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# A Computational Method for Two-Dimensional Quantitative Analysis of Standing Wave Images of Red Blood Cells

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## Introduction

Standing wave (SW) microscopy allows for an improvement in the axial resolution that can be obtained in optical microscopy. In SW microscopy two counter propagating waves interfere producing a SW with antinodal planes that are separated by  $\lambda/2n$  and a FWHM of  $\lambda/4n$  which is the axial resolution, where  $\lambda$  is the excitation wavelength and  $n$  is the refractive index. [1]. Multi-planar SW microscopy, with the addition of a mirror below the specimen, allows for selective plane contour mapping of one concave surface of a red blood cell (RBC) as shown in Figure 1 [2].

We report a computational method to extract SW antinodal plane and boundaries positions (x,y) and extract each pixel intensity value. By doing so, we can create 2D reconstruction of SW RBC images captured at video rate. In future, by utilising the positional information for the each of the antinodal SW planes, we aim to create 3D and 4D reconstruction of the RBCs concave surface. Additionally, applying the extended computational method to SW RBC images we aim to study healthy and diseased erythrocytes and the changes in the concave surface morphology over time.

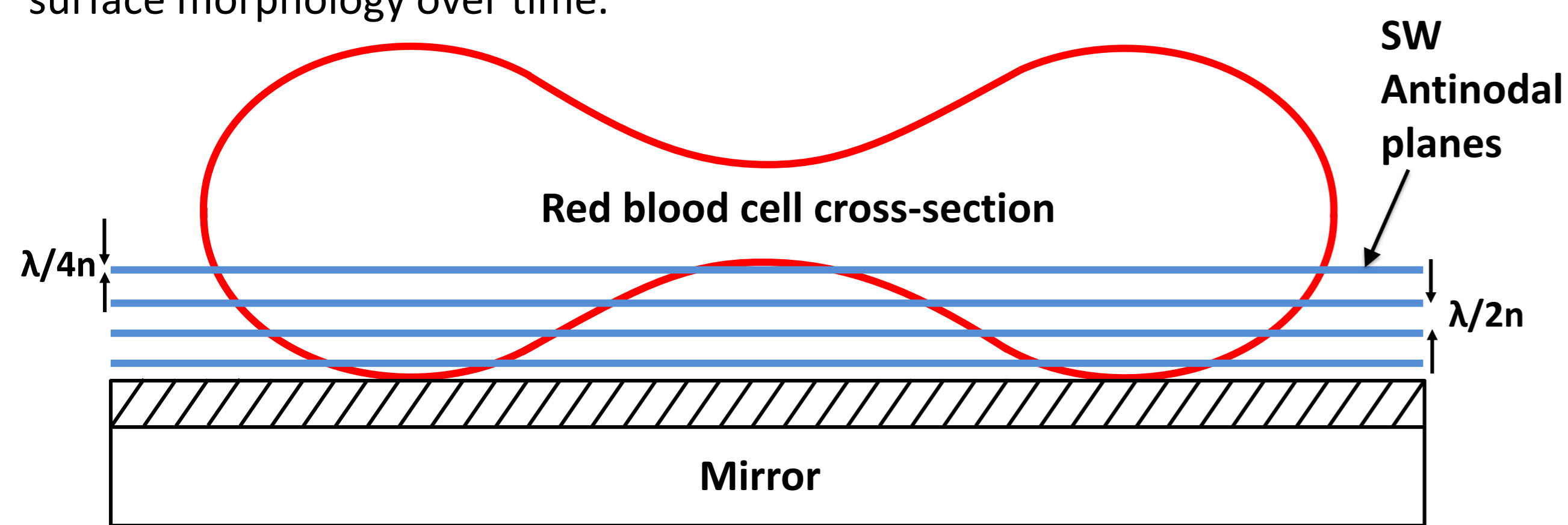


Figure 1- Schematic diagram of a fluorescently labelled RBC adhered to a plane mirror. The incident and reflected excitation light create a SW. The blue lines represent the SW antinodal planes which are spaced at intervals of  $\lambda/2n$ . Where the antinodal planes intersect the specimen fluorescence emission is emitted which result in a 2D contour map of the RBC plasma membrane topography.

## Methods

Mouse RBCs were isolated from the blood plasma and stained with a Dil stock solution (1 mg/ml) at 1 part in 99 of the cell suspension. RBCs were then adhered to the surface of a broadband mirror using Poly-L-Lysine. SW RBC images were acquired using an Olympus BX50 wide-field epi-fluorescence microscope with an 100x/1.40 oil immersion objective and excitation was provided by a 460 nm LED (CoolLED pE-4000). Fluorescence emission was collected at  $\lambda > 515$  nm. The SW image data was captured at a rate of 30 frames per second over a duration of 33 seconds using a Hamamatsu ORCA-Flash 4.0 LT camera.

The raw SW RBC images were processed using the steps in Figure 2.

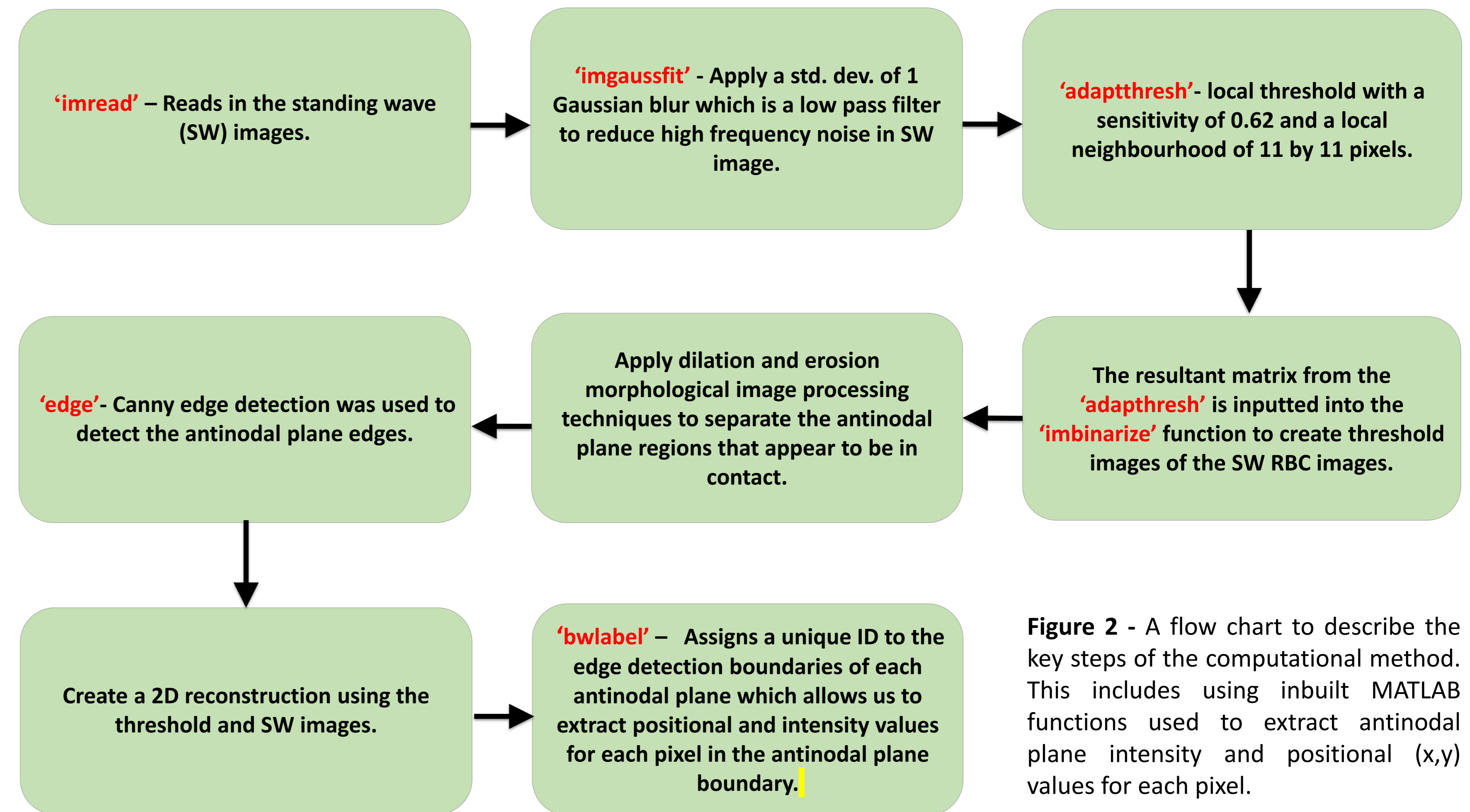
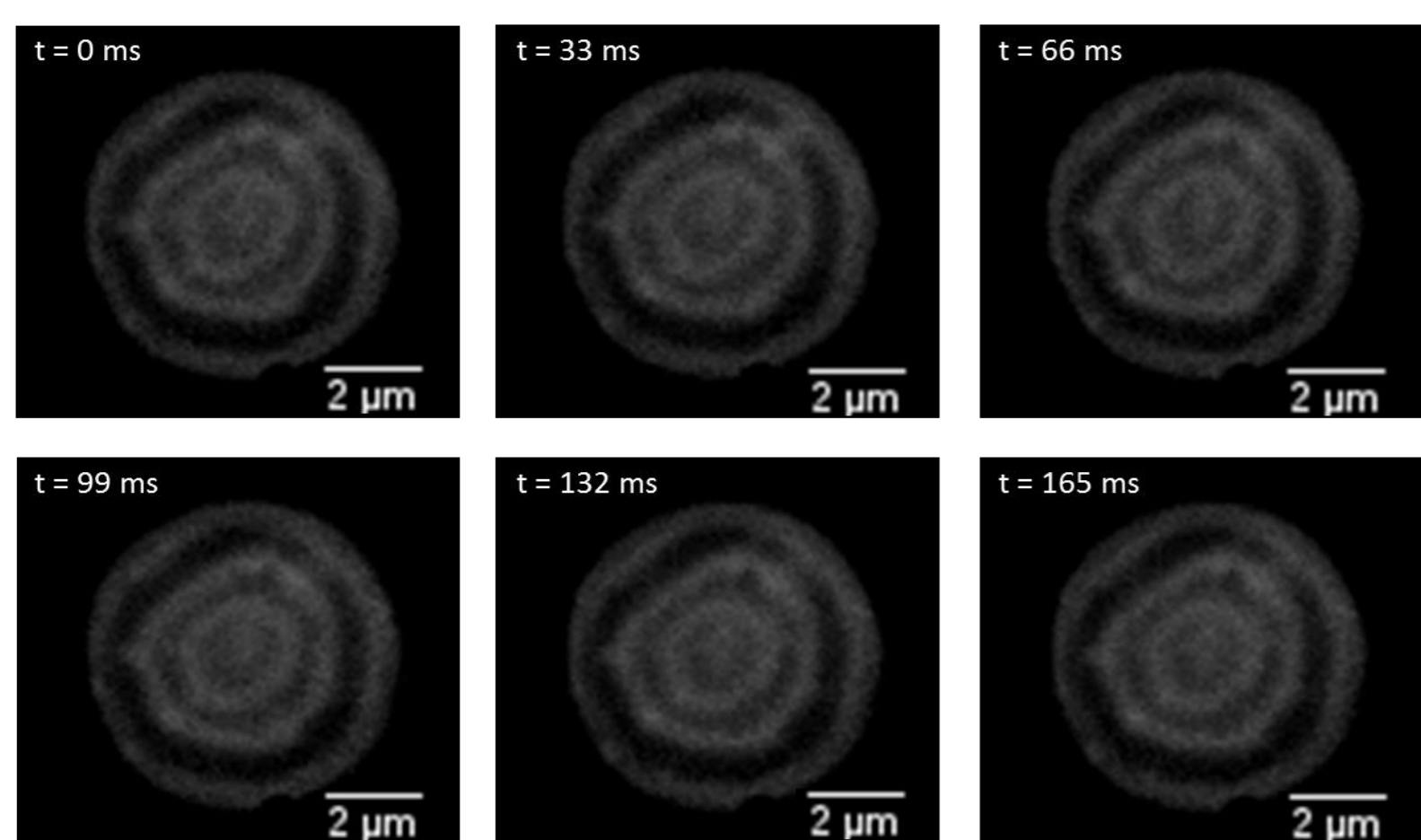


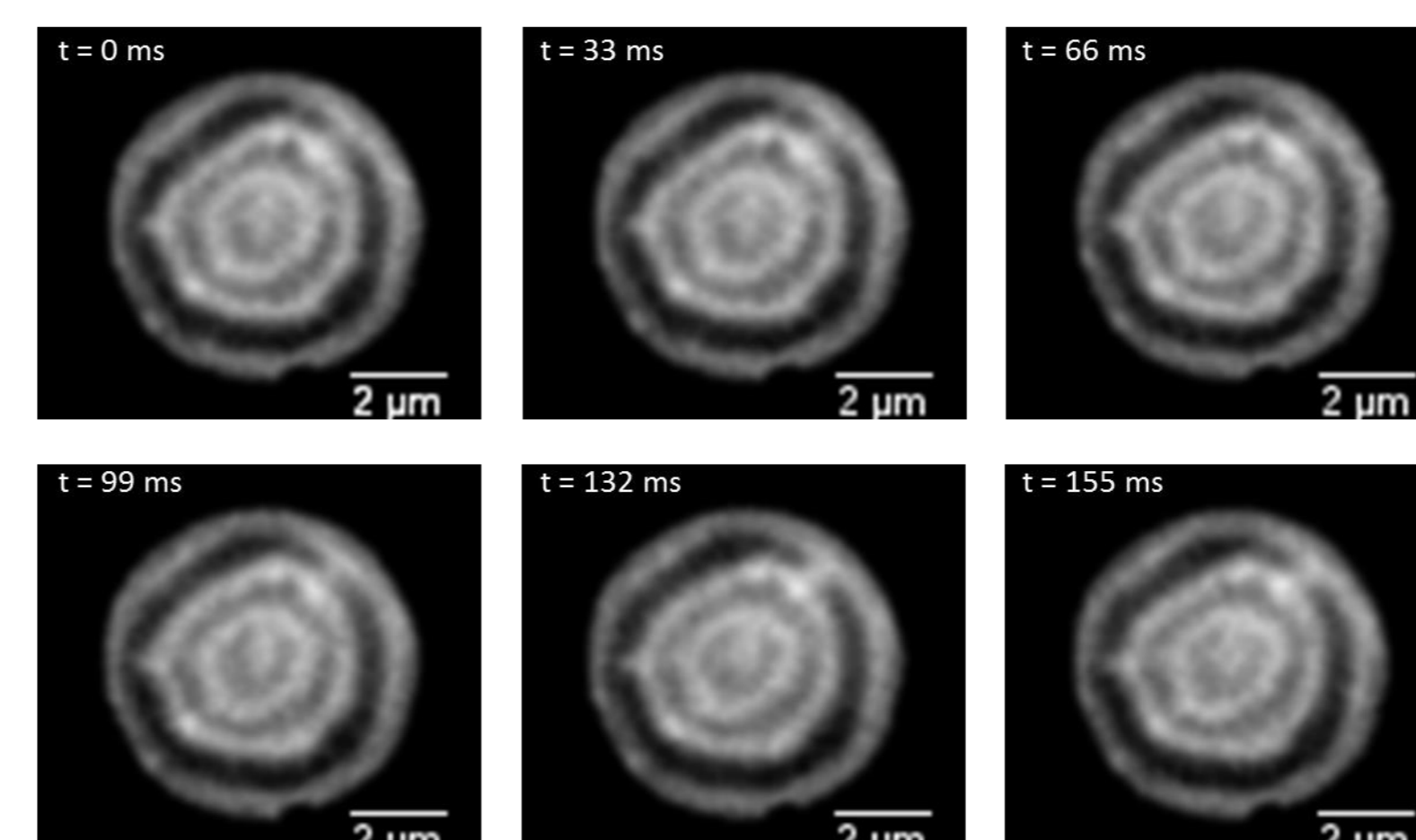
Figure 2 - A flow chart to describe the key steps of the computational method. This includes using inbuilt MATLAB functions used to extract antinodal plane intensity and positional (x,y) values for each pixel.

## Results



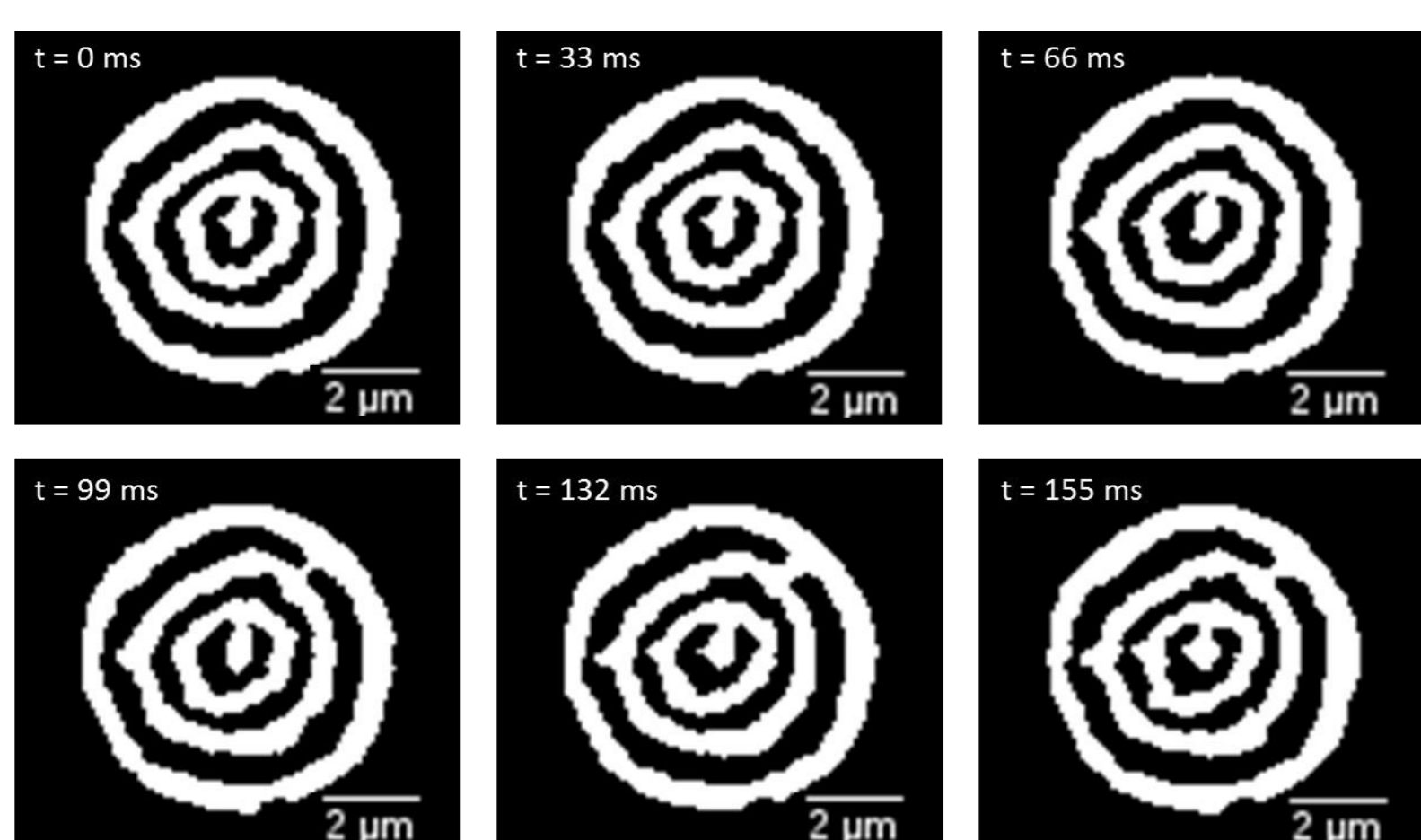
### Image Preprocessing

Figure 3 – The first six RBC images in a movie captured at a 30 Hz frame rate over 33 seconds. In ImageJ, the SW RBC movie was cropped to contain only the RBC of interest. Furthermore, the contrast was adjusted to improve the ability to distinguish between the nodal and antinodal planes.



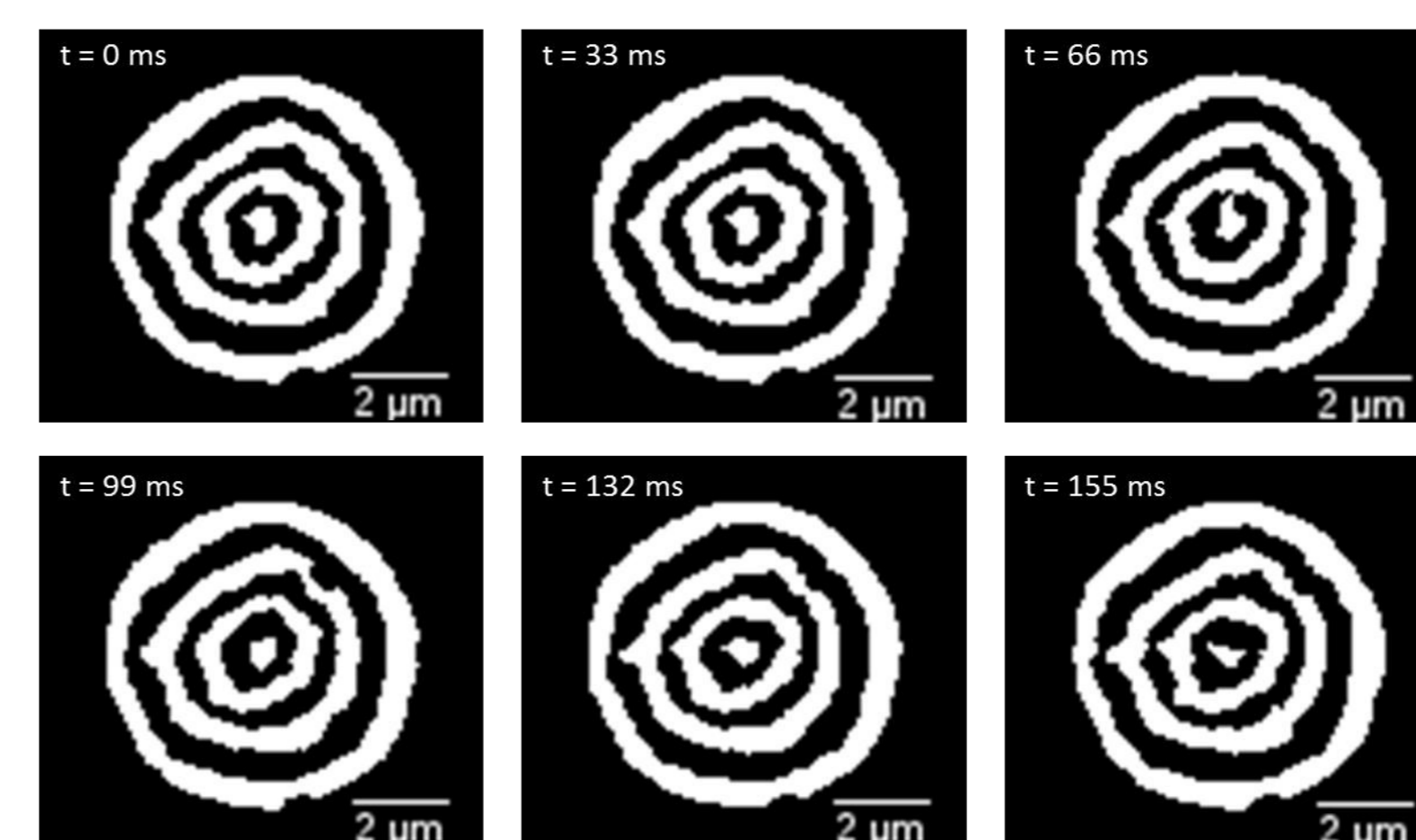
### Gaussian Blur

Figure 4 - In MATLAB, each image is normalised between intensity of 1 (white) and 0 (black). The application of a Gaussian blur removed random noise from each image leaving only the characteristic SW interference pattern. A Gaussian blur with a std. dev. of 1 was applied to each image using the 'imgaussfit' function.



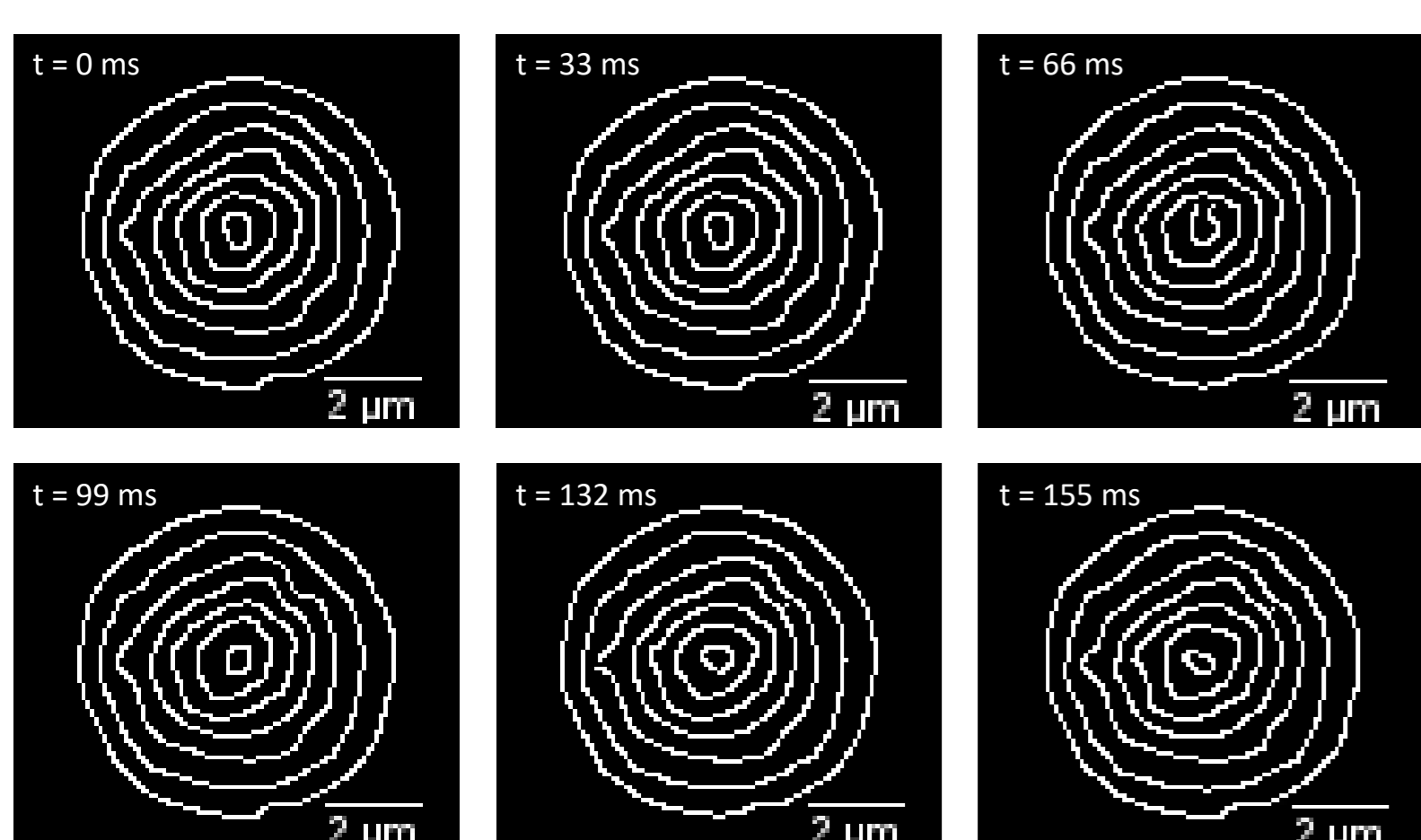
### Thresholding

Figure 5 – Local adaptive thresholding 'adaptthresh' function with a sensitivity of 0.62 and a local neighbourhood size of 11 by 11 pixels was used to determine the local threshold values. The threshold images were created with the 'imbinarize' function. The result is the separation of the antinodal planes from the background and nodal planes.



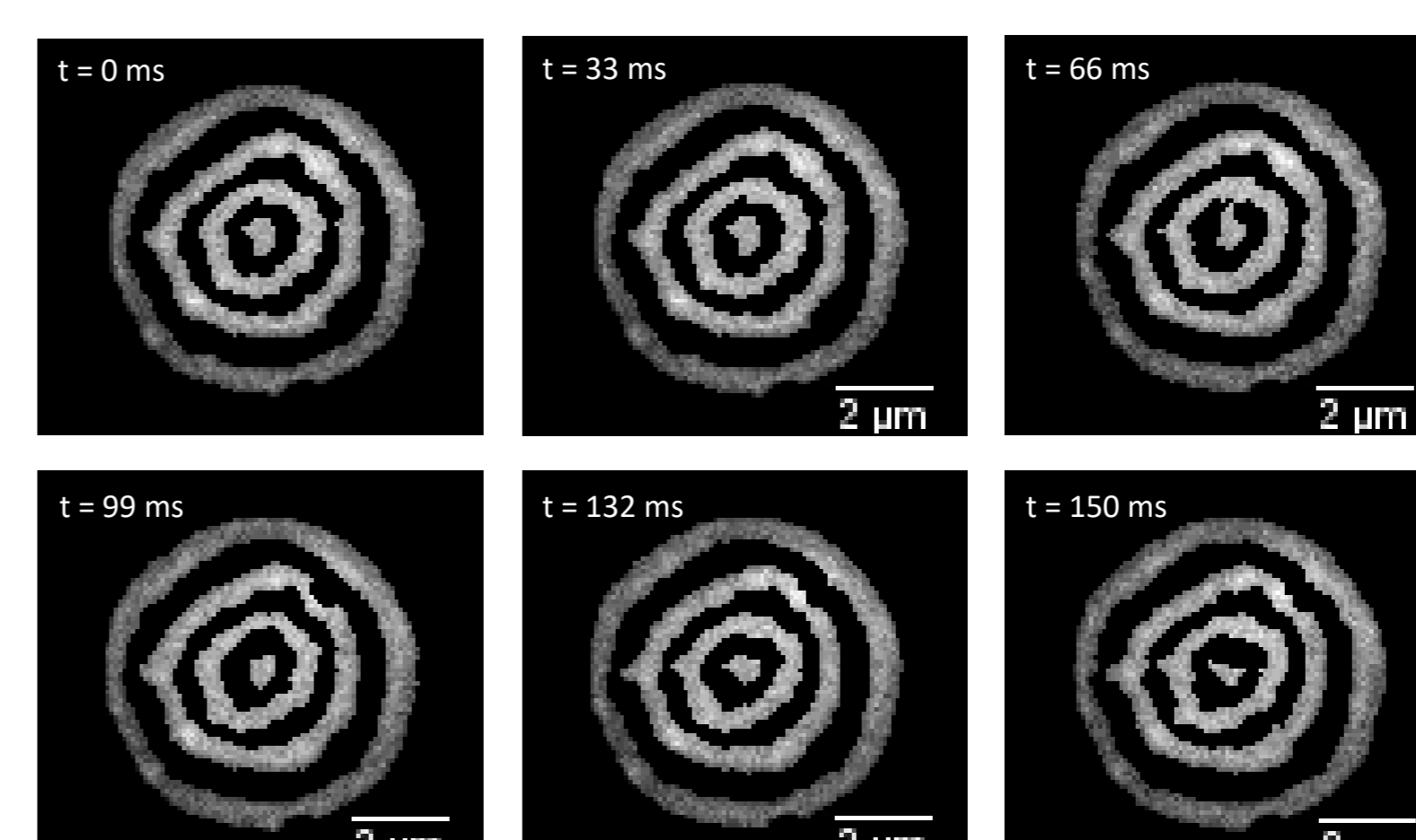
### Antinodal Plane Separation

Figure 6 – The SW antinodal planes were separated using dilation and erosion morphological image processing techniques. Antinodal planes are separated planes where they appear to be in contact and may occur in different axial locations.



### Edge Detection

Figure 7 – Canny Edge detection allows for the boundaries of the SW antinodal planes to be detected and the positions to be determined. This was achieved by using the 'edge' function in MATLAB.



### 2D Reconstruction

Figure 8 – 2D reconstruction of the RBCs in figure 3. The threshold images and the original images were used to create a custom colormap for each individual pixel and produced a 2D reconstruction of the RBC with the appropriate intensity values for each pixel position.

## Conclusion

We report a computational method using MATLAB which can be used to extract 2D SW antinodal planes information such as their intensity and positional (x,y) values for each pixel within the SW antinodal plane.

Furthermore, the intensity and positional information for each pixel is applied to successive SW RBC images captured at video rate and we show that we can create a 2D reconstruction for each subsequent SW RBC images.

## Future Work

Further development of the computational method to apply SW theory to the extracted 2D data to produce a 3D reconstructions of the red blood cells concave surface. Additionally, extending the computational method to produce a 4D reconstruction of the RBC concave surface over time.

Lastly, compare healthy and diseased RBCs using the 3D and 4D computational methods to study the changes in the morphology of the concave surface of a RBC.

[1] B. Bailey, D. L. Farkas, D. L. Taylor, and F. Lanni, "Enhancement of axial resolution in fluorescence microscopy by standing-wave excitation.," *Nature*, 1993.

[2] R. Amor, S. Mahajan, W. B. Amos, and G. McConnell, "Standing-wave-excited multiplanar fluorescence in a laser scanning microscope reveals 3D information on red blood cells."