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APPLICATIONS OF CONTOURS AND PIXEL VALUE SUMMATION IN DETERMINING THREE-DIMENSIONAL SIGNAL INTENSITIES

Anoop Kishore [1,*], Paka Sravan Kumar [2,*], Kodi Triveni [3], Adarsh Gopinathan [4] Nandakumar K [5], Divya Shenoy Purushothama [6,§]

[1,2,3,4,5] Department of Pharmacology

Manipal College of Pharmaceutical Sciences

Manipal Academy of Higher Education

Manipal - 576104, INDIA

[6] Department of Mathematics

Manipal Institute of Technology

Manipal Academy of Higher Education

Manipal - 576104, INDIA

e-mails: [1,*] anoop.kishore@manipal.edu

[2,*] paka.mcopsmpl2022@learner.manipal.edu

[3] kodi.triveni@learner.manipal.edu

[4] adarsh.gopinathan@learner.manipal.edu

[5] nandakumar.k@manipal.edu

[6,§] divya.shenoy@manipal.edu

(∗ these authors contribute equally, § corresponding author)

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Abstract

Contours are two-dimensional plots of three-dimensional data. Contour lines and contour maps are useful mathematical tools for topographic mapping, geology, architecture, weather forecasting and several biological applications. In this paper their application has been extended to determining protein densities from biological samples. The proteins resolved using Western blot technique were subjected to contour-based band boundary determination combined with pixel-value based band density estimation. Additionally, the accuracy of this method has been evaluated by comparing it with the traditional densitometric analysis methods that use rolling disk algorithm that is used for estimating band density and background noise subtraction. It is demonstrated that the contour and pixel value technique is a versatile method for determining signals intensities from biological specimen.

Math. Subject Classification: 54H30, 65D18, 68U10

Key Words and Phrases: contours, 3D-signals, protein density, pixel intensity

1. Introduction

Contour plot is a graphic technique useful for shape analysis and visualizing three-dimensional data in a two-dimensional plot. It is represented using a series of contour lines. The contour lines provide a map of the constant elevation. A contour can be defined by the function

$$f(x, y) = z,$$

where $f(x, y)$ is a set of all points at which the function f takes on the constant value z, [14]. If we plot a graph of the function, it shows all the points that have the height z. A contour line connects a constant z-value on a grid of x and y coordinates. In computing terms, a contour is a line drawn by joining all the points along the boundary of a shape that has the same color intensity.

Contours and contour maps are useful in shape analysis and find applications in several areas including topographic mapping [18], geology, hydrological and ecological applications [11], agriculture [8], mining [1], architecture [16], weather forecasting [4], etc.

The mathematical principles of contour mapping also find several applications in biology including boundary tracking of structures, topological analysis on pathological tissues [2], tracking of cells and cellular components [10], image segmentation [7], drug docking studies, [15], quantification of biomolecules like proteins [19], etc.

For quantification of proteins in biological specimens, a technique known as Western blot is employed. It involves loading proteins on a gel and applying an electric potential to resolve them based on their size and molecular weight. This is known as gel electrophoresis. The separated proteins are then transferred (blotted) to a nitrocellulose or polyvinylidene fluoride membrane. The proteins transferred to the membrane or blots are then detected using specific antibodies that bind to the proteins, [12]. The most common means of quantifying the protein levels involve radioactivity-based or chemical reaction-based methods that generate a signal (radio activity or color) that is proportional to the protein band intensity (Figure 1A), [3]. The critical component of this process involves subtraction of the background signal (noise) levels from the protein band signal using a process known as rolling disk algorithm [20]. It can also be achieved by manual subtraction of background noise.

In this paper we demonstrate an alternative method which involves contour-based determination of protein band boundaries to eliminate background signals and pixel value summation within these boundaries as an estimate of protein band density. Here we used the Python language and the OpenCV library (Open Source Computer Vision Library) to process the blot images and band density computations. This process is versatile, eliminates the need for manual intervention for determining background noise in the signals and can be automated for high throughput processes.

2. **Methods**

2.1. **Visualizing protein bands using Western blot.** The experiments were performed on commercially procured L6 cells (myoblasts obtained from the skeletal muscles of rat) to visualize the protein - AMP-activated protein kinase (AMPK, molecular weight 63 kDa). Bands of AMPK were obtained on a polyvinylidene difluoride (PVDF) membrane using Western Blot by standard procedure, [13]. Briefly, L6 cells were cultured in a cell-culture flask. Upon reaching confluency, the cells were collected, lysed and resolved in SDS-polyacrylamide gel and transferred

to a PVDF membrane. The membrane was incubated with primary antibodies of AMPK, followed by a secondary antibody. The proteins were visualized using IgG–horseradish peroxidase conjugate. The visualized protein bands were captured as an image using a calibrated densitometer (Figure 1A). Three blots of the protein - AMPK were obtained by western blot and were subjected to further analyses.

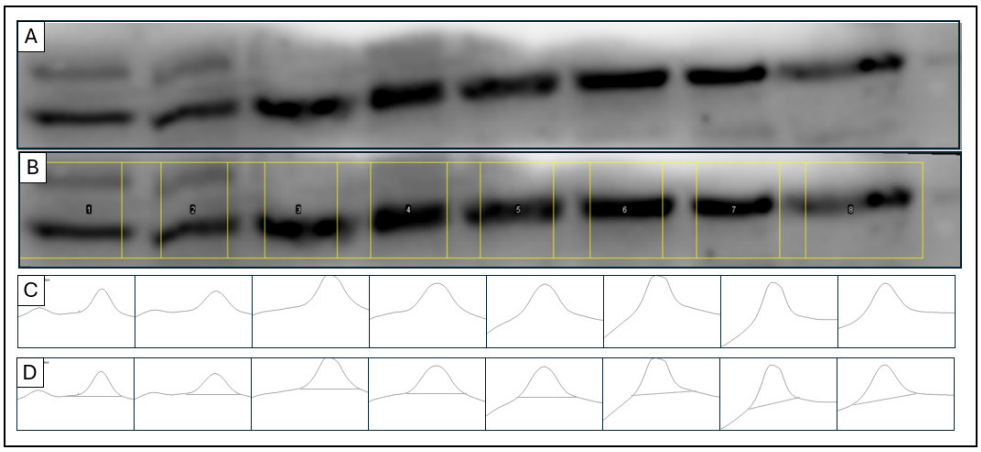

FIGURE 1. Representative bands of the protein AMPK and determination of band density using rolling disk method. A. Protein bands of AMPK (seen as 8 dark bands in a row);B. Marking the region of interest around each band; C. Estimating the density of each band using rolling disk method; D. Thresholds marked for subtracting backgrounds, and calculation of band density.

2.2. **Estimation of protein band densities using rolling disk method.** The ImageJ software (version 1.54j) [6] was used to quantify the densities of protein bands using the rolling disk method. Here, a rectangular frame of fixed dimension is marked manually around each band to obtain the regions of interest (Figure 1B). Then the pixel densities of bands in each region of interest are calculated by the rolling disk method (Figure 1C). Later, the background pixel densities are subtracted from the band density values by marking their threshold manually (Figure 1D).

2.3. **Estimation of protein band densities using contours.** Image processing and data analyses were performed using Python language

(3.12 stable release), on a locally installed interactive coding environment Jupyter Notebook (version 7.0.6). The Python library - OpenCV (Open Source Computer Vision Library, version 4.5.5) [9] was used for processing the Western blot images and band density computations. The other libraries used for processing data included - NumPy (v.1.26.3, for numerical computing) [5], pandas (v.2.2.2, for data processing) [21] and SciPy [version. 1.11.4, for scientific computing] [17].

The methods involved in processing of the blot images and density calculation using OpenCV are depicted in Figure 2.

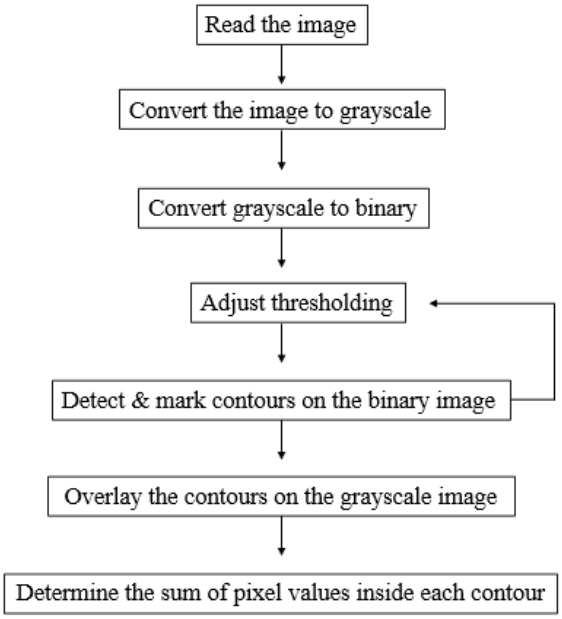

FIGURE 2. Flowchart depicting determination of band densities of proteins using OpenCV in Python.

The process involves first converting the color image (blot) to grayscale image using the cvtColor() function of the OpenCV package (Figure 3B). Next, to eliminate background noise and to set the boundaries of the bands, binary thresholding the grayscale image. This is achieved by setting pixel threshold values using threshold() function. This results in a binary image that shows only the bands on interest (Figure 3C). The thresholding may be suitably adjusted to obtain the appropriate band boundaries. Next, findContours() function is used to mark and draw the contours of the bands on the binary image. Here, the

CHAIN_APPROX_NONE method is applied to retain every contour point, to provide exhaustive representation of each band's boundary. This helps to obtain precise boundaries of each band. Then, these contours are overlaid on the grayscale image to obtain boundaries of the bands (Figure 3D). Finally, the total pixel intensity within each contour is determined. The aggregate pixel values represent the densities of the bands.

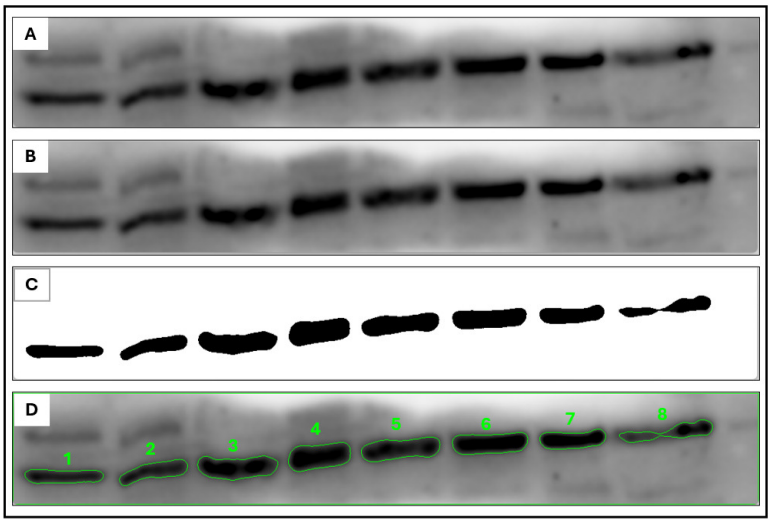

FIGURE 3. Determining the contour points of each band. A. Original image; B. Gray scale image; C. Binary image and with thresholding to obtain contour points; D. Overlaying the contour mask on the grayscale image.

2.4. **Correlation of the band densities obtained using the two methods.** The correlation between the band intensities obtained from both the methods were determined using the pearsonr() function of the stats module of SciPy package. The band intensity obtained thorough the traditional rolling disk method, and the currently demonstrated contour based pixel intensity summation method were provided as inputs in the function. The function outputs Pearson correlation coefficient which measures the linear relationship between two sets of data. The function also provides the p-value which is the probability that two non-correlated datasets would result in the current correlation coefficient. The function also provides the p-value, which is the probability of two uncorrelated

data sets resulting in a correlation coefficient as obtained in the current test. It tests the null hypothesis of no correlation between the datasets.

3. **Results and discussion**

A linear correlation was observed between the band intensities of the protein estimated using rolling disk method and aggregate pixel intensity. The Pearson's correlation coefficient of the band densities between the two methods was determined to be 0.938 with p-value of 0.005, indicating very high positive correlation between the data sets.

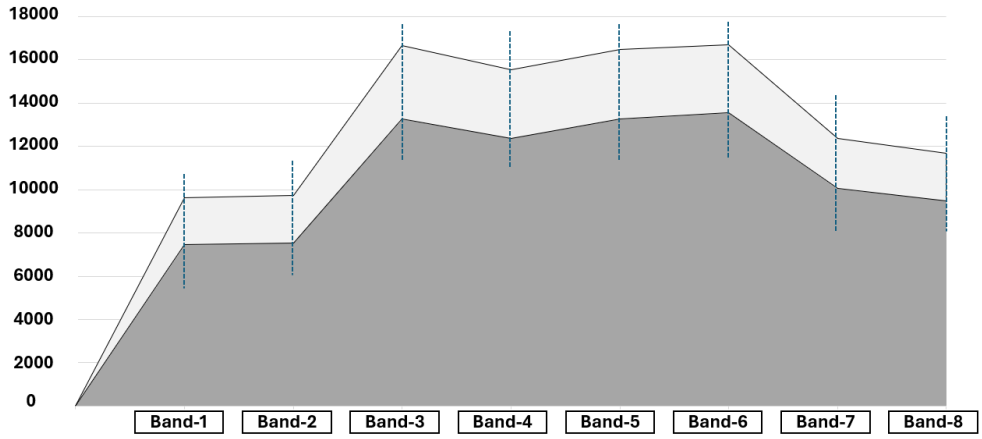

FIGURE 4. The band intensities of the protein using rolling disk method and total pixel intensity method

The band density values obtained using total pixel intensity were 4 times lower than the band densities obtained using rolling disk method (Figure 4). However, the variation in the values does not affect the interpretation of the results since the densities calculated by either method are only arbitrary values and not absolute values.

The pixel intensity based densitometric method has several advantages over the rolling disk method. Firstly, the contour thresholds can be adjusted to mark the most precise boundary of the bands. This process is difficult to achieve using the software based rolling disk method. Secondly, the pixel-based program is faster for large datasets, and can be automated to minimize manual intervention. The rolling disk method is

time-consuming and may not be as scalable or efficient for high-throughput analysis. Therefore, total pixel intensity methods can be powerful tools for estimating signal strengths in biomolecular estimations like determination of protein band densities, DNA and RNA sequences.

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