Standing wave microscopy of red blood cell membrane morphology with high temporal resolution

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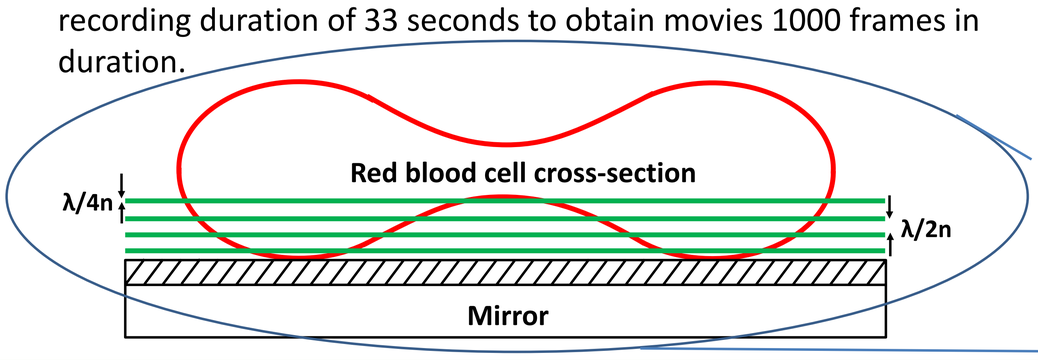
Introduction

Widefield fluorescence microscopy is an integral tool for life science imaging though the achievable resolutions are limited by the diffraction nature of light. One technique to increase the axial resolution is known as standing wave microscopy [1]. The standing wave can be generated by placing a mirror at the specimen plane which causes interference between the incoming and reflected excitation illumination. The axial resolution is reduced to λ/4n as only fluorophores which are in the location of the full width at the half maximum of the antinodes are excited [2] resulting in periodic bands of fluorescence.

Previous investigations using this technique imaged red blood cells upon a mirror using a confocal laser scanning microscope meaning that the temporal resolution was limited [2] and fast membrane movements could not be captured. We present here the first demonstration of widefield standing wave microscopy upon red blood cells with high temporal resolution and an axial resolution better than 100 nm.

Methods

- Red blood cells were isolated and labelled with the fluorescent membrane dye DiI (10 μM). They were then plated onto a broadband reflector under a coverslip and imaged using a 100x/1.4 oil immersion objective lens.
- Illumination was provided by a 550 nm LED with a peak wavelength of 548.8 ± 1.5 nm and using specimen plane powers of 1.71 ± 0.01 mW.
- A camera binning n = 2 was used and an exposure time of 33 ms with a recording duration of 33 seconds to obtain movies 1000 frames in duration.



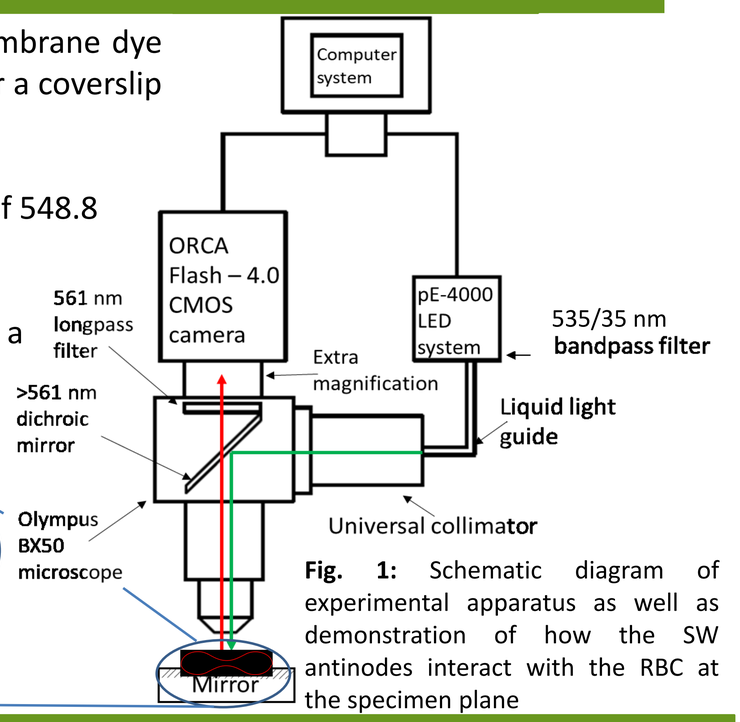


Fig. 1: Schematic diagram of experimental apparatus as well as demonstration of how the SW antinodes interact with the RBC at the specimen plane

Video-rate Standing Wave Imaging of Red Blood Cells

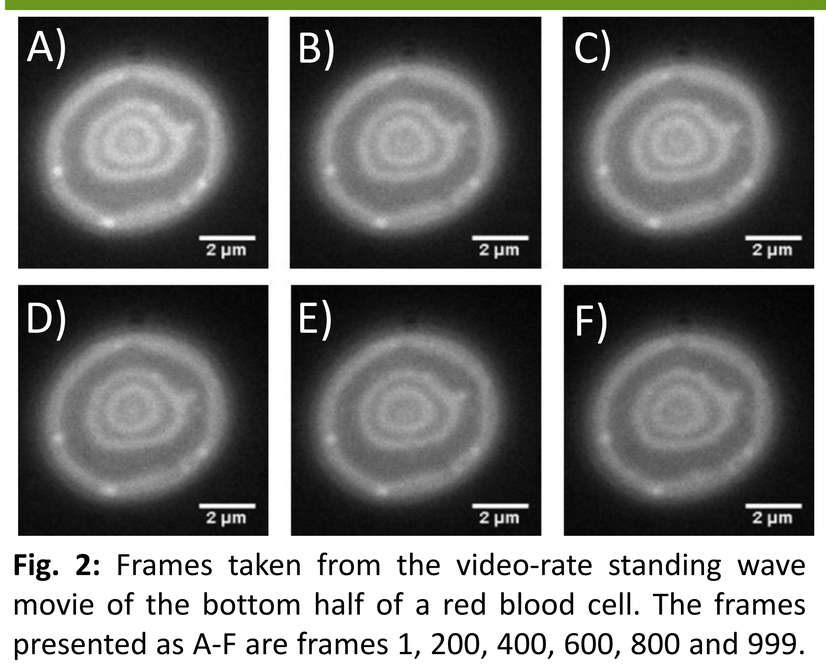


Fig. 2: Frames taken from the video-rate standing wave movie of the bottom half of a red blood cell. The frames presented as A-F are frames 1, 200, 400, 600, 800 and 999. **Using this technique we are able to observe rapid membrane deformations in real time with an axial resolution of 91.4 nm and a lateral resolution of 304 nm.**

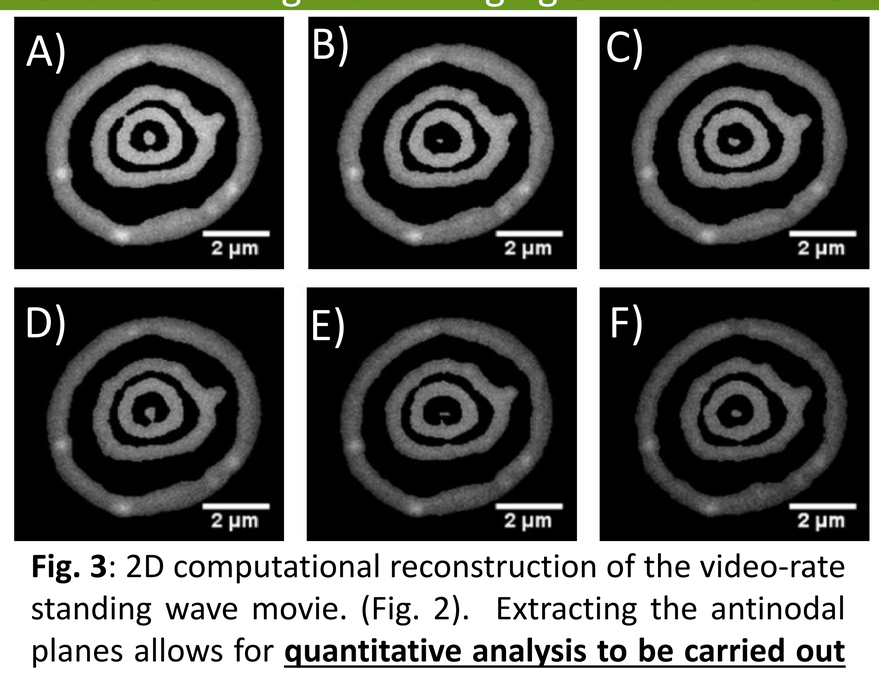


Fig. 3: 2D computational reconstruction of the video-rate standing wave movie. (Fig. 2). Extracting the antinodal planes allows for **quantitative analysis to be carried out on the data as well as easier observation of antinodal plane movements.**

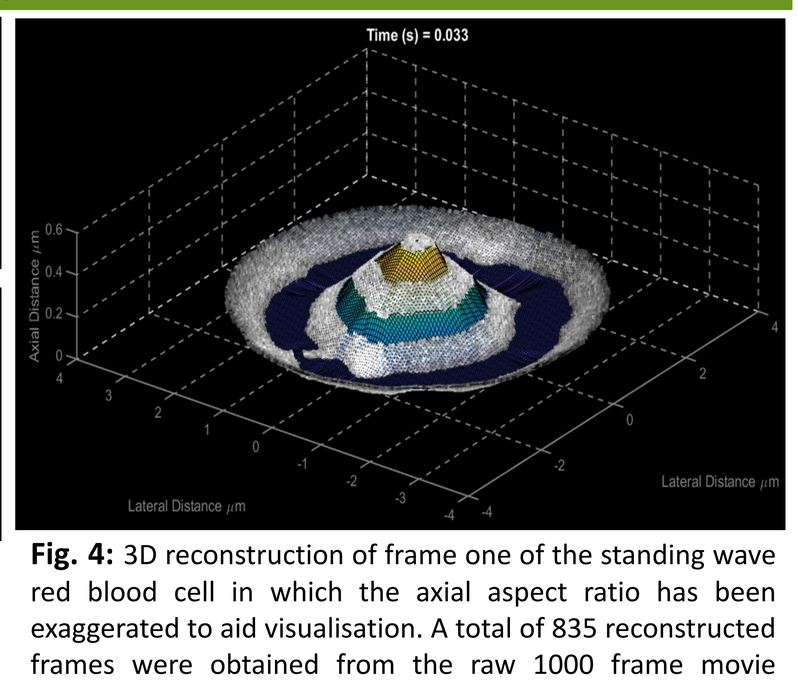


Fig. 4: 3D reconstruction of frame one of the standing wave red blood cell in which the axial aspect ratio has been exaggerated to aid visualisation. A total of 835 reconstructed frames were obtained from the raw 1000 frame movie which allows the **observation of how the 3D membrane topography of the red blood cell changes in real time.**

Photobleaching Rates Compared To Widefield Microscopy

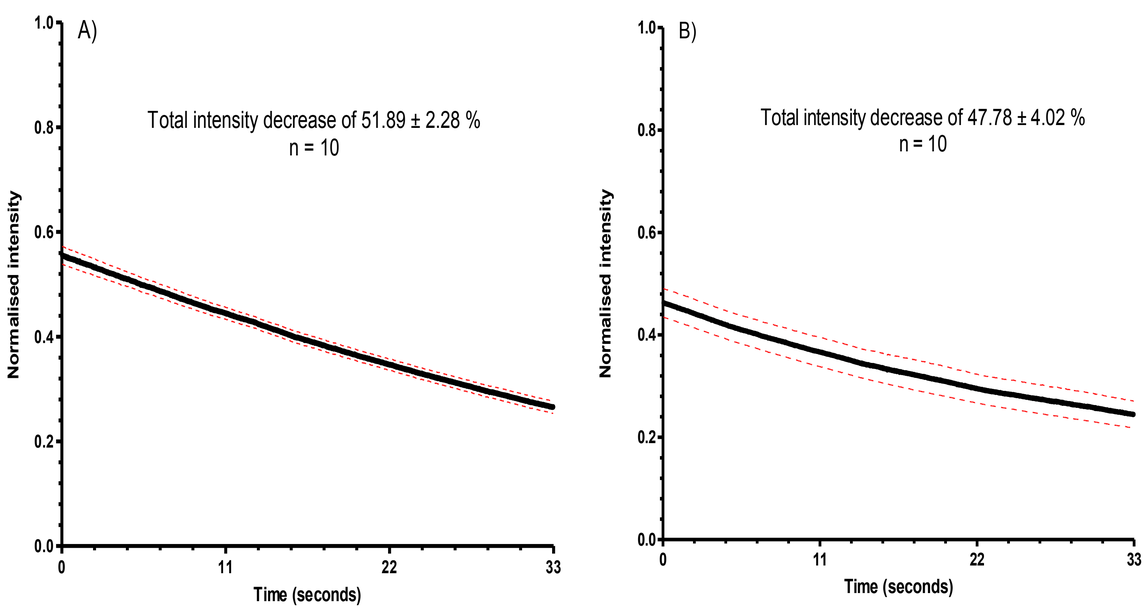


Fig. 5: Average normalised intensity obtained from A) standing wave imaging and B) widefield epifluorescence imaging of red blood cells (n = 10). It can be seen from these graphs that;

1) **Standing wave imaging causes no significantly greater amount of photobleaching than standard widefield imaging.**

2) **Standing wave imaging obtains significantly brighter images due to multiple in focus antinodal planes**

Phototoxicity Compared To Widefield Microscopy

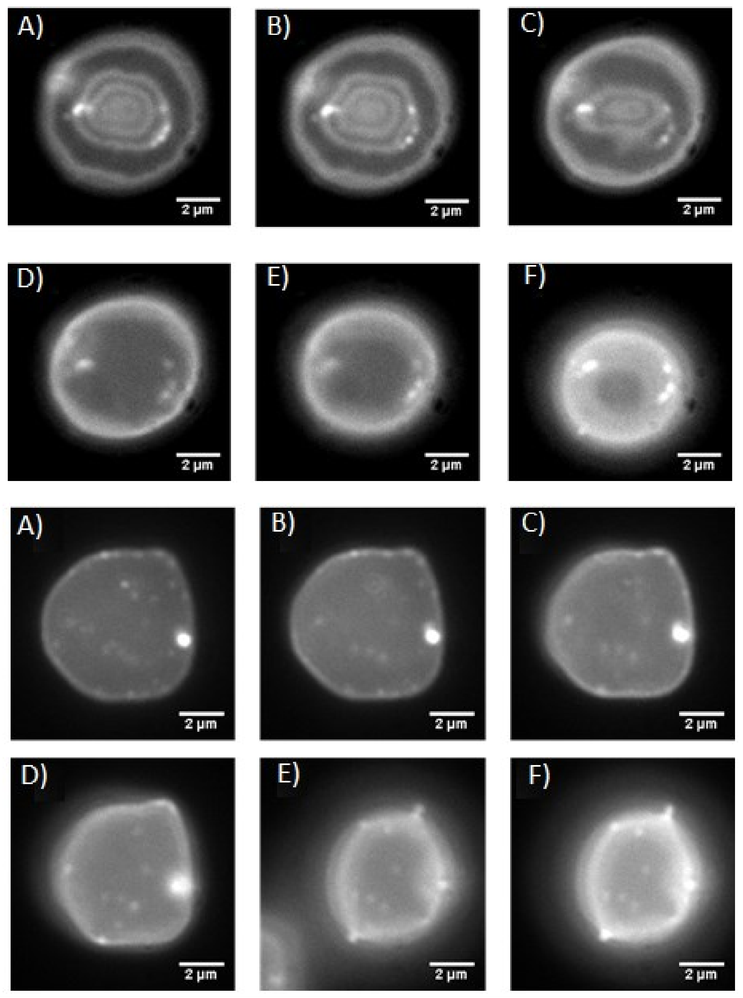


Fig. 6: Standing wave images of a red blood cell. The frames presented as A-F are at time points 15, 360, 720, 1080, 1440 and 1800 s**.** The decay of the red blood cell overtime resembles the process of photo-hemolysis observed under focussed low power laser illumination [4]. It is hypothesised that this process is due to the interaction between reactive oxygen species and the membrane proteins spectrin and band 3 [3].

Fig. 7: Widefield images of a red blood cell at the same timepoints as Fig. 6. It can be seen when compared to Fig. 6 that the red blood cell appears to decay in a similar manner and rate.

This demonstrates that our standing wave technique does not cause any greater amount of photo-toxicity to the specimen.

Conclusions and Future Work

- **This is the first demonstration of widefield standing wave imaging of red blood cells at a video rate with an axial resolution of under 100 nm.**
- **By applying this technique we have been able to observe rapid membrane deformations in real time by simply placing our specimens upon a mirror.**
- **We have also shown that using this technique does not induce any greater amount of phototoxicity or bleaching to the specimen than widefield microscopy.**
- **This work has now been published in Biomedical Optics Express (2018).**
- Future work involves applying this technique in other cell types in order to study morphological changes (blebbing, cell division or migration) and membrane potential imaging in excitable cells.
- We also aim to use multiple wavelengths (excitation or emission) in order to reduce the nodal plane contributions in the images and determine the morphology of unknown specimens by the antinodal plane ordering.

References: [1] B. Bailey et. al., *Nature*, 1993. **[2]** R. Amor et. al., *Sci. Rep.* 2014. **[3]** F. Wong et. al., *J. Microscopy, 2007.*