Imaging Motile Pathogens with Light Microscopy and Cryogenic Electron Tomography

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Introduction

The most prominent vector-transmitted diseases in the first and third world are Lyme disease and malaria, respectively. In both cases the transmitted agents are introduced into the skin from where they eventually disseminate into the blood using active motility. We are interested in deciphering the molecular mechanisms underlying the motility of these pathogens and how they relate to the ultrastructure of the pathogens. Here we provide an overview of the microscopy techniques that we use to achieve these goals.

Malaria

Malaria is transmitted during the bite of an infected female *Anopheles* mosquito (Figure 1) and causes around 500 million infections resulting in 1-2 million deaths per year. Contrary to common belief, the mosquito rarely injects parasites directly into the blood, but rather deposits them in the dermis [1-2]. These parasites, *Plasmodium* sporozoites, are formed in parasitic cysts at the gut wall of the mosquito from where they enter the insect's circulatory system, the haemolymph, and eventually enter the salivary glands [3]. There they migrate through the saliva producing cells and accumulate in the cavities from where they slowly progress through the salivary duct system. Only the parasites that have actively migrated into the salivary canal system can then be transmitted during a bite, when they are flushed out with the saliva [4]. Once in the skin, the sporozoites speed up considerably and move on seemingly random trajectories through the tissue and eventually enter either blood or lymph vessels [1, 5]. The sporozoites that enter lymph vessels accumulate in the draining lymph nodes where they are destroyed, while the sporozoites that enter the blood can reach the liver [1]. In the liver, sporozoites migrate on endothelial cells and pass through resident macrophages and several hepatocytes before invading a hepatocyte to further differentiate into tens of thousands of red blood cell-invading parasites that ultimately cause malaria, (reviewed in Prudencio et al. [6]). Clearly, without their motility, sporozoites, as well as other stages of the parasite, would not be able to successfully infect a host [7]. We are interested in understanding the molecular and biophysical principles involved in parasite migration. The parasite is thought to use an acto-myosin motor linked to the substrate via an integrin-like protein (TRAP) to produce forward motion [7-8]. Purified *Plasmodium* actin filaments are short, measuring only up to 100 nm and have never been visualized *in vivo* in a malaria parasite [9-10]. Similarly, no distinct adhesion sites of the sporozoite to the substrate have been observed. Curiously, as shown in Figure 2, sporozoites move in a circular fashion on flat supports, which facilitates their investigation with light microscopy [11-12]. These parasites are less than 1 μm thick, thus allowing the use of electron microscopy to examine them *in toto* without the need for sectioning. We first developed a set of automated tracking tools that can classify different movement patterns and motility parameters such as average speed, motility pattern, and curvature [11]. This allowed the determination of the effect of known inhibitors of actin dynamics in a highly accurate manner. We currently utilize laser tweezers to probe the adhesion properties and examine the traction forces of parasites in the presence and absence of drugs as well as parasites lacking different proteins that could potentially be important for sporozoite adhesion and migration. We also use surface sensitive light microscopy methods such as reflection interference contrast microscopy as well as total internal reflection fluorescence microscopy to investigate sporozoite adhesion dynamics. This indeed revealed the existence of individual adhesion sites and showed that their dynamic turnover is important for motility. Finally, to investigate the structure of these adhesion sites and reveal the role of actin filaments, we employed cryogenic electron tomography

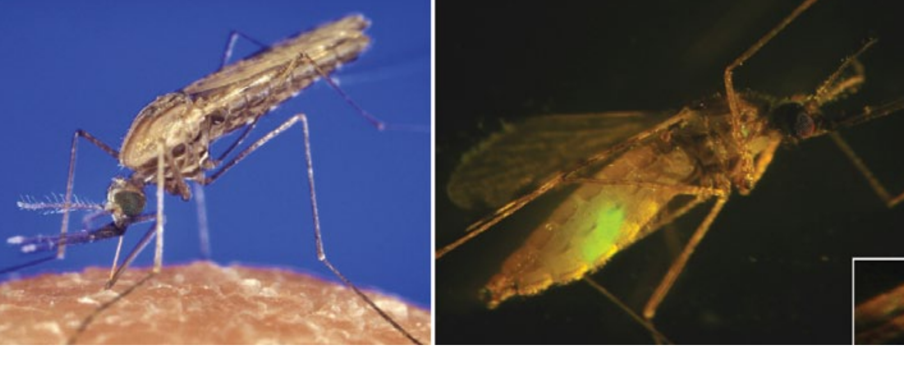

Figure 1: Malaria vectors, *Anopheles gambiae* (left) and Anopheles stephensi infected with green fluorescent *Plasmodium berghei* parasites (right). Note the green drop of saliva at the end of the proboscis indicating extruded parasites (arrow and inset). Images by James Gathany, CDC, and Sylvia Münter, Department of Parasitology, University of Heidelberg Medical School.

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Figure 2: *Plasmodium berghei* sporozoites moving in circles. A sequence of three parasites expressing GFP and gliding on glass (0 s, 10 s, 20 s). The right shows a merged image (the first frame in red; the second in green and the last in blue). Recorded on a spinning disc confocal microscope (Nikon TE 2000-E), using a 63x objective lens. Scale bar = 5 μm.

(CET), which has been successfully used to visualize actin filaments in thin regions of the slime mold *Dictyostelium discoidum* [13-14], as well as in neurons [15]. It has also revealed long actin-like filaments in bacteria [16-17] as well as short FtsZ filaments [18]. For CET, the sample is placed on an EM grid and plunge frozen in cryogenic liquids such as liquid ethane, which immobilizes intact cellular structures within milliseconds [19]. As shown in Figure 3, CET of sporozoites readily revealed their overall architecture at the morphologically complex front end of the parasite, and we first examined the structure of microtubules that are arranged in a cage-like fashion encompassing different secretory organelles. These microtubules revealed a thick microtubule wall that was extended at the luminal side suggesting the presence of a protein in the lumen of the microtubule, probably to stabilize these cytoskeletal structures [20]. Notably, densities were also found in the flagellar microtubules from sea urchin sperm and *Chlamydomonas* [21-22]. In the malaria parasite it is assumed that the stability of microtubules is important for migration and invasion. However, the dynamics of apicomplexan microtubules are difficult to investigate as these are resistant to all known microtubule inhibitors, thus little data concerning microtubule dynamics is available [23]. However, we could not detect filaments resembling F-actin in several dozen tomograms of the sporozoites that were preserved using non-invasive rapid freezing. This could be due to several reasons, one being that filaments might only be formed when parasites are actively moving. We therefore attempted a correlative microscopy approach: first sporozoites were filmed moving on EM grids before these were transferred to a plunger and rapidly frozen. Then the parasites were observed in the electron microscope. However, few sporozoites could be detected that were localized at exactly the same position. To test whether different blotting procedures could overcome this problem, we introduced a pseudo-plunge freezing method whereby we transferred the EM grid back to the light microscope instead of plunge-freezing it after blotting. This proved a rapid method to evaluate different blotting procedures to test the feasibility of a wide range of cells for such correlative imaging approaches. In our case, unfortunately, we found too few sporozoites that

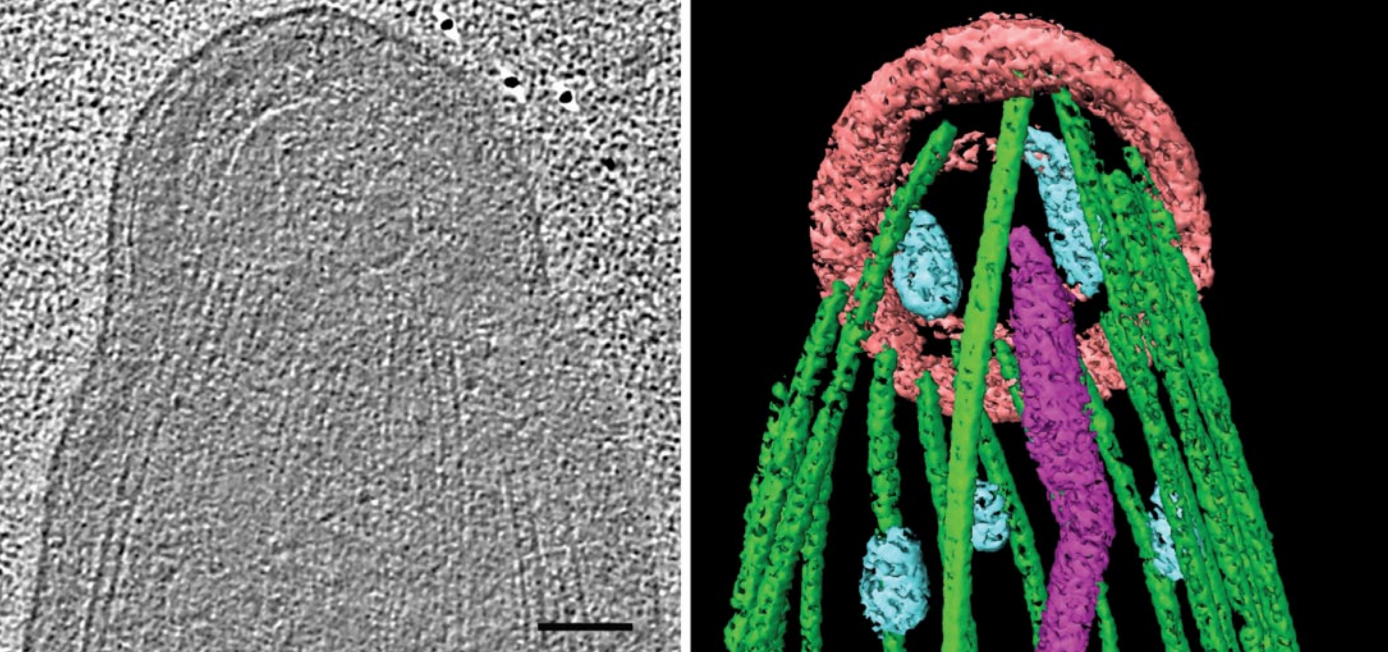

Figure 3: Cryo-electron tomogram of a *Plasmodium berghei* sporozoite. On the left: section through the tomogram showing the apical part of the sporozoite. On the right: surface rendered view of the same area showing: microtubules (green), polar rings - microtubule organising centres (pink), rhoptry (violet), micronemes (cyan). The tomogram was recorded using an FEI CM300 cryo-electron microscope (at MPI for Biochemistry, Martinsried, Germany). Scale bar = 100 nm.

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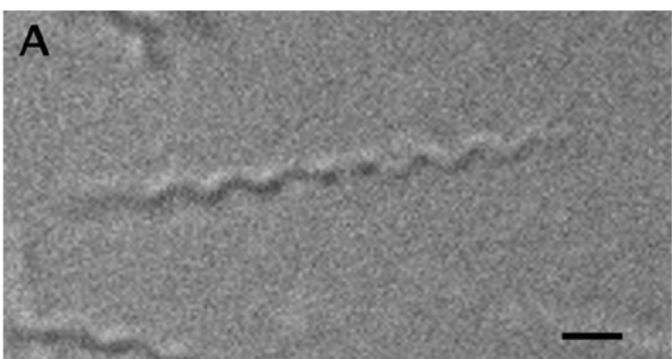
Figure 4: A searching *Ixodes ricinus* tick from Commanster, Belgian High Ardennes (http://www.commanster.eu/commanster.html). Photographs by James K. Lindsey (left) and Richard Bartz (right). Photos from WikiCommons. Both files are licensed under the Creative Commons Attribution ShareAlike 2.5.

were preserved sufficiently enough to successfully implement correlative imaging between light microscopy of living cells and cryo-tomography [24]. Nevertheless, for different research questions an alternative correlative approach will likely prove tremendously valuable when first examining plunge-frozen cells under a cryo light microscope before imaging the same objects with cryo-tomography [25].

Lyme Disease

The causative agents of Lyme disease, *Borrelia burgdorferi sensu lato*, are transmitted during the long bite of an *Ixodes* tick (Figure 4) and cause several tens of thousands of new infections per year in Germany alone. In North America, Lyme disease is mainly caused by *Borrelia burgdorferi,* whereas in Europe *B. garinii* and *B. afzelii* are additional species causing various manifestations of the disease with many more species remaining less well-characterized [26]. *Borrelia* represent one genus within the phylum of Spirochaetes and as such share a similar morphology to other groups of medically important spirochetes, for example *Treponema pallidum* (syphilis). Unlike in other bacteria, spirochetes show a second outer lipid bilayer membrane (the outer membrane sheath) with an external surface layer. The periplasmic space between the cytoplasmic membrane and the outer membrane sheet contains a peptidoglycan layer and periplasmic flagellar filaments that do not protrude from the outer bacterial surface, a prominent feature of the spirochetes [27]. Therefore, the rotation of the flagellar motor leads to the characteristic flat-wave morphology of spirochetes. These morphologies enable the spirochetes to move quickly even in highly viscous solutions. The different *Borrelia* species show different numbers of flagella, but little else is known about their similarity on an ultrastructural level. Using CET we found that, apart from the numbers of flagellar filaments, they appeared indeed largely similar [28]. Nevertheless this study also revealed a number of previously unobserved details: we demonstrated the presence of a surface layer outside the outer membrane sheath; cytoplasmic vesicles at the sites of bacterial cell division; cytoplasmic filaments that resemble those formed by MreB (bacterial actin) and FtsZ (bacterial tubulin) in other bacteria; and long protrusions of the other membrane sheath containing flagellar filaments that probably push the outer membrane out, suggesting that this membrane is rather flexible (Figure 5). We also found numerous



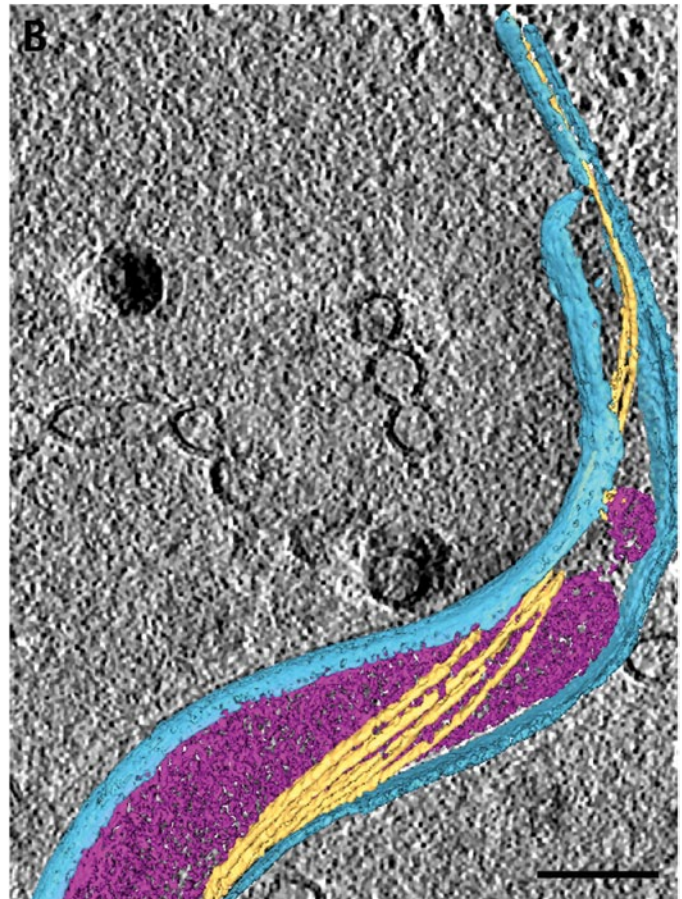


Figure 5: Imaging *Borrelia burgdorferi* cells. A. Phase contrast image. B. Surface rendered structural features of a cryo-tomogram showing: outer membrane (blue), plasma membrane and cytoplasmic cylinder (magenta) and flagellar filaments (yellow). Image recording as described in Figure 3. Scale bars: A = 1 μm, B = 200 nm.

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vesicles budding from the outer membrane, again showing that it does not constitute a rigid cellular wall. These vesicles did not contain cytoplasm, although incubation of *Borrelia* with a borrelicidal antibody against a surface protein leads to the formation of large vesicle-like structures that do contain cytoplasm. We and others also generated structures with less than 5 nm resolution of the flagellar motor from *Borrelia* revealing a number of previously unobserved details of this amazing nanomachine [29-30]. Similar to our work on *Plasmodium* sporozoites [11], we are currently developing methods to rapidly track *Borrelia* to be able to reliably compare motility parameters for imaging *in vitro* in different solutions eventually to provide means to investigate genetically modified *Borrelia* that either lack or overexpress certain genes, with quantitative imaging methods.

Conclusion

Both *Plasmodium* and *Borrelia* represent fascinating biological probes and medically important pathogens. Both can be genetically modified and an increasing genetic toolbox already complements the advanced imaging techniques we have been utilizing and developing over the past years. Thus, there is hope that talented students and post-docs can be attracted to entering these exciting areas of research. They soon might provide insights that will advance our fundamental knowledge and could also contribute to improved treatments for these diseases. MT

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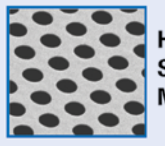
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