Stage-specific detection and characterisation of the malaria parasites

PhD Booklet

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1 Background

Malaria, an infectious disease more ancient than humanity, still imposes a global burden with 240 million annual cases worldwide, as estimated in 2020 [1]. Due to the interplay of various factors, such as the ongoing COVID-19 pandemic, the disruption of measures against malaria and the global climate change, the number of cases has been increasing from 227 million in 2019 again [1]. This circumstance has a large influence on the mortality, if the infection is not promptly and effectively treated, which is often the case in endemic regions [2].
The infection is caused by the malaria parasite, a mosquito-transmitted protozoan. Malaria parasites belong to the *Plasmodium* genus. Five species infecting humans of the genus *Plasmodium* are known: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Among them, *P. falciparum* causes the most deaths. It is widely spread in Africa, New Guinea and in Haiti [3]. Following the mosquito bite and the symptom-free liver stage of the infection, the parasites burst out into the blood stream to start an asexual life cycle. In this 48 hours long intra-erythrocytic cycle, they mature through the ring, trophozoite and schizont forms. At the end of the cycle, they multiply and the merozoites begin the next cycle by invading new RBCs [4]. The intra-erythrocytic cycle has been the subject of intense research because it causes the main clinical symptoms and is the major target of diagnostics and antimalarial treatment [4, 5].
Despite the rapid progress in treatment and diagnosis, malaria is still a burden in African, American, Eastern Mediterranean, South-East Asian, and Western Pacific regions [1]. The recent increase in deaths by 13% compared to 2019 due to the COVID-19 pandemic requires a better understanding of the alterations in RBC structure during the maturation of malaria parasites. Tracing the morphological and optical changes may contribute to the development of new and more effective antimalarials. If such changes are characteristic to the intra-erythrocytic stages of malaria, they could improve diagnosis by NNs.
Since most endemic areas suffer from low resources, they require simple diagnostic methods. While the standard methods, such as light microscopy of stained samples and PCR (Polymerase chain reaction) offer reliable diagnosis, they are time-consuming and require trained experts. Light microscopy on Giemsa-stained blood smears is still the gold-standard diagnostic method used worldwide. Autonomous computer assisted recognition of malaria infected RBCs using neural networks (NNs) has the potential to overcome these deficiencies, if a fast, high-accuracy detection can be achieved using low computational power and limited sets of microscopy images for training the NN. Starting from simple categorisation into healthy and infected cells, newly developed NNs now offer an intriguing opportunity to accurately classify all malaria blood stages based on characteristic alterations of the host RBCs induced by the malaria parasite. This approach has the potential to boost the accuracy of light microscopy-based classification and to reduce human error.

2 Objectives

Pioneering studies have clearly demonstrated the potential of NNs for automatised malaria diagnosis [6–11]. However, this approach still requires substantial improvement in terms of sensitivity, specificity to different malaria species and stages, as well as robustness against the imperfectness of microscopy images. The other challenge is to improve the performance while keeping the computational costs low. Thus, the motivation of my PhD work was to develop a neural network-based method to support and facilitate the diagnosis of malaria in thin blood films. The basis for this objective was a thorough investigation of the morphological and optical changes in RBCs during the intra-erythrocytic cycle to find characteristic properties, which could be taught to the network. To achieve this goal, I planned to prepared a data base consisting of single RBC images, measured with three different imaging techniques (Atomic force, fluorescence, and light microscopy). Following this step, my aim was to develop a NN and test its applicability on cell images and one-dimensional cuts representing characteristic features of the stages.

3 Novel scientific achievements

During the first phase of the project, I studied the morphological and optical changes in RBCs during the maturation of malaria parasites with atomic force, fluorescence, and light microscopy. From these measurements, I determined height profiles and fluroescence patterns characteristic to the intra-erythrocytic stages [Publication 1]. The atomic force and fluorescence microscopy experiments were carried out in the laboratory of the Semmelweis University with the participation of the author and Miklós Kellermayer, while the sample preparation and light microscopy measurements were performed in the BME-TTK Malaria Research Laboratory by the author.
Based on the results of the morphological and optical analysis, I developed a neural-network based stage-specific detection of malaria, which can quantify the intra-erythrocytic stages with and accuracy of $> 96\%$. To my knowledge, no application has been presented before that can detect all malaria stages in images obtained with three different imaging techniques with such precision. The development of the network was assisted by János Török.

The most important results obtained in this work are summarised in the following thesis points.

1. High-parasitemia cultures for in vitro analysis of *P. falciparum* under physiological conditions
I tested two methods for the immobilisation of RBCs on a glass surface in liquid environment by exposing them to cantilevers of different spring constant. Further-

more, I calculated the required concentration of RBCs to obtain a single cell layer on the sample surface and confirmed the calculated value by tests with various concentrations. With the determined settings, I successfully imaged healthy RBCs under physiological conditions. Based on these experiments, I derived a sample preparation routine for imaging RBCs under physiological conditions, which can also be applied for imaging of the autofluorescence in living cells. These results provide the basis for the studies of optical properties in RBCs infected with *P. falciparum*.

2. Exploring characteristic features of malaria-infected RBCs
To create a data base for studies of morphological and optical properties of malaria-infected RBCs, I recorded all atomic force and fluorescence microscopy images used in this thesis. I designed an algorithm to extract single cells from microscopy images and evaluated its performance. I found that the algorithm can detect cells with a precision of more than 95%. Furthermore, I showed that the presented detection method is not limited to a certain type of imaging technique. This was a crucial step for the analysis of single RBC images, which showed that the morphological and optical changes during the maturation of the parasite are reflected in the height and fluorescence intensity profiles of the host cell. Furthermore, I revealed diagnostic patterns in the topographical structure of infected erythrocytes, showing close correlation with their fluorescence map. I showed that atomic force and fluorescence microscopy can be used to locate hemozoin crystals based on the topographical and optical features. These observations revealed connections between the structure and the hemozoin content of RBCs, and their consequences on the optical properties. I further confirmed statistic significance between average profiles characteristic to the intra-erythrocytic stages. Additionally, I showed that malaria-infected RBCs under physiological conditions emit fluorescence patterns similar to the patterns I observed in thin blood films. **Publication 1**

3. Reduction of dimensionality as a tool for feature selection in RBCs boosts the stage-specific classification of *P.falciparum*
I showed that in malaria-infected RBCs, the most important features are associated with the presence or absence of the parasite. I determined the position of the parasite by using the gravitational centre, which is shifted by the presence of the parasite. I further tested the influence of background noise on the position of the gravitational centre and showed that removing background significantly improved the localisation method. In the next step, I used this method to reduce the two-dimensional images of single RBCs to the parasite cut, which goes through the parasite, and three additional cuts spanning 90^o and $\pm 45^o$ with it. The experiments revealed that this method for parasite localisation works with high accuracy on atomic force, fluorescence, and light microscopy images, providing the characteristic properties of RBC in the form of four one-dimensional cuts.
To find a suitable network for the stage-specific classification of RBCs, I tested

various architectures of networks. In the next step, I used the network with the highest performance for the classification of single RBC images and the cuts characteristic to the parasite features. I found that the network reaches a sensitivity of $> 87\%$ on the 2D images. By the smart reduction of data dimension to $2n$ with a careful selection of features, I significantly boosted the performance of the NN-based classification to $> 96\%$, independent of the microscopy technique. I demonstrated that the characterisation method I developed in my work captures the most important features of extracted, single RBCs from microscopy images and provides a reliable tool for an automatised stage-specific recognition of malaria. **Publication 2**

4. The malaria stage classifier
I developed a software package for the neural network-based stage-specific detection of malaria. I wrote a user friendly interface and implemented dimension reduction method introduced in the previous point. In the algorithm, I implemented the cell detection introduced in the second point. I further wrote a documentation, detailing the use and implementation of the package. In addition, the package provides a pre-trained NN for the blood stage classification and further offers the possibility to retrain the NN with additional data. **Publication 3**

4 Publications related to the thesis points

1. **Preißinger, K.**, Molnár, P., Vértessy, B. G., Kézsmárki, I. & Kellermayer, M. Stage-Dependent Topographical and Optical Properties of Plasmodium Falciparum-Infected Red Blood Cells. *J Biotechnol Biomed* 4 (3): 132-146 (2021). doi: 10.26502/jbb.2642-91280040 **IF: 5.3**

2. **Preißinger, K.**, Kellermayer, M, Vértessy, B. G. Kézsmárki, I. & Török, J. Reducing data dimension boosts neural network-based stage-specific malaria detection. *Sci. Rep.* 12(1):1-14 (2022). doi: 10.1038/s41598-022-19601-x **IF: 5.5**

3. **Preißinger, K.**, Kézsmárki, I. & Török, J. An automated neural network-based stage-specific malaria detection software using dimension reduction: The malaria microscopy classifier. *MethodsX* Accepted **IF: 2.2**

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