# Development of a cell microarray chip system for early and accurate malaria diagnosis

## — Finding one parasite in 2 million erythrocytes for elimination of malaria —

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**[Translation from *Synthesiology*, Vol.10, No.1, p.33–40 (2017)]**

Early and accurate diagnosis of malaria is needed to prevent the spread of this parasite. To this end, we developed a novel microarray chip system for the detection of malaria, and evaluated it in Africa. A chip with approximately 20,000 microchambers was developed to detect malaria parasites (hereafter called a cell chip). Leukocytes were removed by filtration columns from whole blood cells. An erythrocyte suspension containing fluorescent nuclear staining dye was dispersed onto the cell chip surface and washed, creating an erythrocyte monolayer in each microchamber that contains more than 2 million erythrocytes. Malaria parasite-infected erythrocytes are then detected using a fluorescence detector. Accurate and rapid detection of the parasites with high sensitivity was achieved by the developed system.

*Keywords* : Malaria, diagnosis, cell microarray chip, field work

## 1 Background of research

Malaria is an infectious disease transmitted by anopheles mosquitoes, and is one of the three major infectious diseases of the world. Every year, about 2 hundred million people are infected and 430 thousand people die. As a global strategy for suppressing malaria, the development of a quick and accurate diagnostic method is given as one of the most important issues.[1] Malaria diagnosis is done by microscopic observation of thin blood films stained with Giemsa stain (the Giemsa microscopy) and this has been the gold standard for over 100 years. This is an excellent method that not only detects malaria, but also enables diagnosis of the infection rate (severity). It is conducted in the following steps: (1) a drop of a patient's blood is collected, (2) a thin blood film is prepared on a slide glass, (3) the thin film is stained using the Giemsa solution, and (4) malaria parasites in the stained erythrocytes are observed through the microscope. However, accurate diagnosis cannot be made without a technician skilled in this method. Also, time is necessary (normally, about 30 min to 1 h), and it is a labor-intensive work since several thousands to several ten thousands of erythrocytes must be observed. Therefore, quick diagnosis is difficult with this conventional gold standard method, and early diagnosis of patients with low infection rates is impossible.

In the current medical settings of developing countries, rapid diagnosis tests (RDT), which employ immunochromatography as the principle and can be conducted by easy operation in a short time (20 min), are widely used. However, the detection limit of RDT is equivalent to the analysis of Giemsa microscopy, and incidences of false-positives and false-negatives are common. Therefore, it is used as a preliminary screening method prior to the definitive diagnosis by the Giemsa microscopy. It is not possible to calculate the infection rate with RDT (i.e. diagnosis of infection only), and this is one of the disadvantages. Recently, new diagnostic methods utilizing the flow cytometer and polymerase chain reaction (PCR) have been developed, but the sensitivity is insufficient for early diagnosis, and several hours are required before the results are obtained, respectively.[2]–[6] To prevent infection by early detection of malaria, development of a new diagnostic method with high sensitivity, accuracy, quickness, and easy operation is demanded. The characteristics of each diagnostic method are shown in Table 1.

We focused on the microchip technology to conduct analysis at high throughput, ultra-high sensitivity, and with ease for individual cells.[7]–[10] In this paper, we shall describe the "scenario" in which this technology was applied to develop a device that enabled highly sensitive diagnosis in an actual malaria-endemic region (Fig. 1).

## 2 Development of the cell chip for malaria diagnosis

In starting the development of a malaria diagnostic method, we considered it necessary to be able to measure the infection rate (what percentage of a patient's erythrocytes is infected with malaria parasites) that showed the severity of malaria, as well as the presence of infection, in order to diagnose the

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Original manuscript received February 3, 2017, Revisions received February 21, 2017, Accepted February 21, 2017

**Table 1. Comparison of malaria diagnostic methods**

| | Giemsa microscopy | Rapid diagnosis test (RDT) | Polymerase chain reaction (PCR) | Flow cytometer | Cell chip |
|---|---|---|---|---|---|
| Detection limit | 0.01 % | 0.01 % | 0.0005 % | 0.0005 % | 0.00005 % |
| Detection time | 60 min | 20 min | 6 h | 5 min | 15 min |
| Difficulty level | Ultra-high | Low | High | Low | Low |
| Cost※ | Low | Medium | High | Ultra-high | Medium–High |

※Does not include cost of labor

disease at the medical settings of developing countries and then to engage in effective treatment. That was because there were possibilities of delayed early diagnosis, and increased risk of appearances of drug-resistant parasites if drugs were prescribed according to symptoms only such as fever. Therefore, we did not select improving the existing methods such as PCR and immunochromatography that merely detect the presence of infection. That is, we thought the method of counting the number of parasites was good, considering the facts that the Giemsa microscopy was considered the gold standard, was most universally used, and had high accuracy, while the PCR and immunochromatography methods had problems of producing false-positives and false-negatives. Among the existing methods, the method of counting cells included the flow cytometer method, and it was possible to calculate the infection rate. However, it was necessary to increase the number of measured cells to increase the measurement precision, and if one attempted to detect the low infection rate of one parasite in several million erythrocytes, 100 or more parasites had to be counted (refer to https://www.bc-cytometry.com/FCM/immunologyFCM_02.html). Taking into consideration the ratio of one in several million cells, it meant that over a hundred million erythrocytes had to be counted, and this would lengthen the detection time and would not lend to a quick diagnostic method. Moreover, it was too expensive for practical application if it were to be used or sold in African developing countries. In such conditions, our research group decided to utilize microfabrication technology used in the fields of MEMS and μTAS, to apply the technology of individually placing the cells on plastic substrate microarrays to erythrocytes, to arrange as many erythrocytes in a monolayer, to seek out erythrocytes infected with malaria parasites, and then to calculate the infection rate.

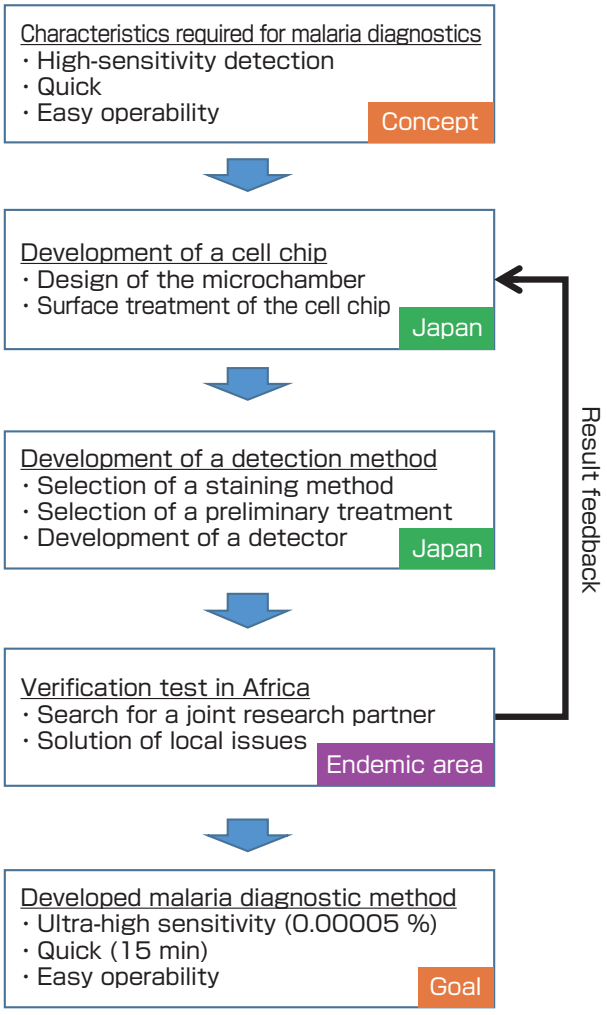


**Fig. 1 Flowchart of how this research was carried out (scenario)**

While details will be explained later, when erythrocytes infected with malaria parasites and their nuclei stained by fluorescence are placed on a cell chip, the erythrocytes are deposited in a monolayer in the microchamber, and it becomes possible to detect the parasites only. The goal was set of detection limit equivalent or higher than the PCR method, and we considered fabricating a polystyrene cell chip with about 20,000 microchambers. For the optimization of the cell chip, the major premise was to control the number of cells to be measured at a constant number to facilitate the calculation of the infection rate. The flow cytometer method is designed to calculate the number of measured cells by counting the erythrocytes with a detector while allowing the cells to flow along, but our goal was to control the number of cells at a certain level quickly and accurately without counting, or in other words, to accurately arrange a certain number of individual cells by advancing and developing a new design of the conventional cytobiological handling technology. When we came up with the design of cell chips

that might enable the arrangement of a certain number of cells without complicated maneuvers, we intuitively knew this method was a breakthrough. After numerous discussions with joint researchers, we thought it would be possible to arrange a large number of cells in a monolayer in each microchamber by controlling the diameter and the depth of the microchambers. We also thought that the optimal diameter of the microchambers would hold about 100 erythrocytes, and this took into consideration that if one parasite was present in the chamber, the infection rate would be 1 %, and it could also be easily used in the following culture and reaction such as in drug resistant tests. That is, to conduct a drug resistance test by a conventional method, it was necessary to use whole blood, but using this cell chip, it was only necessary to target the cell in the chamber in which the infection was found, and if there were 100 cells, there would be very few problems in conducting the test. Considering the above factors and the size of the erythrocytes, the diameter was set as 105 µm. Since we were also aiming for detection limit equivalent or higher than the PCR method, we determined the number of necessary microchambers. The final designed cell chip has 20,994 microchambers, and each individual chamber has a diameter of 105 µm and a depth of 50 µm (Fig. 2).

Since there was a possibility that infected erythrocytes would not be seen if the erythrocytes were stacked up on top of each other in the microchambers, it was necessary to arrange them in a single layer to enable analysis by microscope or a microarray scanner. When the erythrocytes were statically placed for 10 min on the cell chip, the erythrocytes gathered in the chamber by gravity. However, it was found that when the surface of the cell chip was gently washed with physiological saline, only the erythrocytes of the lowermost layer that adhered to the bottom of the chamber remained, the others were washed away, and the single-layering of erythrocytes was achieved (Fig. 3). The number of erythrocytes in each chamber was almost constant at 130 ± 6 cells, and it became possible to control the erythrocytes to a certain number without counting. This cell chip allowed analysis of about 2.7 million erythrocytes at once, and we obtained the detection limit equivalent to or higher than the PCR method.

The design of a microchamber that allowed monolayer arrangement of erythrocytes by a simple maneuver was done by trial-and-error using various shapes (such as diameters and depths). Recently, the flow of fluid within the microchamber was simulated by a computer, and it was shown that there was almost no flow at 10 µm from the bottom.[7] This provided theoretical support that the erythrocytes of the lowermost layer would not be removed by the washing procedure. In the future, we believe this simulation technology will be useful for the design and optimization of the microchambers of different sizes. Moreover, the important factor in monolayer arrangement of erythrocytes in the microchamber is the hydrophilization of the cell chip surface by oxygen plasma treatment. This treatment will increase the adhesion of erythrocytes to the chamber bottom, and monolayer formation is more easily controlled. The hydrophilization of plastic substrates is Yamamura's specialty, and he has conducted hydrophilization treatment optimized for various

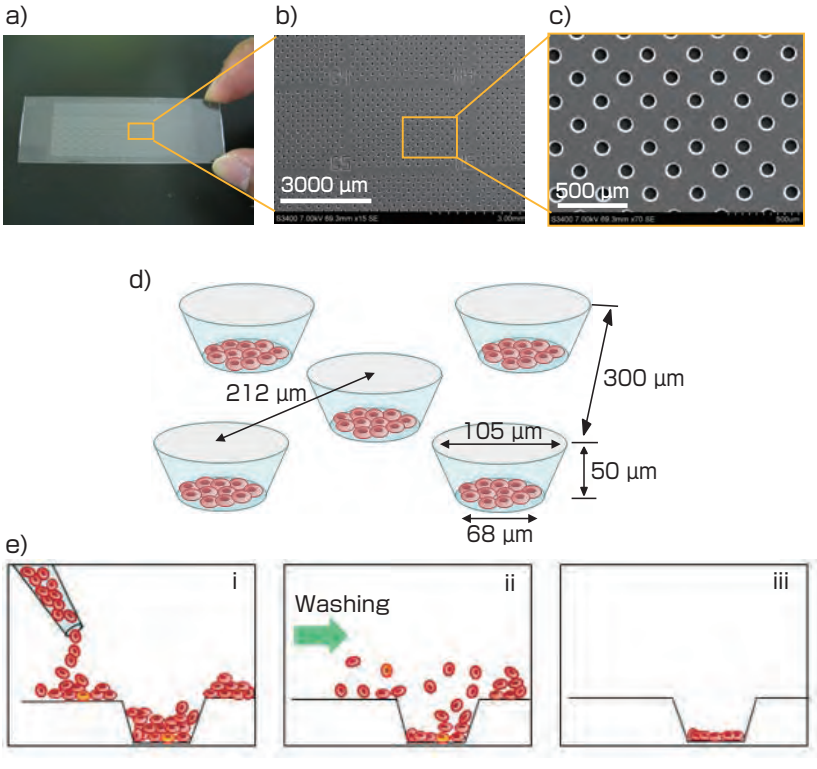


**Fig. 2 Structure of the cell chip and single layering of erythrocytes in the microchamber[8]**
(a) The size of the cell chip is about the same as the slide glass. (b, c) SEM image of the cell chip. The cell chip is made of polystyrene, and has 20,944 microchambers. It is formed by arrangement of 112 clusters (14 × 8) composed of 187 microchambers. (d) The microchambers have diameters of 105 µm and depths of 50 µm, and are separated from each other by 300 µm. The bottom of the chamber has a diameter of 68 µm, and is cone-shaped. This shape is appropriate for single-layering the erythrocytes. (e) The process by which the erythrocytes become arranged in a monolayer in the microchamber is shown: (i) erythrocytes are placed on the cell chip, (ii) erythrocytes that are stacked on top of each other are removed, and (iii) erythrocytes are arranged in a monolayer.

cells before this project was started. Hence, we were able to conduct optimization for erythrocytes smoothly.

## 3 Development of the method to detect malaria parasites on the cell chip

With the development of the cell chip, it became possible to arrange the erythrocytes in a monolayer and it became easy to keep the number of cells to be measured at a certain number. Next, we investigated the parasite detection method that was suitable for the cell chip. For parasite detection, the basic concept was to keep the maneuver as simple as possible. Therefore, it was not desirable to have methods that required imparting membrane permeability using surfactants or others, multiple washing procedures, or antibodies that specifically identified the parasites. We considered using the characteristics that erythrocytes do not have nuclei and that malaria parasites are parasitic only to erythrocytes. That is, we decided to stain the nucleic acid (DNA) that is the index of the presence of a nucleus using a fluorescent reagent, and to flag the erythrocyte with a fluorescent signal as an infected erythrocyte.

The concept of the above detection method was to keep the maneuver as simple as possible, and we set the requirements for the nucleic acid staining method as follows: parasites will be kept alive (no need for fixing), staining can be done easily and quickly (no need for washing), and detection can be done by a fluorescent method at high sensitivity. We compared almost all commercially available fluorescent stains that stained DNA and RNA (regardless of membrane permeability). As a result, we found that the SYTO reagent had excellent membrane permeability and stable staining, and we selected SYTO21 that had low background.

To apply this method to actual malaria patients, it was necessary to remove the leucocytes that were nucleated cells from the whole blood. Often, it was difficult to use the centrifuge in malaria-endemic areas due to lack of stable electric power supply. Therefore, we used several types of push columns that did not require centrifuge, and compared the amount of necessary blood and the removal rate of leucocytes. As a result, we confirmed that 99.9 % or more of leucocytes (standard WBC: 3200–9000/μL, RBC: 3.6–5.0 million/μL) could be removed from the whole blood using columns of silicon oxide nanofibers made by Panasonic Corporation. This push filter could be used for patients with sickle cell disease that was prevalent in the malaria-endemic areas.

As a detector, we used the CCD camera system (EZBLMLH01T) with a fluorescent detector from Panasonic. To complete the cell chip scanning in five minutes, it was equipped with a 480 nm semiconductor laser, objective lens, an optimized fluorescence detection filter, and an XYZ axis automatic positioning stage. To detect the parasite, it is possible to detect the fluoro-positive erythrocyte by setting the cell chip in the detector and pressing the “Analyze” button. The resolution of this device is 1.1 μm. It is possible to detect the parasite-infected erythrocyte specifically from the form of fluorescence. The malaria-infected erythrocyte was set as having 1.3 times or more and 7.5 times or less of fluoro-signal intensity compared to non-infected erythrocytes. It was programmed to detect as noise when the aspect ratio (vertical to horizontal ratio) of the fluorescent spot was 2.8 or more, or when the surface area was 4.8 $\mu m^2$ or less or 45 $\mu m^2$ or more. These figures were actually determined by comparing the blood samples of many malaria patients by microscopic observation of cell chip and Giemsa stained images, identifying the source of fluorescence (leucocyte, platelets, fragments of broken cells, etc.), and by specifically detecting malaria parasites only. The workflow from blood sampling to parasite analysis is shown in Fig. 4.

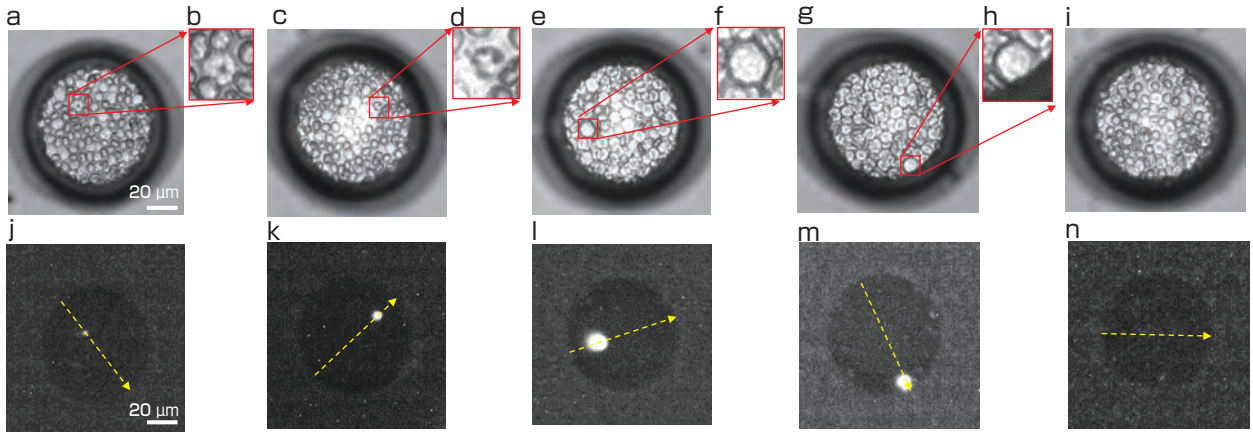


**Fig. 3 Separation of the leucocytes and infected erythrocytes in the microchamber**
(a,c) The bright field microscope images of infected erythrocytes in the microchamber. (b, d) Enlarged photographs of the infected erythrocytes, and (e, g) the bright field microscope images of cells in whole blood in the microchamber. (f, h) Enlarged photographs of the leucocytes, and (i) a bright field microscope image of non-infected erythrocytes in the microchamber. (j–n) Fluorescent images of cells in each chamber stained with SYTO21 and photographed with a CCD camera. It can be seen that the signal intensities and sizes differ greatly between malaria parasites and leucocytes.

## 4 Analysis using the cell chip in the endemic area

Gulu District, Uganda is a highly malaria-endemic area, and the research base for the verification test was established in this district. The research base was Lector Hospital, and Professor Horii of Osaka University and Professor Mita of Juntendo University were conducting field research to develop vaccine and to study drug-resistant malaria, and we were able to accompany them. We were extremely grateful since it takes time and effort to find joint research partners from scratch, including the signing of agreement for joint research. We conducted the microscopic observation to analyze the Giemsa stained image of blood samples of 41 patients who visited the hospital. We found parasites in 37 patients, and the infection rate was from 0.0039 % to 2.34 %. Analysis was done using the cell chip for the same samples, and the infection rate was 0.0033 % to 2.39 % (Fig. 5). The values of infection rates calculated by the Giemsa microscopy and the cell chips were almost the same, and showed positive correlation ($R^2 = 0.9945$).

In reality, there were major problems in obtaining the above data. First was the issue of the power source. Gulu is the second largest city in Uganda after its capital Kampala, but it was subject to frequent power outage. Since the power outage could continue for half a day or more, a high quality uninterruptible power supply system was necessary. Also, the transportation of supplies was difficult. Since about two travel days were necessary from Japan to Gulu, reagents that required refrigeration could not be used. When the machineries were checked in with the airlines, they were

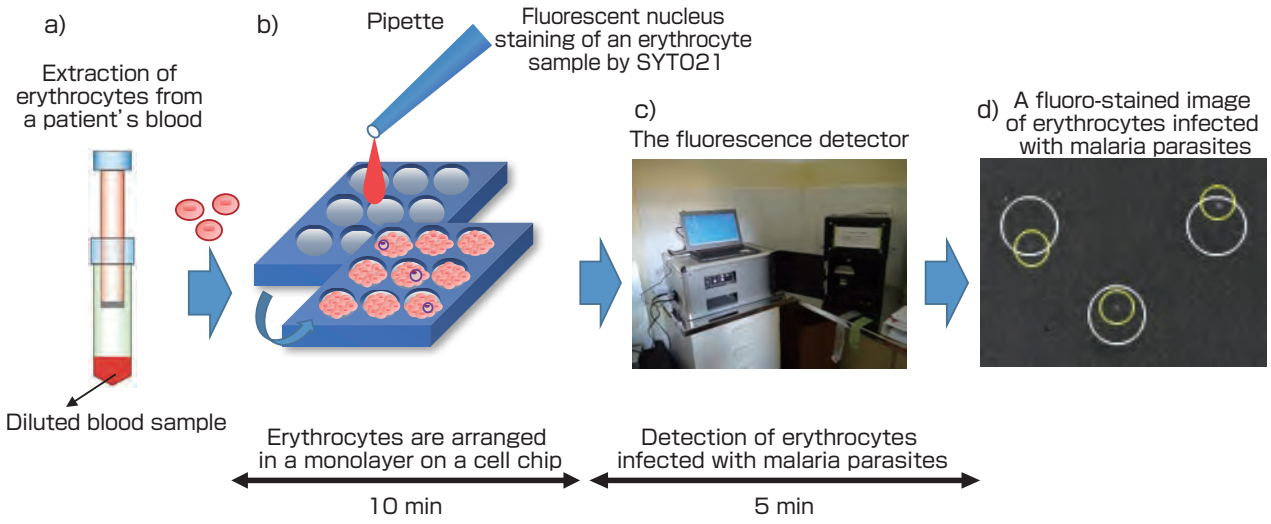


**Fig. 4 Workflow of the detection of erythrocytes infected with malaria parasites, using the cell chip[8]**
(a) Erythrocytes are isolated from the patient's whole blood using push columns. (b) Erythrocytes are stained with fluorescent nucleus staining reagent (SYTO21), to stain the nuclei of malaria parasites. Then, erythrocytes are placed statically on the cell chip to form a monolayer in the chamber. (c) Malaria parasites are detected by detecting the fluorescence. (d) Number of detected parasites is counted by automatic image analysis. It is possible to calculate the infection rate since the number of erythrocytes in the chamber is constant.

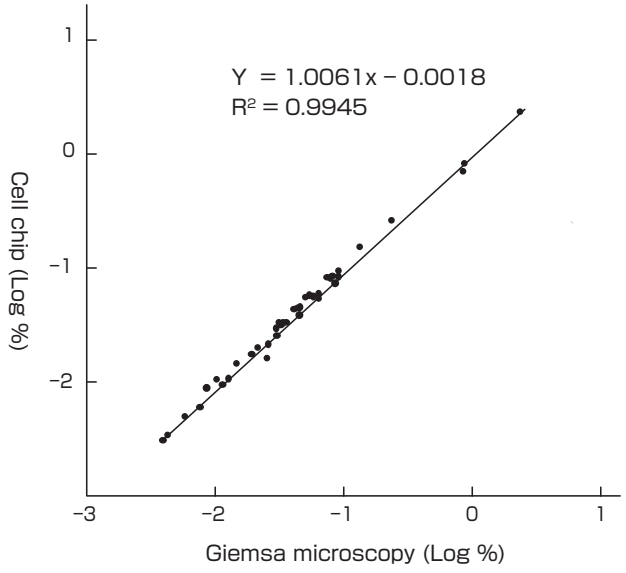


**Fig. 5 Measurement of the infection rate using cell chips in the malaria-endemic area[8]**
Using the cell chip (vertical axis) and Giemsa microscopy (horizontal axis), the infection rates of patients infected with malaria were calculated, and comparative analysis was conducted for Uganda

handled roughly and were broken, and there was no way of repairing them locally. We became painfully aware of the importance of selecting reagents that were stable at room temperature, downsizing the machines and devices, and obtaining reliable transportation routes.

When the above problems were solved, we thought all we had to do was to set up the experimental apparatuses and collect data. However, at the start of field research, we received almost no blood samples of patients. This was puzzling because there were many malaria patients visiting the hospital which was our joint research partner. When we asked the joint research physician, we found that the flow of action from obtainment of informed consent for research by the local physicians from the patients, the sampling of blood samples, and the transportation of samples to us was not functioning smoothly. We understood that the hospital was always full of patients and busy. It was necessary to talk to the personnel to have them understand the importance of this research. The blood samples of the 41 patients that we received after experiencing such hardship are very valuable. To publish this diagnostic system to the internationally recognized World Malaria Report, we need to obtain data of about 500 cases. From the perspective of hospitals in Japan, it may seem easy to gather 500 cases, but we face hardship in Africa.

## 5 Discussion and future prospects

Our cell chip technology has the potential of detecting one malaria parasite from 2.7 million erythrocytes (infection rate of 0.00005 %). In the research up to now, we have succeeded in detecting the parasites from blood of infected patients at an infection rate of 0.0033 % in the malaria-endemic area, and this surpassed the detection limit of the Giemsa microscopy (0.01 %) that is the current gold standard. That is, it is possible to detect the early stages of infection that cannot be detected by the Giemsa microscopy. The figure 0.0033 % is about two digits higher than 0.00005 %, but this is because most patients visit the hospital at advanced stages of infection. If we can obtain blood samples at early stages immediately after infection, we believe detection at a lower infection rate will be possible. On the other hand, for the problem of false-positives, out of the 41 samples, detection was not possible even by the cell chip method in four samples that was not detected by the Giemsa stain method. There were samples that turned out positive by RDT and PCR methods, and it was shown that false-positives can easily occur. For false-positives by the PCR method, cases where parasite DNA remained in the blood after treatment of malaria had been reported.[11] One of the major characteristics of the result of the cell chip is that false-positives do not occur since the malaria parasite that is actually inside the erythrocyte can be detected and visualized.

Currently, the treatment of malaria is done by artemisinin-based combination therapy (ACT). Appropriate diagnosis is important for reducing the cost and to prevent the occurrence and dispersal of drug-resistant malaria by preventing the overuse of ACT.[12] That is, the development of a high-throughput and high-sensitive diagnostic method contributes in controlling malaria, by maximizing the use of existing treatment drugs without necessitating new drug development that requires enormous amount of time and cost. The diagnostic method using the cell chip is highly sensitive, quick, and easy to handle. Moreover, the supplies needed for this diagnosis (cell chips, SYTO21, push columns, etc.) are relatively inexpensive (2 USD or less), but there are issues that the detection device is expensive (8,000 USD), bulky and inconvenient to carry, and is difficult to use in fieldwork such as at villages and elementary schools.

Prototype detectors are being developed for downsizing and cost reduction (WHO aims for 1 US or less for cost of a test per person), and we hope to create a device that can be used in wide ranging fields and is affordable in malaria-endemic regions, and we will work toward commercialization. In the future, by advanced diagnosis of malaria, we hope to enable treatment at early stages of infection without inducing drug resistance, and to contribute to the suppression of malaria. For advanced diagnosis, ultra-high sensitive detection of malaria parasites is insufficient, and the development of a “highly functional diagnostic cell chip” that can identify five parasite species and can check whether there is drug resistance is essential. Currently, we are engaging in this development.

## Acknowledgements

The result of this research was obtained by the support of the following: the Ministry of Education, Culture, Sports, Science and Technology; Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science), Japan Agency for Medical Research and Development; and Global Health Innovative Technology Fund. For the development of the detection device, we thank the people of the Biosensing Development Division, Sensing Solution Development Center, Technological Headquarters, Automotive & Industrial Systems, Panasonic Corporation. For the field testing in Uganda, we are thankful to the people of the following: Lector Hospital, Gulu, Uganda; Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University; and Seminar for Tropical Medicine and Parasitology, School of Medicine, Juntendo University.

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## Discussions with Reviewers

### 1 Overall

**Comment (Hiroaki Tao, AIST)**

This paper is about the development of a device for early detection of malaria infection, which is one of the three major infectious diseases of the world. It explains the concept of a

diagnostic method to be used locally in Africa, the selection of reagents and development of a cell chip that is original technology to realize the concept, comparison with other methods such as the Giemsa stain method that is the gold standard, the problems and solutions in using the device for a verification test in Africa, and the future prospects. It will be good reference for researchers who are working on the R&D of diagnostic devices for practical use in society, and it is significant as a paper to be published in *Synthesiology*.

**Comment (Noboru Yumoto, National Cerebral and Cardiovascular Center)**

This paper describes the process of developing an accurate, highly sensitive, quick, and easy-to-operate diagnostic device, by utilizing the microchip technology in malaria diagnosis. To solve the issues that were difficult to solve by conventional diagnostic methods, it progresses all the way to local field testing with a clear scenario and international industry-academia-government collaboration. I think it is suitable as a paper for *Synthesiology*.

## 2 Clarification of the scenario

**Comment (Hiroaki Tao)**

I think this research was done (scenario) under the strategy of advancing the diagnostic method by repeating the four processes of "concept of diagnostic method" → "R&D of device and reagent to realize the concept" → "verification test in Africa" → "discovery of new technological development needs," and then practical use in society. I think the overall research will be better understood if you show this as a diagram at the end of Chapter 1. Why don't you talk about the simulation of fluid flow within the chamber and the hydrophilization treatment to control the adhesion of the lowermost cell? I think it will be good to state that you have theoretical support by simulation rather than designing only by experience, and that the simulation technology is useful in the optimization and design of chambers of different sizes. Also, other cell chips have been designed so that only one cell would fit in each chamber. I think you should describe the advantage of your cell chip compared to those.

**Comment (Noboru Yumoto)**

For research papers of *Synthesiology*, the requirement is the authors' originality for the scenario and the element constitution (selection and integration). While the current manuscript allows grasping of the overall image, it is rather difficult to understand how the authors synthesized the elements based on the scenario. I think the key to the realization of the scenario is the development of the cell chip. I think this was realized by combining the technological elements such as the selection of the substrate, design, processing, and surface treatment, and I feel there isn't much description of those elements. Also, for the field test, I think you should explain what issues were set forth by WHO and what you were trying to solve, and how you found the partners of joint research.

**Answer (Muneaki Hashimoto)**

• I added the flow chart of the scenario as Fig. 1.

• I added the technological elements for fabricating the cell chip as much as possible. For simulation, I added an explanation to the final paragraph of Chapter 2 citing a recently published paper, and stated that it would be useful in the future. I additionally wrote about hydrophilization treatment in the same paragraph. In this cell chip, about 100 erythrocytes are arranged in one microchamber, and if the infection rate is 1 %, one signal is detected in one chamber. Therefore, one of the aims of this cell chip is that when the infection rate is at a certain level, it is not necessary to analyze multiple chambers, and that will be its advantage. I added this point to the text. For the selection of the substrate, we actually tried several plastic materials, but the results were not much different, and therefore, it was not mentioned in this paper.

• For the issue raised by WHO, priority was cost. For the cell chip, some progress can be made on the running cost, but the problem is the expensive detection device. The one that we are developing jointly with companies have the same detection principle, but I think it will clear this cost issue. WHO states that the cost of analysis per person must be 1 USD, and I clearly wrote down this point in Paragraph 3, Chapter 5.

• On how we found our joint research partner, I added some lines to Paragraph 1, Chapter 4.

## 3 Comparison with existing methods

**Comment (Hiroaki Tao)**

I think the readers will better understand the overall picture of the current malaria diagnostic methods and the characteristic of this method, if you create a table of the principles, advantages, and disadvantages of this cell chip method along with Giemsa stain, immunochromatography, PCR, and flow cytometer methods.

**Answer (Muneaki Hashimoto)**

I newly created Table 1.