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# Counting Platelets: a Novel Image Analysis Algorithm for Detecting and Counting Platelet Adhesion on an Assay Pattern

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# Counting Platelets: A Novel Image Analysis Algorithm for Detecting and Counting Platelet Adhesion on an Assay Pattern

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**Abstract**

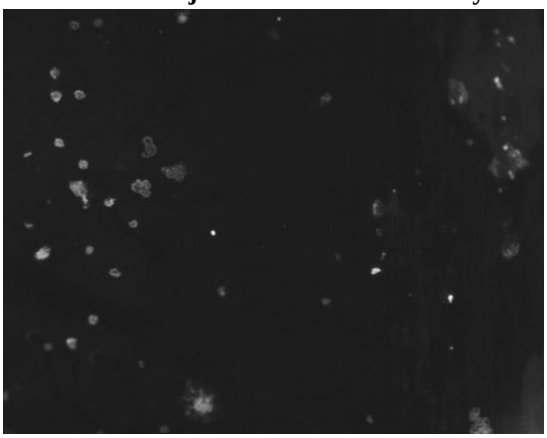
Changes in platelet function is a major complication in patients with cardiovascular disease. The authors have previously designed a portable, easy to use, low cost biochip for monitoring of anti-platelet therapy in cardiovascular disease patients [1]. This method directly measures platelet function by measuring platelet adhesion to platelet-specific protein patterns using a simple, optical counting technique. The patterned surfaces are designed so that a single platelet adheres to a single protein spot and for imaging purposes, the proteins patterned on the surface are labelled with a fluorescent dye, as are the platelets that adhere to the patterned surface. Following image acquisition, the occupied (i.e. covered by a platelet) protein spots in each sample must be enumerated. The result, calculated as percent adhesion, constitutes a direct and straightforward measurement of platelet adhesion and, therefore, platelet function.

**Keywords:** Imaging, Image Processing, Biomedical, Biology, Computer Vision

## 1 Introduction

In the work presented in this paper, a customised algorithm for the enumeration of platelets has been developed and validated. This type of biological testing algorithm has been designed by the authors in previous work [2] and has proved extremely effective in aiding the automation and accuracy of quantitative analysis. Data was collated and tested in a simple implementation of the design. The resultant images from this method also contain more useful information than just the percentage adhesion and were presented along with the adhesion results. It is expected that this algorithm will not only have a high impact on platelet function testing [3] but also on cell and bacteria capture assay which use a similar approach to cell counting.

**Figure 1 – An unprocessed sample of Platelets, contrast adjusted for reader visibility**



# 2 State of the Art

Adhesion assays measure platelet function by measuring the fluoresced platelet adhesion to platelet-specific fibrinogen protein patterns[4]. These patterns are stamped onto a glass surface to form a substrate image. Following sampling, protein spots are typically enumerated by hand and the result is calculated as a percentage of the total assay. Adhesion Assays have typically been used in biomedical research fields to gauge the potency and efficiency of anticoagulant agents. Given that single free-floating platelets tend to adhere to individual protein spots in the fibrinogen assay[5], the number of protein spots to which platelets have adhered is an appropriate measure of the free-floating platelet level in a given blood sample. Low platelet adhesion can be indicative of low platelet count due to illness or thrombotic disorders in the patient, or whether drugs to treat such disorders are performing adequately.

Where methods reported elsewhere in the literature have implemented the process of automatically enumerating the number of adhered platelets within adhesion assays, these methods were either undocumented in the corresponding papers [6] or applied to a niche case or application of adhesion assays [7]. A documented public method using open source libraries and tools has yet to be presented, furthermore the use of additional data produced by the automatic platelet counting process, such as average platelet clump size, does not appear to be the focus of investigation in the literature.

## 2.2 Sample Collection

The fluorescence microscopy platelet images were obtained using an inverted microscope (Olympus IX81) equipped with a CCD camera (Hamamatsu C4742-80-12AG) and a xenon lamp as the light source. Images were collected with a 20X objective (excitation filter BP492/18; emission light was collected through a filter cube, U-MF2, Olympus). An image of the protein array on the surface was compared to a corresponding image of adhered platelets, which was taken after the blood sample was placed on the array.

# 3 Algorithm Overview

The algorithm was developed and implemented in Python 3.6 with Open CV 3.2, this allowed for the automated calculation of the percentage platelet occupancy. The Python Tkinter library was used in development of a user-friendly graphical interface. The steps in the process are outlined graphically in Figure 2.

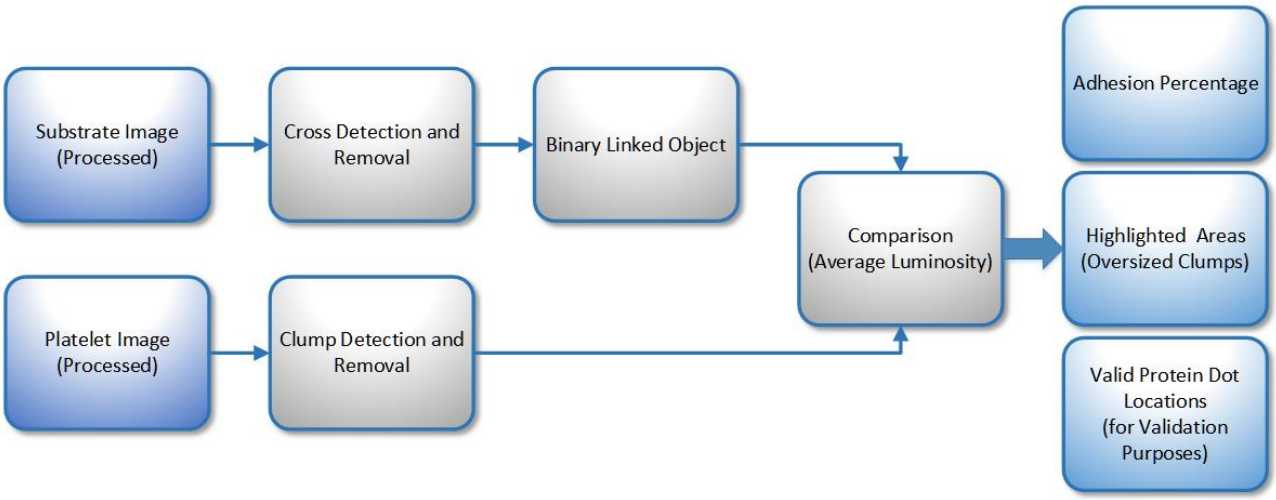



**Figure 2– A basic overview of the Platelet Detection Algorithm**

Prior to detection stages, both platelet and substrate images were converted from the source format into a greyscale

image. The greyscale images were equalized using Contrast Limited Adaptive Histogram Equalisation (CLAHE) [8], which was selected as a technique to account for the bright spots in some samples.

# 4 Cross Detection

After the substrate image was equalised using CLAHE, the noise floor of the image became consistent and the low and high elements were more predictable allowing the image to be binarised using a threshold of 35%. The resulting image had a morphological process applied to it with a large (20x20 pixel square)[9] kernel used as an operator.

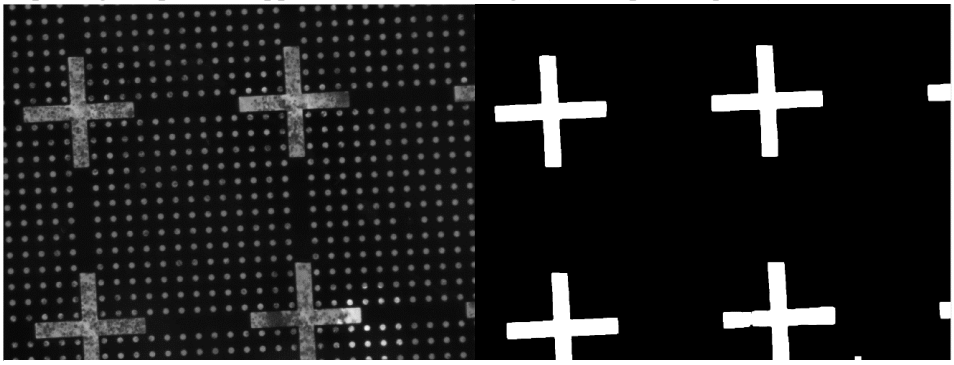


**Figure 3– Processing stages for Cross Removal**

This process ensures that only large elements remain in the binary image, specifically the 'crosses' which denote the boundaries between protein arrays. The result was corner-filtered and the remaining points aggregated locally and used to separate out relevant sample areas, however this was left out of the final version of the algorithm in order for the output data to remain aligned with the data from the manual method.

The heuristic for acquiring the threshold values for both Cross and Clump removal was a combination of a simple (single variable) parametric sweep of the threshold function and visual inspection of the output, as the intensities in the image are very dependent on the pre-processing stage of the algorithm, the laboratory protocol and lab equipment used during sampling, it is anticipated that new thresholds would need to be generated on a study-by-study basis. As such, these parameters were included as inputs into the final function and UI.

The resultant image, (seen in Figure 3) was used as a mask to subtract these boundary indicators from the substrate image, which allowed for a more accurate detection of the location of individual protein 'dots' within the protein array. The remaining 'cross-less' image can be seen in Figure 4.

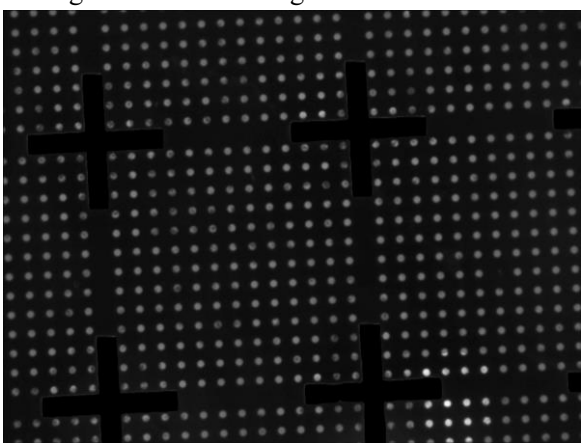


**Figure 4– Result from Cross Detection and Removal process**

# 5 Clump Detection

The platelet image was processed in a manner similar to the substrate image using equalisation via CLAHE, binarisation to a threshold and the application of a morphological process. Given that the average intensity of the platelet image was low and prone to gaps in the sample leading to bright (high intensity) areas in the image, the regions of interest remained dark but identifiable after applying CLAHE. A threshold of 20% was used to generate the binarised image. A morphological process was similarly applied but with a small (4x4-pixel) kernel used as an operator. The detection process can be seen in Figure 5.

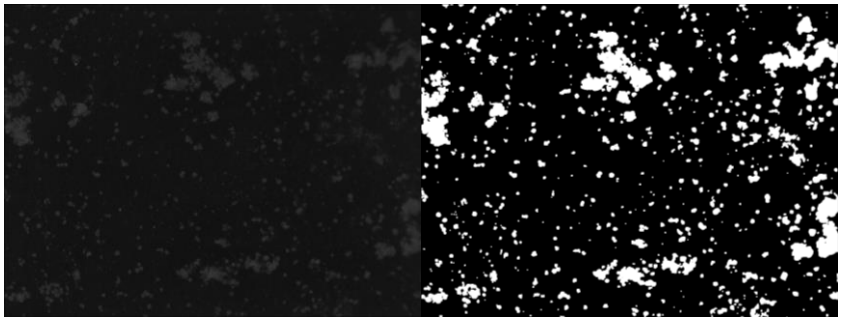


**Figure 5 - The platelet clump detection process**

## 5.1 Clump Size Evaluation & Removal

For accurate platelet adhesion detection, simply separating the areas of interest from the background is not sufficient, given that platelets have a tendency to clump around the boundary indicators of the protein array. It was found that this clumping effect would typically invalidate any nearby 'dots' on the protein array. Further processing was necessary to determine the size of a given collection of platelets, and a threshold was needed to separate valid size from invalid ones. The contours within the binarised platelet image and the respective sizes of each contour were found through the application of Suzuki's well-established border following algorithm [10], this algorithm uses a raster scan to categorize pixels within a binary image as border pixels, and then determines contours based on the generated 'value' and sequence of said border pixels. Using this approach, platelet clumps whose area exceeded a given size were rendered out to form a mask of all invalid clumps in the image. This process also allowed for the size of the platelet clumps to be evaluated given that the size of a single protein 'dot' was known to be 6µm in diameter. The results of the size evaluation and removal can be seen in Figure 6.

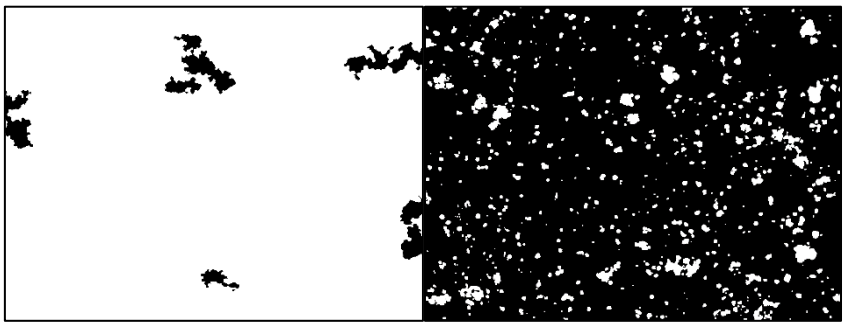


**Figure 6 – Processing stages for Clump Removal**

# 6 Adhesion Calculation

The points found by the binary-linked objects determines the location of each individual element of the protein arrays present in the substrate image. These points were evaluated in the corresponding platelet image based on the intensity of the local area of pixels around the points. A distance calculation ensured that the pixels being checked were within the area of the protein dot and not outside the circular boundary (i.e.: the corner areas of the square area). The mean intensity of these pixels were calculated for each key point in the image. The threshold for deciding

if a platelet had adhered to a given element was determined by the manual process, in which a dot with more than half of its surface illuminated would be considered adhered. Lastly, the number of valid adhered dots was compared to the total number of detected BLOBS and percentage adhesion was calculated according to the formula below.

### 6.1 Formula 1 – The number of adhered points as a percentage of the total points

$$\boldsymbol{Adhesion}(\%) = \frac{\boldsymbol{Valid}}{\boldsymbol{Total}} \times \mathbf{100}\%$$

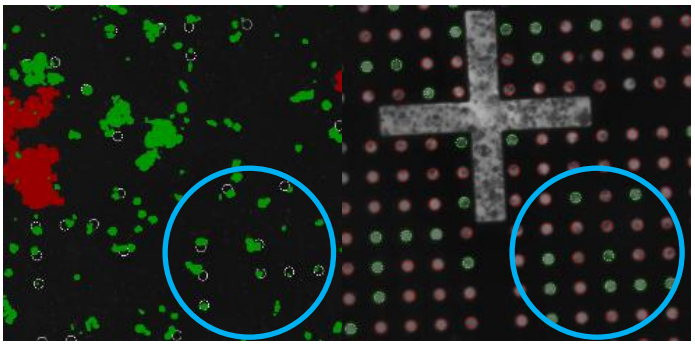


**Figure 7 - Image output (right) displaying valid dots in green and valid adhesion of platelets (left) in white, a region of adhered points can be seen circled in blue**

## 7 Results and Comparison

After applying the algorithm to several sample batches, the average error in adhesion was found to be approximately 0.025% with peaks up to 0.166%. However, failures based on a criterion of 100 or more missed valid protein dots found an average failure rate of approximately 20%. A small subset of results is shown in Table 1. The total points are retained to ensure a large enough test area. Sample 1111 is detected by the application as a failed sample and was included to demonstrate erroneous output detection. Sample input and output images are shown in Figure 1.

### 7.1 Table 1 – Results from sample processing via Platelet Detection algorithm

| Sample ID | Adhered | Total | Adhesion % | Missed Valid | Manual Adhesion % | Error % |
|---|---|---|---|---|---|---|
| 1111 | 21 | 381 | 5.511811024 | 130 | 4.109589041 | 0.341207349 |
| 1112 | 39 | 461 | 8.459869848 | 5 | 8.369098712 | 0.010845987 |
| 1113 | 78 | 450 | 17.33333333 | 40 | 15.91836735 | 0.088888889 |
| 1114 | 64 | 522 | 12.2605364 | 3 | 12.19047619 | 0.005747126 |
| 1115 | 73 | 524 | 13.93129771 | 20 | 13.41911765 | 0.038167939 |

## 8 Conclusion

Investigation into the failed samples revealed that the algorithm was appropriately ignoring invalid points caused by various factors such as imperfect appliance of the protein to the substrate, blurred or bloomed points and other anomalies inherent in the application of the microarray. However, the most common cause of missed valid points was high variance in the local contrast of both the substrate and the platelet image. It would be possible to increase

the robustness of the application by reapplying the method to failed samples with modified thresholds.
Failure rate aside, the results provide comparable accuracy with the manual method but with automation and additional data, including size calculation and area of interest highlighting for ease-of-use and further data analysis. A prototype application was developed with several user-friendly features to facilitate its use by scientists without technical inclinations. A feature was built in to the prototype application to allow for the visual validation of each point should the user wish to do so. The function operates at approximately 1.39μs per sample on a mid to high end workstation without the aid of optimisations such as GPU acceleration or multithreading. This means that with the prototype application allowing the algorithm to be applied to all relevant images within a given folder; entire batches of samples can be processed and evaluated in a matter of minutes. Given that manual enumeration can take minutes per sample to process, the method outlined in this paper is capable of performing analysis at a much greater efficiency than the existing manual approach and can provide the user with potentially beneficial additional data.

## Acknowledgements

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