Correlative Scanning Electron Microscopy (SEM)-Fluorescence Microscopy reveals the organisation of the red blood cell membrane skeleton in healthy and diseased states

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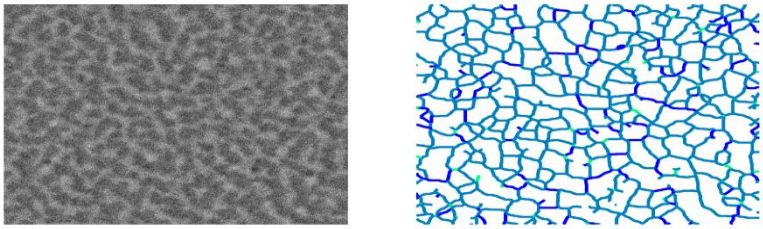
Red blood cells (RBCs) undergo remarkable deformations as they traverse the circulatory system, passing through narrow capillaries and sinusoidal slits within the spleen. There is enormous interest in the physical properties of the RBC membrane that allow such flexibility and durability. In particular, the organisation of the protein network (comprised of a meshwork of spectrin molecules converging on actin junction points) that lies underneath the RBC membrane bilayer is thought to be critical for physiological and pathological processes.

We present here a correlative Scanning Electron Microscopy (SEM)-Fluorescence Microscopy method to examine the cytoplasmic side of RBC membranes that have been sheared open. This novel imaging method has been made possible by recent improvements in secondary-electron detection capabilities in the latest generation of SEM instruments that overcomes the poor electron signal of biological samples that have previously prevented direct imaging. Our method involves high-resolution imaging of membranes coupled with image analysis using custom software written in Python to trace the protein network. Correlative fluorescence imaging was used to detect particular subsets of RBCs within a mixed population. Parallel imagining of the behaviour of the RBCs in a microfluidic device called a Human Erythrocyte Microchannel Analyser (HEMA) (Gifford, Frank et al. 2003) was used to measure surface area and volume. With these tools, we sought to understand how the protein network changes with induced variation in RBC shape and volume, how the network develops during reticulocyte maturation, and how it is affected by malaria infection.

Examination of RBC membranes of osmotically swollen cells revealed that the membrane permits a maximum increase in the average network branch length of approximately 5%. In contrast, cells infected by the malaria parasite, *Plasmodium knowlesi*, show an increase in membrane branch length of 4.5%, while an increase of almost 8% occurs upon infection with *Plasmodium falciparum*, highlighting the extent to which *Plasmodium* parasites alter the organisation of their host RBC membrane.

We also probed the process of reticulocyte maturation. We observed a ~20% decrease in volume in mature RBCs compared with CD71+ reticulocytes. It is currently held that this volume decrease is accompanied by a corresponding compression in the protein network, however our measurements show that the membrane branch length is decreased by only ~2%. This is consistent with the maintenance of a similar surface area to volume ratio and similar deformability. The data indicate that contraction of the network does not drive the volume change, and that instead the protein network in the RBC membrane is dynamically rearranged during maturation. Consistent with this suggestion we observed ruffles, furrows and endocytic pits in SEM images that may reflect spectrin degradation and loss of membrane by exosome expulsion. Our correlative SEM/ fluorescence imaging coupled to microfluidics-based rheology is providing new insights into RBC physical properties.



RBC Skeleton and trace performed by algorithm 'skan'