very important as normal healthy people can demonstrate positive ANA with low end point titre. So, a high titre sample indicates that the person would likely to have such diseases.

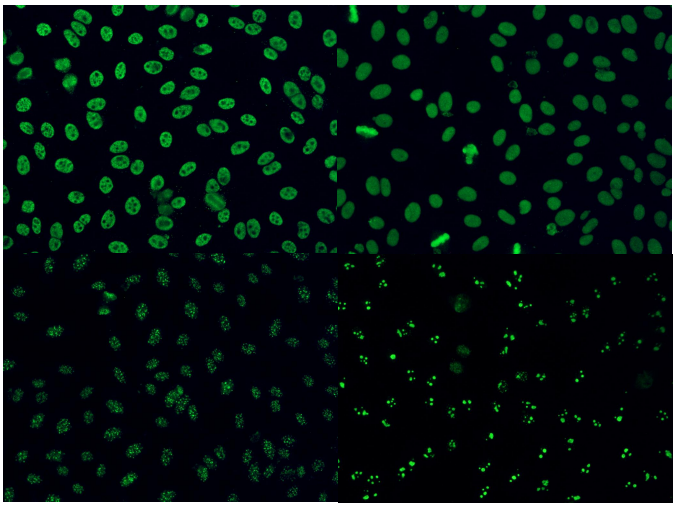

Fig. 1. Some patterns of strong positive ANA samples. First row: Speckled and Homogeneous; Second row: Centromere and Nucleolar.

Traditionally, positive sample titres are determined by serially diluting the samples using chemical reagent. However, this method is considered costly. Obviously it requires more chemical reagent and glass slides to create the titrated samples. Furthermore, more time is required for scientitsts to examine these under a microscope. So, the researchers posed the question as to whether this process can be made more economical and automated.

One of the initial attempts to address the posed question was done by Hollingsworth *et al.* [7]. The basic idea of their work is to use a set of standard samples with known end point titre and manually compare the fluoresence signal intensity of a sample being examined with this set. The end point titre of the sample is determined as the end point titre of the standard sample having similar fluoresence intensity. By this approach, the end point titre of the sample can be determined without serial dilution which requires large amounts of expensive reagents.

The Hollingsworth *et al.* work becomes the basic foundation of several single-dilution titration methods which have been reported in the literature [12], [3], [6]. Technically, the fluorescence signal from each sample is measured in terms of a new measurement unit. This unit quantifies how much simulated titration is required before the sample becomes negative. Based on this unit, a standard curve is constructed using a set of samples produced by serially diluting a high end point titre sample. Given the value of measurement unit, the corresponding end point titre can be determined using the standard curve. In short, the end point titre of a positive specimen is determined as follows: (1) Quantify the fluorescence signal in terms of the new measurement unit; (2) Use the constructed standard curve to determine the end point titre. Despite the similarity in these approaches, each approach has differences.

These differences can be categorised into two groups: (1) How the chemical titration is simulated; (2) How to detect whether the sample is positive or negative. Table I summarises these differences amongst the current approaches. PolyTitre uses a series of image processing techniques to simulate the chemical titration. Although this process is automated, it still requires human intervention to decide whether the digitally titrated sample is still positive or has been negative. This is because the image processing techniques used only provides a crude way of simulating the chemical titration. Different from ImageTitre, PolyTitre uses a specialised unit to quantify the fluorescence signal. A filter is fitted between the microscope and its light source. A control pad is connected to the filter, and is used to increase or decrease the amount of light illuminating the slide in a stepwise manner. A human operator will then count the number of decrements or increments until the sample becomes negative. Despite its good performance, this approach still requires human intervention. Our approach closely resemblances [5]. This approach assumes that the effect of camera exposure time is proportional to the sample end point titres. For example, in order to take a good image, a high titre sample requires shorter exposure time, while low titre sample requires longer exposure time. Although this method is fully automated, varying exposure time would then increase the scanning time because the system needs to take a sample image multiple times with various exposure times. In this paper, we prospose a tiration method which digitally simulates the effect of camera exposure time on images. This means, every sample image is only taken once with the same exposure time. Furthermore, unlike ImageTitre, this simulation uses a realistic camera model based on sensitometry [4]. Using this method, the processing time will be much faster than in [5].

The rest of this paper is organised as follows. Section 2 presents the proposed digital titration method. Empirical experiments and discussions are presented in Section 3. Finally, conclusions and future work are discussed in Section 4.

II. Proposed digital titration system

The proposed system simulates the chemical titration effects by simulating the effect of camera exposure time on an image. This can be done throught the proposed HD curve which models the effect of camera exposure time on photographic negative films. Once the titration simulation has been developed, each sample can be serially diluted by iterating the simulation process and this process stops when the sample becomes negative. The system counts the number of iterations required in order to make the sample becomes negative. We call this number as Image Titration End point unit (ITE unit). Figure 2 illustrates the process to determine the ITE unit of a sample image.

Assuming that there is relationship between the ITE unit and the sample end point titre, a standard curve Φ can constructed for each batch of samples. The standard curve Φ is defined as a function $\Phi : \Theta \rightarrow ITE$, where Θ is end point titre. The system uses samples produced by serially diluting a very strong positive sample to construct the standard curve. Then, the ITE unit for each dilution level is used to construct the curve. Once this has been done, given an ITE unit of a sample with unknown end point titre, its end point titre can be determined using the standard curve.

In this section, the discussion begins with how the ITE unit is calculated, and a sample end point titre calculation discussion follows after that.

A. Simulating exposure time by using exposure-density curve model

Back in 1876, Hurter and Driffield studied the relationship between the density of silver produced in negative film and the amount of exposure time [8]. This relationship can be depicted as a characteristic curve which overally has an "S" slanted shape (refer to Figure 3). The curve, usually called as "HD curve", is characterised by a parameter γ which is the slope of the curve's linear part.

As each negative film usually has its own HD curve, such a curve can be constructed by exposing the negative film with different exposure time. In this work, this curve will be modeled mathematically.

Bhukhanwala and Ramabadran [1] used a logistic model to mimic this curve. Their goal was to simulate the impact pixel intensity value on the exposure time. This model was then used by Li et al [9] to manipulate images in terms of their exposure time. One of the shortcomings in this model is that the curve codomain which is the pixel intensities is expressed in linear manner.

Similarly to Bhukhanwala and Ramabadran, Geigel [4] used the HD curve of which the information is adapted from the

TABLE I
COMPARISONS BETWEEN THE CURRENT AND PROPOSED METHODS

Approaches	Chemical titration simulation process	Samples positive-negative detection method
ImageTitre [12]	Image processing (Software-Automatic)	Manual
PolyTitre [3]	Light Intensity (Hardware-Manual)	Manual
Patent DE2008001894 [6]	Camera (Hardware-Automatic)	Automatic
Proposed Method	Image processing (Software-Automatic)	Automatic

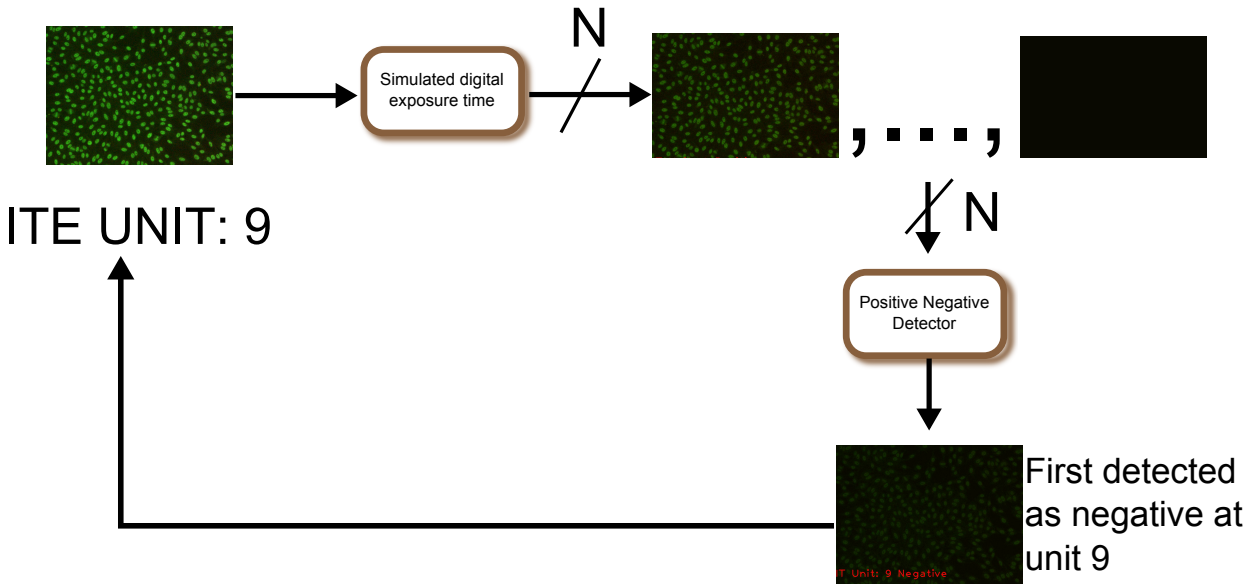

Fig. 2. The system diagram for calculating ITE unit of a positive sample. N: the number of stop point which will be discussed in Section II-B

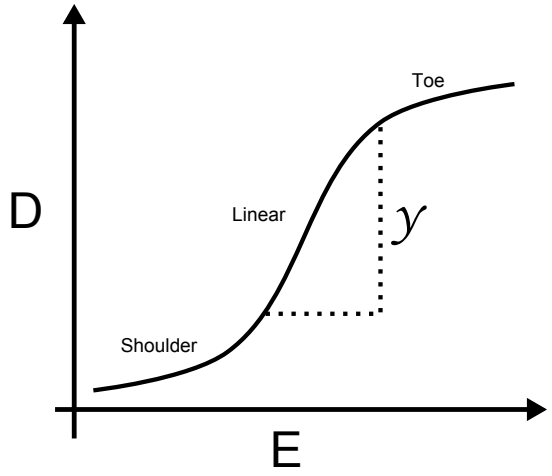

Fig. 3. An illustration of an HD curve. The curve normaly has three parts: shoulder, linear and toe. D: density; E: exposure time. These axes are represented on a logarithmic scale.

real photographic hardware, in order to simulate the effect of exposure time on images. By using this information, he was then able to develop a virtual darkroom which digitally simulates the photographic process. Unlike Bhukanwala and Ramabadran, both axes in the HD curve were assumed to be non-linear.

Inspired by these works, we propose an HD curve model which combines both Geigel and Bhukhanwala and Ramabadran approaches. The proposed model is based on Bhukhanwala and Ramabadran and it assumes non-linearity on both of the curve axes. The proposed HD curve is then presented as follows.

$$D = \frac{D_{max}}{1 + e^{-\gamma x}} \tag{1}$$

where D is density; D_{max} is the maximum density value; γ is the curve constant; and x is $log_{10}(E)$ in which E is defined as exposure.

The exposure of an image is defined as follows [4].

$$E = It \tag{2}$$

where E is exposure (flux-seconds); I is irradiance (flux); t is exposure time (seconds)

Let E_1 be the exposure of time t_1 and E_2 be the exposure of t_2. Assuming that I_2 is the same as I_1 then E_2 can be expressed as the following.

$$E_2 = E_1 \frac{t2}{t1} \tag{3}$$

From Equation 1, E_1 can be calculated:

$$E_1 = 10^{-\frac{1}{\gamma}(\frac{D_{max}}{D_1} - 1)} \tag{4}$$

Finally, D_2 can be determined as follows.

$$\begin{aligned} D_2 &= \frac{D_{max}}{1 + e^{-\gamma x_2}} \\ &= \frac{D_{max}}{1 + e^{-\gamma log_{10}(E_2)}} \\ &= \frac{D_{max}}{1 + e^{-\gamma log_{10}(E_1 \frac{t2}{t1})}} \end{aligned}$$

From the above equation and Equation 4, one can calculate D_2 if one knows D_1, t_1 and t_2. The above derivation is similar to [9] with pixel intensities being replaced by density.

According to Geigel [4], density can be calculated using reflective density equation.

$$D_R = log_{10}(\frac{1}{R}) \tag{5}$$

where R is the fraction of incoming flux (I_i) and the reflected flux (I_r) (i.e. $\frac{I_r}{I_i}$). From this equation, the range for R is [0,1]. If we assume that R is the pixel values of the negative image then R can be calculated from the original image as follows.

$$R(x, y) = 1 - \frac{I(x, y)}{255} \tag{6}$$

where, $R(x, y) \in [0, 1]$, is the reflectivity of the negative image at location (x,y); $I(x, y) \in [0, 255]$, is the grey value of positive image at location (x,y).

By using equations 5 and 6, D_1 can be calculated from a given sample image.

B. Image Titration End point (ITE) Unit

As depicted in Figure 2, the ITE is defined as the number of times the exposure time is decreased in order to make the positive sample becomes negative. The rate of exposure time decrement is expressed in terms of stop points. The exposure time at every stop point halves from the previous one.

In this work, we use 24 stop points. This means, the ITE unit value ranges from 1 to 24.

C. Standard curve construction

The standard curve can be created using a very strong positive sample serially diluted 2 or 4-fold. After that, the system calculates the ITE unit of each image at different dilutions. By using a curve fitting method, these ITE units are used to construct the standard curve. Since from our observations, the curve is non-linear, we use logarithmic function as the standard curve which can be expressed as follows.

$$\Phi_{ITE}(\Theta) = \alpha ln(\beta\Theta) \tag{7}$$

where Θ is the titration level; α and β are the constants; Φ_{ITE} is a function $\Phi : \Theta \rightarrow ITE$.

D. Positive-negative detector

As suggested by its name, the positive-negative detector decides whether or not the given sample is positive. The decision can be made based on various image features. In this work, we simply use the global features such as the image greylevel mean and standard deviation. Despite these features simplicity, the promising overall results presented by the proposed approach justifies that these features are sufficient for this application.

III. Experiments & Discussions

First we were interested to see how the proposed HD curve mathematical model affects individual greylevel value at different stop points. To do that, an image which has values from 1 to 254 (left to right) depicted in Figure 4 was used. The exposure time was varied by using twenty four stop points. Figure 5 depicts the result. The first linear line represents the plot of greylevel values at the first stop point. At this stop point, T_1 is equal to T_2, hence there was no change.

As we can see from the figure the non-linearity becomes more apparent as shorter exposure time is used (i.e. $T_2 << T_1$). In addition, each individual greylevel value has different response for each stop point. The biggest influence can be observed at the middle range of the greylevel values, and the influence becomes less and finally negligible at both lowest and highest values. This behaviour follows the HD curve characteristic which has three parts: shoulder, linear and toe.

Fig. 4. The image used in the first experiment. This image was constructed using a row vector containing grey level values [1..254].

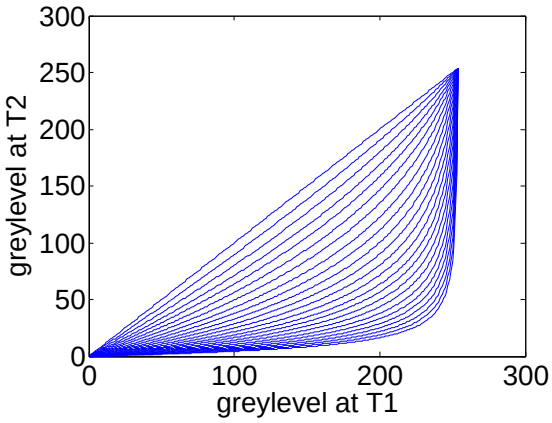

Fig. 5. The effect of the proposed HD curve model on image in Figure 4. The linear line is when $T_1 = T_2$. The line bends down as T_2 is set shorter than T_1.

Next, we were interested in observing the proposed approach accuracy. Since, there were no benchmark datasets

available, 134 positive samples with various end point titres were collected between August 2010 and September 2010 at the Sullivan Nicolaides Pathology Laboratory, Taringga, Australia. The images were taken using an Olympus Microscope BX41 with Color View II camera. The samples consist of 47 Homogeneous, 46 Speckled, 16 Nucleolar, 8 Centromere, 1 Cell Cycle Dependent (CCD), and 16 mixed patterns. As for the truth label, the end point titres were determined using a manual method with error tolerance $\pm$ 4-fold. According to the manual method 69 samples were determined as low end point titres and 65 were high end point titres.

As we did not have access to the commercial products such as PolyTitre or ImageTitre, a simple 2 dilution (2D) approach was used as the baseline method. Unlike the proposed approach, the two dilution approach constructs the pattern extinction curve by using average fluoresence intensities of images taken from two dilution levels. In this case, each sample was diluted at 1:40 and 1:320. The images were taken for each dilution, and the image intensities were averaged. A simple regression calculation was applied to construct the curve. Finally, the end point titre of the sample was determined by applying a simple thresholding method on the exticntion curve. We used the 2D approach as the baseline method because the 2D approach should have a reasonably good performance as it considers information extracted from two dilution points.

The proposed approach was applied on two screening dilutions, 1:40 (SD1:40) and 1:80 (SD1:80). The purpose of this was to see whether the present of the prozone effect [10] affected the overal proposed approach performance.

A. Standard curve

A strong positive sample was chosen from the dataset to construct the standard curve used in the experiment. The sample was serially diluted 4-fold from 1:40 to 1:2560 (i.e, 1:40, 1:160, 1:640 and 1:2560). Figure 6 depicts the constructed standard curve plot.

Table II presents the experiment result. Generally, the SD1:80 approach outperforms the other two approaches. However, 2D performs best if the error tolerance is set to zero. This superior performance goes down as the error tolerance is widened. From our observation this is because the 2D approach relies on whether the extinction curve can be constructed correctly. The method tends to have more error when the curve is not constructed correctly due to low image quality (i.e. the presence of artifacts, air bubbles and blurring caused by out of focus imaging). Moreover, as SD1:80 performs better than SD1:40, this suggests that the existence of the prozone effect [10] affects the proposed approach performance.

Figure 7 depicts the titre agreements between the results taken from the manual method and SD1:80 titre results. There are no positive samples which are determined to be more than 4-fold dilutions apart from the truth label. This means, it is safe to say that the proposed approach with screening dilution 1:80 has a comparable performance with the manual method which assumes 4-fold dilutions error.

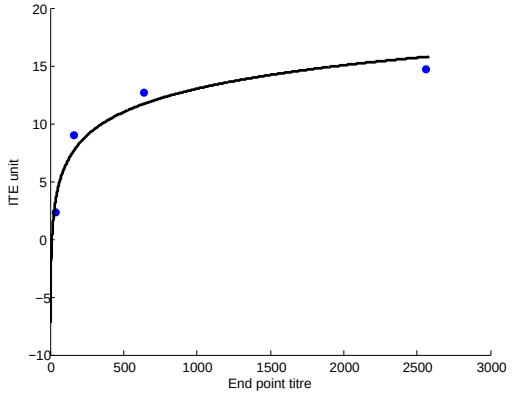

Fig. 6. The standard curve used in the experiments. The curve was constructed using only four points in which each point represents the ITE unit of the selected sample at a particular dilution level. The standard curve equation is: $\Phi_{ITE} = 2.94ln(0.084\Theta)$

TABLE II
COMPARISON RESULTS. SD1:40:PROPOSED METHOD WITH 1:40 SCREENING DILUTION; SD1:80: PROPOSED METHOD WITH 1:80 SCREENING DILUTION; 2D: 2 DILUTION METHOD (BASELINE); EQUAL: PERCENTAGE OF SAMPLES WHICH EQUAL TO THE TRUTH LABEL; 2-FOLD & 4-FOLD: PERCENTAGE OF SAMPLES WHICH FALL WITHIN $\pm$ 2-FOLD AND 4-FOLD DILUTIONS FROM THE TRUTH LABEL, RESPECTIVELY. SPEARMAN CORRELATION SHOWS THE TITRATION RESULTS AGREEMENT WITH THE MANUAL METHOD.

Approach	Equal	2-fold	4-fold	Spearman correlation
SD1:40	28.36	70.90	91.05	0.7937
SD1:80	21.64	**79.85**	**100**	**0.8595**
2D	**36.84**	74.43	96.24	0.8441

TABLE III
PERFORMANCE OF SD1:80 BASED ON SAMPLE STRENGTH. TENDENCY IS CALCULATED USING TWO TAILED BINOMIAL HYPOTHESIS TEST.

Titre level	Equal	2-fold	4-fold	Tendency
Low titres ($<$ 1:640)	14.50	**88.41**	100	Higher
High titres ($\geq$ 1:640)	**29.23**	70.77	100	Lower

A further investigation on SD1:80 reveals that the method performs better on high end point titre samples when there is no error. However, when error is presence, the method tends to have a better performance on low end point titre samples. As depicted in Table III, all samples are correctly determined with the maximum error tolerance of $\pm$ 4-fold. In addition, when there is error, low end point titre samples tend to be determined higher titre than what they should be, and vice versa. This can be seen from Figure 7 and confirmed by two tailed binomial statistical tests.

IV. CONCLUSIONS & FUTURE WORK

IIF method on HEp-2 cells test has been the hallmark in ANA tests due to its sensitivity and the large range of

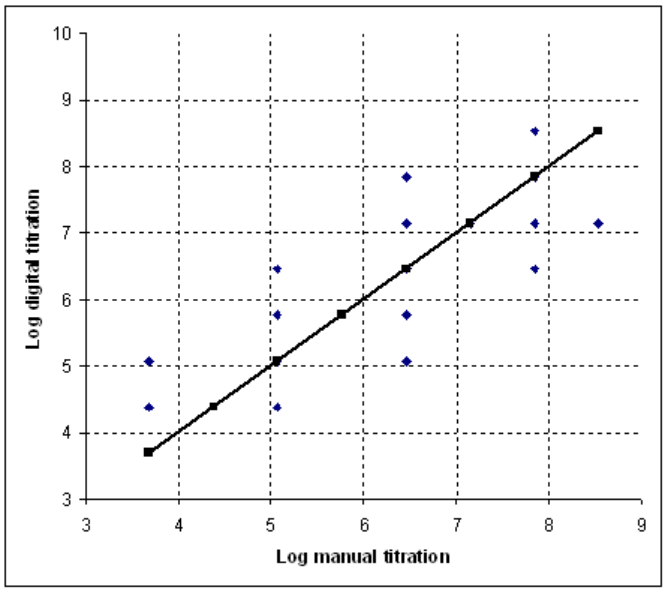

Fig. 7. Comparison between the manual method and the SD1:80 results.

antigens that can be expressed. One of the shortcomings in this method is that it requires human subjectivity to interpret the test results. Furthermore, much reagent is required in order to determine the positive samples strength. The method presented in this work is specifically tailored to determine the strength (i.e. end point titre) of positive samples using only a single dilution. Unlike the other single dilution methods, the proposed approach offers two main advantages: (1) Requires much less human intervention; (2) Has a more realistic digital photography simulation.

This work has two main contributions in immunofluorescene area: (1) A mathematical model of exposure-density curve which is suitable for a digital titration purpose; (2) The digital titration method which can be described as follows: First, each sample is serially diluted by iterating the digital titration simulation until the sample becomes negative. The number of iterations required in order to make sample becomes negative is defined as Image Titration Endpoint (ITE) unit. After that, a standard curve which gives the corresponding ITE unit from a given end point titre is constructed from a very high end point titre sample serially diluted 4-fold. Finally, the system determines the end point of each positive sample by first determining the sample ITE unit and use the standard curve to get the corresponding end point titre.

As shown in the presented experiments, the proposed digital titration method had a very good agreement with the manual method. The proposed method achieved 100% agreement with the manual method when the required error tolerance was assumed to be $\pm$ 4-fold dilutions. This promising result would then make it possible to use the proposed method for routine clinical IIF ANA tests. The next step for this work will be to adapt and apply the proposed method to other immunofluorescence tests such as Micro Immunofluorescence (MIF) for Chlamydia and IIF for Anti Neutrophil Cytoplasmic Antibodies (ANCA) tests.

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