

A Method to Extract Spots from the image of the ELISA (Enzyme-Linked Immunosorbent Assay) Spot Assay

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Abstract- ELISA (Enzyme-Linked Immunosorbent Assay) Spot Assay is a method widely used by immunologists to enumerate cytokine-producing cells within a specific cell population. The result of ELISA is presented in an image containing numerous color spots. We present a method to extract and to count the number of spots. The proposed method is based on color analysis. Since CIE $L^*u^*v^*$ space have linear perceptibility of color differences, we convert the RGB space to $L^*u^*v^*$ space. The system is trained to obtain the standard color of the spots and get the color difference image in $L^*u^*v^*$ space. According to the feature of the spots we design a special matched filters to filter out the noise and enhance the spots. Finally a binary image is obtained. In the binary images, pixels in the spots have gray scale 255 and the others are 0. Our design makes it easy to analyze the perimeter and size of the spots in addition to counting them in the binary image.

Keywords: ELISA, Spot, Segmentation, CIE.

I. INTRODUCTION

ELISA Spot Assay is designed to detect cells that produce cytokines [1]. Cytokines are proteins readily secreted by immune cells upon stimulation by antigens they recognize. Test wells are coated with anti-cytokine antibody before cells were added. A certain number of cells and antigen are added to the precoated wells. During incubation, cells are stimulated to secrete cytokine. The secreted cytokine is captured by the precoated antibody. After washing, an enzyme-conjugated secondary anti-cytokine antibody and the substrate are added in sequence. A color reaction (red in this case) specific to cytokine-secreting cells occurs as a result of enzymatic reaction. Each red spot represents one cytokine-secreting cell. Since there can be hundreds of spots in a well of 70 mm diameter, counting the spots is labor intensive even one uses a dissecting microscope. The goal of this work is to produce a binary image containing pixels only in the spots.

The first step in the presented method is color space conversion. It linearizes the perceptibility of color differences and provides a uniform color space. The second step is to train the system by using the color of the spots to obtain an image of color differentials. The third step is to apply the matched filter to identify the spots and remove the undesired noise. Finally, the resulted image in step three is thresholded according to the histogram and gets the binary image containing only pixels in spots.

In the next section, we describe these steps. The results are shown in Section 3.

II. METHOD

1. Color Space Conversion

The spot colors provide good information for extracting the area of the spots. There were used to be over 40 color difference formulas before that CIE (Commission

Internationale de l'Eclairage) recommended two standard color difference formulas: the CIE $L^*a^*b^*$ and the CIE $L^*u^*v^*$ for surface and lighting industries [2]. They attempted to linearize the perceptibility of color differences. They provided a uniform color space. We have chosen the $L^*u^*v^*$ space [3]. While video cameras use RGB representation for colors. We must convert the representation from RGB to $L^*u^*v^*$. The CIE also has recommended the use of other color-coordinate systems, derived from CIE XYZ. We convert the representation from RGB to XYZ space using Eq. (1).

$$\begin{bmatrix} X \\ Y \\ Z \end{bmatrix} = \begin{bmatrix} 0.412453 & 0.357580 & 0.180423 \\ 0.412453 & 0.357580 & 0.180423 \\ 0.019334 & 0.119193 & 0.950227 \end{bmatrix} \begin{bmatrix} R \\ G \\ B \end{bmatrix}. \quad (1)$$

$L^*u^*v^*$ is based directly on CIE XYZ. The non-linear relations for L^* , u^* , and v^* are given below:

$$L^* = \begin{cases} 116\left(\frac{Y}{Y_n}\right)^{1/3} - 16 & \text{if } \frac{Y}{Y_n} > 0.008856 \\ 903.3\left(\frac{Y}{Y_n}\right) - 16 & \text{if } \frac{Y}{Y_n} \leq 0.008856 \end{cases}, \quad (3)$$

$$u^* = 13 \cdot L^* (u' - u'_n),$$

$$v^* = 13 \cdot L^* (v' - v'_n),$$

$$u' = \frac{4X}{(X + 15Y + 3Z)}, \quad (4)$$

$$v' = \frac{9Y}{(X + 15Y + 3Z)}$$

where u'_n and v'_n refer to the reference white X_n , Y_n and Z_n . The daylight standard D_{65} was used as reference illuminant. The non-linear relationship for Y is intended to mimic the logarithmic response of the eye. Now we have converted the spot image in RGB space denoted as $f(x, y)_{RGB}$ to $L^*u^*v^*$ space denoted as $f(x, y)_{L^*u^*v^*}$.

2. Train the system and obtain an image of color differentials
To train the system to recognize the standard color of spots, we use a user interface method to select the spot area denoted as $A(x, y)$. We used all pixels in the spot area to obtain the standard color denoted as $(\mathbf{m}_L, \mathbf{m}_u, \mathbf{m}_v)$. We calculate the average for $A(x, y)$.

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$$\begin{aligned} \mathbf{m}_L^* &= (\sum A(x, y)_L^*) / N \\ \mathbf{m}_u^* &= (\sum A(x, y)_u^*) / N, \\ \mathbf{m}_v^* &= (\sum A(x, y)_v^*) / N \end{aligned} \quad (5)$$

where N is the number of pixels in the spot area. The only user interface required is to obtain the standard spot color $(\mathbf{m}_L^*, \mathbf{m}_u^*, \mathbf{m}_v^*)$ in this step. No more other user activities are needed in the following steps. The difference between two measured colors can be expressed using the CIE color difference formula.

$$\Delta E_{uv}^* = ((\Delta L^*)^2 + (\Delta u^*)^2 + (\Delta v^*)^2)^{0.5}. \quad (6)$$

We substitute $f(x, y)_{L^*u^*v^*}$ and $(\mathbf{m}_L^*, \mathbf{m}_u^*, \mathbf{m}_v^*)$ to Eq. (6), then we can obtain the color difference image, $\Delta f(x, y)$, which is the difference between each pixel of $f(x, y)_{L^*u^*v^*}$ and the average spot pixel $(\mathbf{m}_L^*, \mathbf{m}_u^*, \mathbf{m}_v^*)$.

3. Designing the Matched Filter

The spots have an approximate shape of circle. In fact, the intensity in a spot almost follows a 2-D Gaussian function as Eq. (7) in the following

$$g_1(x, y) = e^{-(x^2+y^2)} = e^{-r^2}. \quad (7)$$

In Eq. (7) r is the radial distance measured from the center of the spot. If we define R as the radius at which the intensity drops to one-half of its maximum value, we can write the spot profile function as

$$g_1(x, y) = e^{-(r/R)\ln(2)} = e^{\ln(2-2r^2/R^2)}. \quad (8)$$

We can simplify Eq. (8) as

$$g_1(x, y) = 2^{-(x^2+y^2)/R^2}. \quad (9)$$

Now let us consider the detection of an arbitrary signal $s(t)$ in additive Gaussian white noise. If the signal is passed through a filter with transform function $H(f)$, the output signal $s_o(t)$ is given by

$$s_o(t) = \int H(f)\{S(f) + \mathbf{h}(f)\}e^{(j2\pi ft)df}, \quad (10)$$

where $S(f)$ is the Fourier transform of $s(x, y)$, and $\mathbf{h}(f)$ is the noise spectrum. By Schwartz's inequality, it can be proved that the filter $H(f)$, that maximizes the output SNR (Signal to Noise Ratio), is given by $Ho(f) = S^*(f)$ [4,5]. We apply inverse Fourier transform for both $Ho(f)$ and $S^*(f)$. Since the input signal $s(t)$ is a real value, $ho(t) = s(-t)$. Hence, in a 2-D case $ho(x, y) = s(-x, -y)$. The optimal filter must have the same shape as the intensity profile. In other words, the optimal filter is given by

$$h(x, y) = s(-x, -y). \quad (11)$$

We substitute Eq. (9) for Eq. (11)

$$h(x, y) = g_1(-x, -y) = g_1(x, y), \quad (12)$$

where $g_1(x, y)$ is a symmetry function, thus $g_1(x, y) = g_1(-x, -y)$. This optimal filter with the impulse response $h(x, y)$ is commonly known as the matched filter[6,7]. To obtain a zero mean filter, $g_1(x, y)$ is subtracted by the mean of the filter.

$$g(x, y) = g_1(x, y) - m = 2^{-(x^2+y^2)/R^2} - m, \quad (13)$$

where m is the mean of the filter.

The radius sizes of spots are ranged between 4 and 16 pixels. We only use pixel sizes 4, 8, 12 and 16 as the radius for designing the kernel of the matched filters. Using more matched filter kernels could get more accurate results, but also takes more computing time. Fig. 1 shows two of the 4 kernels of matched filters. After applying matched filter, Most of noises could be removed and thus the spots were enhanced. It is useful for the following step.

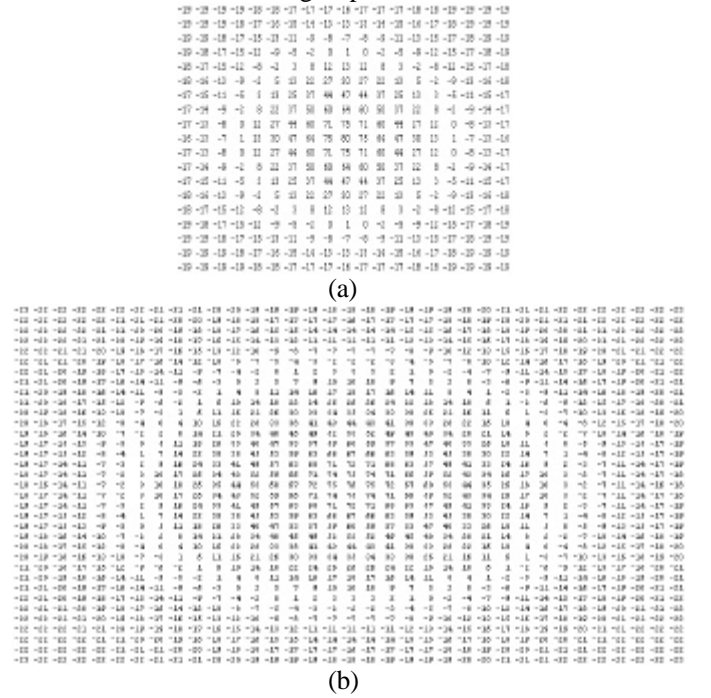


Fig. 1. 2-D matched filters used in our proposed method. The radius size is (a) 4, and (b) 8.

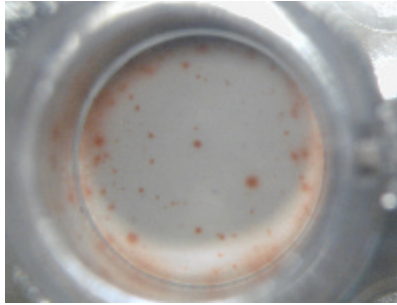
4. Obtain the Binary Image

Finally, we threshold the resulted images based on the histogram analysis to get binary images. Now we can easily to count the number of spots, size of spots, and measure perimeter

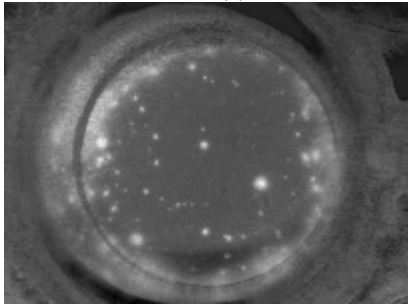
III. RESULTS

In this section, several images were tested and the results obtained by the proposed method are presented. The input images are color images in Jpeg format of size 1600 by 1200 pixels (Fig. 2-x(a), where x means 1, 2, and 3). Fig. 2-x(b) shows the distance measure. The results after matched filtering were applied are shown in Fig. 2-x(c). The results after threshold are shown in Fig. 2-x(d). The borders of spots Fig. 2-x(d) are overlaid to the initial image are shown in Fig. 2-x(e). The proposed methods were implemented on a PC with a Pentium III (800 MHz) CPU running on Windows 2000 operating system. The overall execution time for a 1600x1200 pixels image took less than 5 minutes. The radius sizes used for filter kernel were 1,2,3 and 4, respectively. It took a quit long time for a 1600x1200 image. Thus we reduce the resolution from 1600x1200 to 800x600 and 400x300 for those images, respectively. The 800x600 images took about 25 seconds. The radius sizes used for filter kernel

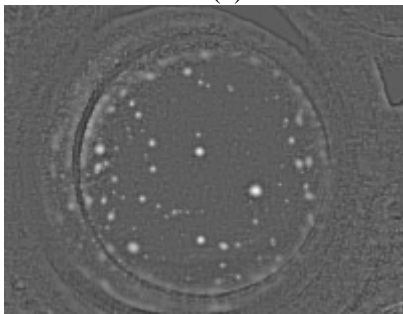
are 2,4,6 and 8, respectively. The execution time for 800x600 images is much faster than the previous case. In the case of 400x300 images, it took about only 3 seconds. The radius sizes used for filter kernel are 1,2,3 and 4, respectively. The results of 1600x1200, 800x600, and 400x300 have almost the same quality, but the execution time is significantly different. Because the larger images the larger the radius sizes are needed for matched filter kernels. Since the operations of 2-D convolution need large computation time, we suggest using 400x300 or 800x600 pixel images instead of 1600x1200.



2-1. (a)



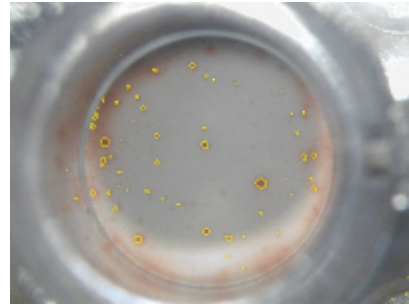
2-1. (b)



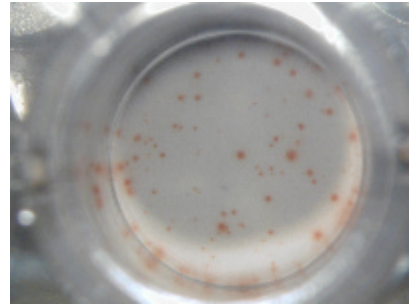
2-1. (c)



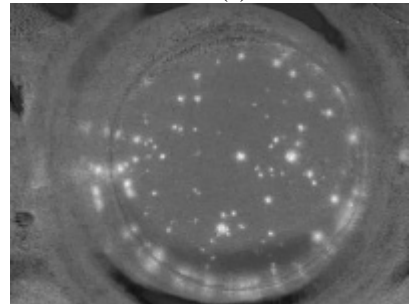
2-1. (d)



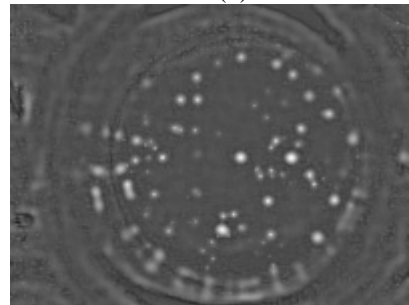
2-1. (e)



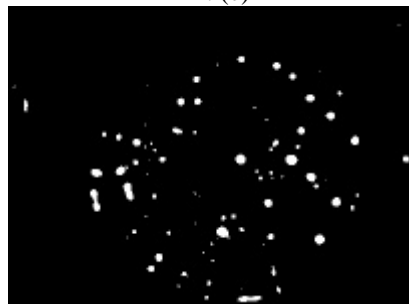
2-2. (a)



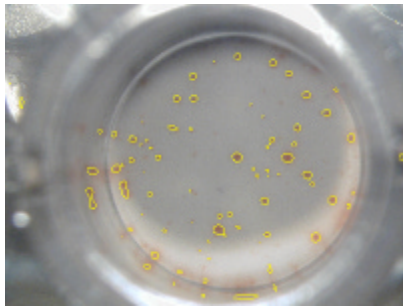
2-2. (b)



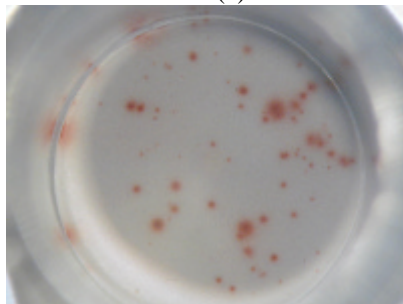
2-2. (c)



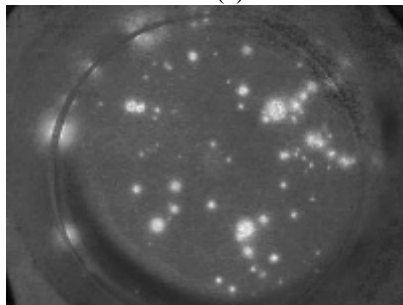
2-2. (d)



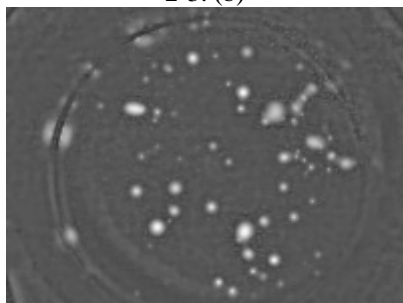
2-2. (e)



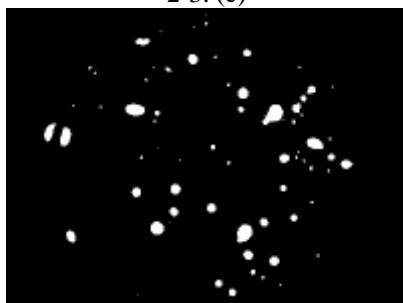
2-3. (a)



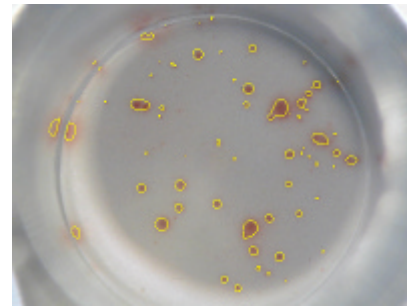
2-3. (b)



2-3. (c)



2-3. (d)



2-3 (e)

Fig. 2. 2-x(a) are the tested images for proposed method. 2-x(b) are the color distance images of 2-x(a). 2-x(c) are the results after matched filters. 2-x(d) are the final results. 2-x(e) are the final results overlying the border of 2-x(d)

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