Received: 28 October 2024 | Revised: 13 December 2024 | Accepted: 23 December 2024

DOI: 10.1111/jmi.13384

Journal of Microscopy RMS

INVITED REVIEW

Imaging malaria parasites across scales and time

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Funding information
Chica and Heinz Schaller Foundation

Abstract

The idea that disease is caused at the cellular level is so fundamental to us that we might forget the critical role microscopy played in generating and developing this insight. Visually identifying diseased or infected cells lays the foundation for any effort to curb human pathology. Since the discovery of the *Plasmodium*-infected red blood cells, which cause malaria, microscopy has undergone an impressive development now literally resolving individual molecules. This review explores the expansive field of light microscopy, focusing on its application to malaria research. Imaging technologies have transformed our understanding of biological systems, yet navigating the complex and ever-growing landscape of techniques can be daunting. This review offers a guide for researchers, especially those working on malaria, by providing historical context as well as practical advice on selecting the right imaging approach. The review advocates an integrated methodology that prioritises the research question while considering key factors like sample preparation, fluorophore choice, imaging modality, and data analysis. In addition to presenting seminal studies and innovative applications of microscopy, the review highlights a broad range of topics, from traditional techniques like white light microscopy to advanced methods such as superresolution microscopy and time-lapse imaging. It addresses the emerging challenges of microscopy, including phototoxicity and trade-offs in resolution and speed, and offers insights into future technologies that might impact malaria research. This review offers a mix of historical perspective, technological progress, and practical guidance that appeal to novice and advanced microscopists alike. It aims to inspire malaria researchers to explore imaging techniques that could enrich their studies, thus advancing the field through enhanced visual exploration of the parasite across scales and time.

KEYWORDS
history, in vivo imaging, live cell imaging, malaria, microscopy, *Plasmodium*, superresolution

1 | INTRODUCTION

In the sweltering heat of late 19th-century Algeria, amidst the shadows of colonial rule, a French military physician, Charles Louis Alphonse Laveran, stood at the frontier of a scientific breakthrough of remarkable magnitude. Equipped with nothing but his simple light microscope (Figure 1) he gleaned at blood samples from patients afflicted by a disease that was long thought to be the result of exposure to 'bad air' (mala ariae). Amongst the sea of red blood cells, he spotted pigmented bodies and filiform elements that moved in wave-like patterns identifying for the first time the causative agent of malaria.[1,2] This microscopy-based discovery marked the commencement of the history of the investigation of the malaria parasite and the targeted pursuit to counteract its proliferation and spread.

Since this moment we have made significant progress in imaging parasites of the *Plasmodium* species at ever finer detail. To achieve this, the community has implemented a plethora of imaging and labelling methods that seem to have grown exponentially over the last decades. In the following, I attempt to explain and contextualise early and recent technological advances and how they have impacted the field of malaria research.

Several seminal reviews on the topic of imaging malaria parasites have focused on specific stages, tissues, and technologies[3–9] and provided an overview of the impact of imaging on important discoveries of *Plasmodium* biology at large.[10] Here I will more specifically highlight the historical context and focus on the pioneering studies adapting new light microscopy technologies to the study of the parasite's life cycle (Figure 2), while citing original references wherever possible. Imaging has widely contributed to our understanding of parasite biology so I focus on the very first studies implementing a new imaging approach to blood and other life cycle stage and the resulting benefits. This review will span biological scales ranging from entire organisms down to molecular resolution. I will present labelling techniques and confocal, live cell, intravital, superresolution microscopy, as well as a brief introduction to computational image analysis. Upon opportunity I will share some practical considerations, according to my experience, about the respective imaging technology. This account of how the *Plasmodium* imaging field developed aims to provide some historic context so the reader can appreciate the progress that has been achieved. Further it seeks to encourage scientists in the field to apply emerging cell biological methods to their research question.

2 | 'BEGINNING TO SEE THE LIGHT' – WHITE LIGHT MICROSCOPY

In 1880 Laveran was able to identify the disease-causing blood stage parasite using a simple bright-field microscope, that is, measuring the absorption of white light

FIGURE 1 Monocular microscope belonging to Professor Laveran alongside parasite drawings. Photographs kindly provided by the Musée du Service de santé des armées, Val-de-Grâce, Paris. On the right an aquarelle by Laveran of blood stage parasites drawn during a course at the Institut Pasteur, Paris.

FIGURE 2 Milestones in microscopy and malaria imaging. Timeline highlighting important inventions and discoveries throughout the history of biological light microscopy (left) and important milestones in imaging and cell biological discoveries in the malaria-causing parasite (right). Timeline is not to scale. Author names are shown for single author studies. Nobel Prize awarded discoveries are in bold.

by unstained blood samples from malaria patients. He uncovered asexual blood stages, which replicate within the blood, as well as the sexual stages, which were later show to be relevant for transmission (Figure 1). Around 1895 Sir Ronald Ross discovered 'pigmented bodies' within the midgut of mosquitoes using a similarly simple microscope. He later identified these structures as the oocyst stage of the malaria parasites, thereby demonstrating that the disease was transmitted through a mosquito vector.[11] As was later uncovered, oocysts are formed when the ookinetes, which are themselves products of the fusion between male and female sexual stages, penetrate the midgut epithelium. Yet transmission light microscopy has limited contrast and consequently various strategies that exploit the different physical properties of light, have been leveraged to reveal cell organisation. Phase contrast microscopy, a variant of dark-field microscopy, which was awarded the Nobel Prize to Frits Zernike for his discovery in 1932, visualises how cellular structures affect the phase of the light wave, rather than the absorption.[12] Later Differential Interference Contrast (DIC) microscopy was established, which visualises how specific cell regions affect light polarisation and yields a particular good contrast at membrane interfaces using Nomarski prisms. Importantly, the optimal performance of those imaging techniques relies on properly adjusted illumination, already described 1893 by August Köhler.[13] Almost 130 years later Köhler illumination still should be verified occasionally, including on simple bench-top microscopes used to inspect parasite cultures, to achieve optimal image quality.

2.1 | Transmission, dark-field and differential interference contrast microscopy

Key advantages of using white light for illumination, aside from the capacity for label-free imaging, is the very low phototoxicity, compared to, for example, more energy-intensive excitation of fluorophores. This makes it compatible with live cell imaging at very short time intervals and over extended periods. Consequently, several key cellular events within the parasite's life cycle have been visualised employing this technique. Using dark-phase microscopy Meir Yoeli visualised already in 1948 that sporozoites, are 'circling swiftly and gracefully in eel-like movements'.[14] Sporozoites are the parasite stages that emerge from the oocyst and, after migration to the salivary gland of the mosquito, can be injected into the host skin. From there they use gliding motility to reach the blood stream. Motility further plays a role in the unusual traversal of liver cells prior to the final invasion and a pre-erythrocytic round of replication.[15,16] By now gliding motility, which already starts within oocysts, has also been studied extensively in other life cycle stages such as ookinetes, which invade the mosquito midgut.[17–19]

Other life cycle transition stages that can be readily observed with white light microscopy are egress from the host cell.[20] During exflagellation male gametocytes produce reproductive microgametes while bursting out of the red blood cell. Those can fuse with mature female gametocytes to subsequently form the ookinete.[21] The rapid beating of the microgametes clearly sparked the curiosity of Laveran as he wrote: 'filiform elements resembling flagella which moved with great vivacity, displacing the neighbouring red blood cells; from then on I no longer had any doubts about the parasitic nature of the elements I had found in the malarial blood'.

This foreshadows key benefits of time-lapse microscopy that will be discussed later in detail (see chapter on time-lapse imaging). A more thorough quantification of this event was made possible by the development a transmission microscopy based assay,[22] which later allowed the seminal discovery of the essential external cues triggering the development of blood stages into the mosquito stages.[23,24] Imaging egress from the red blood cell led to the description of the PKG2 mediated pathway causing sequential rupture of the parasitophorous vacuole and the red blood cell membrane alongside the development of chemical tools to arrest parasite development.[25–27]

During egress multiple daughter cells, called merozoites are released, which can invade new red blood cells, although they have a very short half-life once extracellular. As a result, studying the process of reinvasion in a more quantitative manner has benefited from the implementation of dedicated purification protocols, high-speed white light imaging, and a combination with an automated imaging platform.[28,29] More recently DIC time-lapse imaging of merozoites revealed that gliding motility can also occur in merozoites.[30] Surprisingly recent white light imaging data, combined with some fluorescent tagging, suggested that the egg-shaped merozoites invade their host cell with the wide end first, which refutes the invasion models presented until now.[31]

2.2 | Hemozoin and polarisation microscopy

Polarisation microscopy relies on the special optical properties of anisotropic materials to reveal structural and molecular information. The most notable structure of malaria parasites regarding its optical properties must be the hemozoin, a haeme-based crystal formed by parasites when digesting haemoglobin. Aside from its paramagnetic properties it is very highly refractive leading to distinctly

dark regions within mature blood stage parasites. While this reliably indicates the position of the digestive vacuole those refractive properties can also lead to issues. In some microscopy set ups the excitation light can be reflected onto the detector if not filtered extensively, which can obscure the specific fluorescent signal (personal observation). Further, when using very high-power lasers as in STimulated Emission Depletion (STED) microscopy the illumination of the hemozoin crystal can lead to 'vaporisation' of the cell (see also chapter on superresolution).[32] Whether the hemozoin aggravates phototoxicity during live cell imaging is unclear. Intriguingly, the crystal is strongly birefringent, that is, an anisotropic material that shows very different refractive indexes depending on the polarisation and angle of the incident light. In live polychromatic polarisation imaging the hemozoin then manifests as 'Coloured brilliantly birefringent granules'.[33] As already noted in 1986 this makes the hemozoin crystal a prime subject for the sensitive polarisation microscopy, which has been explored in the context of diagnosing placental malaria.[34,35]

2.3 | Colorimetric dyes and stains

While white light microscopy can readily capture the more dramatic life cycle transitions it usually fails to reveal intracellular organisation of this small parasite in sufficient detail. One approach to generate intracellular contrast is the use of colorimetric dyes, the most prominent one being the ubiquitous Giemsa stain.[36] It was developed in 1904 by Gustav Giemsa building on the work done by Dimitri Romanowsky and Bernhard Nocht leading to the formulation of Eosin Y, Methylene Blue, and Azure B, we still use today.[37,38] Methylene Blue was previously discovered as biological stain in 1881 by Paul Ehrlich and he showed its utility for staining malaria parasites 10 years later.[39–41] At that time he already carried out preliminary in human trials to test its potential use as antimalarial and today methylene blue is still under consideration as such.[42,43] Methylene Blue staining also enabled the first description of Maurer's clefts in 1902,[44] which were much later identified as unique parasite derived compartments used for protein transport into the host erythrocyte[45,46]

While Laveran initially had no access to Methylene Blue, Giemsa, or Romanowsky stains, Ronald Ross' discovery of the mosquito stages of the parasite was critically aided by staining with crystal violet. It was also known as gentian violet and synthesised by the Charles Lauth in 1861 as one of the first synthetic derivatives of aniline. Nowadays mercurochrome, which only appeared 1919, is used to reliably stain oocysts inside the midgut of infected mosquitoes.[47] Since then, however, colorimetric dyes, which were so foundational for the chemical industry, have not been developed much further in the context of biological application, who has since then rather focused on fluorophore-based labelling (see chapter on fluorophores).

2.4 | Outlook and conclusion

While white light microscopy is traditionally associated with its use in diagnostic applications, such as observing Giemsa-stained blood smears, it retains significant value for malaria research. Despite being less specific than fluorescence-based techniques, white light microscopy provides essential contextual information about the parasite and its environment, making it a valuable companion to other imaging methods. Although it has historically been limited to 2D imaging, recent innovations, such as holotomographic imaging, have expanded its capabilities. This technology enables 3D cell reconstruction through interferometric detection.[48] It has more recently found application in malaria research allowing measurement of alterations of the host cell structure by divergent *Plasmodium* species and dynamic analysis of cholesterol transport in parasite-infected red blood cells.[49,50] The weak contrast of white light microscopy has also been a key limitation for automated image analysis and necessitated time-consuming manual annotation. However, recent advances in machine learning and neural networks have greatly improved automated segmentation and tracking of cells (see chapter on image analysis),[51–53] mitigating this issue. Those advances in image analysis and progress in 3D data acquisition combined with label free imaging of living parasites at high speed and over prolonged time will continue to raise new applications of white light microscopy in malaria research.

3 | 'CONTRAST, PLEASE!' – FLUORESCENT MICROSCOPY AND FLUOROPHORES

Aside from the pursuit of better resolution, microscopist also consistently strive for optimal signal-to-background ratio. The lack in contrast in white light microscopy has been addressed by leveraging the phenomena of fluorescence described by Sir George Stokes in the mid-19th century.[54] Intriguingly, Stokes based his discovery on an observation made by Sir John Herschel 7 years prior describing:

'The solution of quinine … exhibits … under certain incidences of the light, a beautiful celestial blue colour'.[55]

Thereby, in a strange twist of history, linking the discovery of fluorescence to the first ever used antimalarial, which was already isolated 1820 from the Cinchona bark.[56] The technological breakthrough that allowed the exploitation of this physical phenomenon was the construction of the first fluorescent microscope by August Köhler in 1904.[57] Its widespread applicability was enabled later by the development of the epifluorescence microscope by Philipp Ellinger and August Hirt in 1929,[58] further enhanced by the introduction of the dichroic mirror by Bas Ploem in 1967.[59]

3.1 | Immunofluorescence

The first synthetic fluorophore that found widespread usage was fluorescein, invented in 1871 by Adolf Baeyer.[60] It was most notably used in the context of antibody labelling required for immunofluorescence assays that were originally devised in the mid-20th century.[61,62] In the context of malaria research the first immunofluorescence assays were carried out in the 1960s and used Globulins isolated from infected host organisms to investigate cross-reactivity between erythrocytic and exo-erythrocytic parasite stages as well as between various species.[63–65] While serological assays, that is, using serum from infected hosts as antibody source, were highly predominant at first, the development of continuous culture of *P. falciparum* motivated the production of the first monoclonal antibodies against specific protein targets.[66,67] Since then, immunofluorescence staining has been an invaluable tool to dissect the cellular organisation of the malaria parasite.

Fluorophore chemistry, however, has significantly progressed since the appearance of fluorescein. Fluorophores are now covering the entire spectrum from ultraviolet to infrared wavelengths and are available coupled to a range of antibodies, nanobodies, and 'functional' proteins such as streptavidin. Notable examples are the Alexa Fluor dye series, and more recently the significantly improved Alexa Fluor Plus series, but also the Atto dye family.[68] Their high photostability, brightness, and possibility of selecting the optimal excitation and emission wavelengths for your specific microscopy setup, using, for example, an online spectrum viewer, have all but eliminated fluorophore quality as a limiting factor for classical immunofluorescence techniques.

Key limitations for immunofluorescence assays in malaria research that remain are sample preservation (see chapter on sample preservation) and the availability of high-quality primary antibodies. Albeit some notable attempts have been made to compile resources, like through MR4,[69] *Plasmodium* targeting antibodies are lagging far behind the commercially availability of the ones against model organisms. Therefore, when studying conserved proteins one can screen commercially available antibodies raised against other species for crossreactivity. Otherwise, the production of custom antibodies against a *Plasmodium* spp. protein of interest should be a serious consideration.

3.2 | Genetically encoded fluorophores

One of the biggest milestones in cell biology must be the discovery of green fluorescent protein (GFP), allowing the direct visualisation of a selected protein without any preparation of the cells in 1994.[70–73] Exactly around this time the first transfections of *Plasmodium* species with transgenes were achieved, laying the molecular basis for the implementation of GFP tagging.[74–79] This was shortly followed by the first expression of GFP in parasites[80,81] and the first fusion of GFP with a specific targeting sequence that allowed visualisation of the apicoplast, the stunningly unique parasite organelle, which still fascinates the field to date.[82]

No different than for other branches of cell biology fluorescent protein tagging has been an invaluable source of insight and the basis for fluorescence time-lapse microscopy in malaria research (see chapter on time-lapse imaging). Since the early days fluorescent protein engineering has spurred a tremendous diversity of proteins having a range of properties with FPbase.org currently listing over a thousand different fluorescent protein species.[83] Only a tiny fraction of those have been tested in *Plasmodium* species let alone compared with each other.[84,85] How a fluorescent protein will perform in terms of photostability, brightness, stability, and maturation is challenging to predict and usually requires some empirical evidence. This is illustrated by the observations that contrary of what to be expected from studies in mammalian cells,[86] the red fluorescent protein (RFP) variant RedStar performs significantly worse than mCherry and tdTomato in *Plasmodium*.[85] While tdTomato is expected to be significantly brighter than mCherry, based on comparative analysis in HeLa cells,[87] there was no improved performance in asexual blood and liver stages.[85] Nevertheless, tdTomato has proven as an excellent marker protein, for example, for gametocyte stages under the right promoter.[88] Other red fluorescent proteins remain poorly explored. While mRuby was used to visualise parasites in mosquito stages and mKate2 as cell marker for competitive growth assay, the authors give no indication whether the choice of their red fluorescent protein was critical to the success of their marker cell line.[89,90] A recent promising example of a bright RFP is mScarlet.[91] Its successful application as reporter for protein export and as cell marker for

transfection suggests favourable fluorescence properties in parasites.[92,93] In the green spectrum GFP, particularly monomeric enhanced GFP (mEGFP), has remained a staple in FP-tagging. More recently monomeric NeonGreen (mNG), which is unlike GFP derived from *Branchiostoma lanceolatum*,[94] has also in *Plasmodium* delivered on its promise to be particularly bright and photostable.[30,89,95] This makes it an interesting alternative to mEGFP, while users must consider that resources like anti-GFP antibodies will not be compatible with mNG.

Additionally, there are entirely different classes of fluorescent proteins such as photoactivatable, photoswitchable, and photoconvertible ones. Their use has been rare in *Plasmodium* but Dendra2, which can be converted from a green to a red emission spectrum by UV-illumination has successfully been used to show that membrane associated proteins can be transported to already preformed Maurer's clefts.[96] Different classes of FPs also enable different types of microscopy-based techniques including Fluoresecence Recovery After Photobleaching (FRAP), Förster Resonance Energy Transfer (FRET), Fluorescence Correlation Spectroscopy (FCS), or Fluorescence Lifetime Imaging (FLIM) whose background and applications lie outside the scope of this review.

3.3 | Fluorescent stains

Despite their astounding versatility and insightfulness fluorescent proteins carry some intrinsic limitations. Firstly, they necessitate transgenic manipulation, which is more tedious in *Plasmodium* than many other organisms. Secondly, they are relatively less bright and photostable than chemical fluorophores limiting the signal-to-noise ratio that can be achieved and prohibiting some superresolution microscopy techniques (see chapter on superresolution microscopy). Those issues can be mitigated using what I will call fluorescent stains, that is, small fluorescent molecules that bind to specific target sites within cells. The list of fluorescent stains, their historic background, and use cases in *Plasmodium* are too vast to discuss exhaustively but have been extensively reviewed.[97,98]

The very first known fluorescent stain was Acridine Orange synthesised in 1891 by Heinrich Caro at the chemical company Badische Anillin- und Soda Fabrik (BASF) although its use in a biological context did not occur before 1940.[99] It binds primarily to nucleic acids and depending on whether it binds RNA or DNA has different spectral properties. The first use of Acridine Orange in *Plasmodium* was reported in 1959, suggesting some increased detection sensitivity in patient samples over conventional methods such as Wright's stain.[100,101] Analysis of the colour spectrum and nuclease treatments led to the speculation that the *Plasmodium* DNA is indeed double-stranded.[102] The use of Acridine Orange as a more sensitive diagnostic tool was explored, but several practical considerations such as the necessity of a fluorescent set up have prevented its more widespread application in the field.[103–106] Interestingly, Acridine Orange displays some affinity for membranes notably staining highly mobile vesicles within live infected erythrocyte, which might be Maurer's clefts.[107,108] Stains with significantly higher specificity for DNA are the Hoechst stains synthesised in the 1970s,[109] closely followed by DAPI produced by Otto Dann and colleagues.[110] Due to the ease of use of these UV-excitable stains, they are to this day one of the most widely used stains in cell biology and often complement immunofluorescence assays. Due to the absence of DNA in human erythrocytes Hoechst-staining also became very early a reliable method for flow cytometric sorting of infected red blood cells.[111] In some case the variable affinity of Hoechst dyes for the minor groove of AT-rich sequences and therefore some chromatin states can be a concern.[109] In those cases intercalating dyes like DRAQ5 or SYBR green, which fluoresce in the infrared and green spectrum, respectively, can be used instead.[112] For quantitative assessment of DNA content, however, RNAse treatment of parasites is essential prior to SYBR green staining to remove the signal caused by residual RNA binding.[113]

Another cell component that is biochemically very distinct from nucleic acids, and proteins, are lipids and membranes. One of the first dyes to be recognised for its potential to fluorescently stain lipids in cells was Nile Red quickly followed by the description of carbocyanine dye, such as DiO and DiI, and later the Bodipy series.[114,115] Many of those dyes, including directly labelled phospholipids, have been tested in *Plasmodium* with a notable absence of nuclear envelope staining, until recently.[97,116,117] Bodipy C5-ceramide has been particularly efficacious in, for example, revealing host and parasite membrane dynamics throughout the intraerythrocytic development.[96] NBD-cholesterol staining of liver stages was critical to demonstrate that the parasite needs to scavenge some lipids from the host cell.[118]

Further there is a growing family of organelle or cytoskeleton specific dyes that have been tried in *Plasmodium* such as Mitotracker, frequently used as live/dead stain,[97] fluorophore-coupled phalloidin,[119,120] or Taxol-based microtubule stains like TubulinTracker.[121] Stains such as ER-tracker, Lyso-tracker, and Peroxisome-tracker still need to be explored in more detail. Since all those stains were largely developed for use in mammalians, their application in *Plasmodium* must be properly evaluated (see below). Last but not least sensor molecules, most notably calcium sensing molecules, like Fluo-4 AM, have been instrumental to visualise the implication of calcium

signalling in key life cycle events such as egress from and invasion into host cells.[122]

3.4 | Challenges and practical considerations

Despite the finite list of targets, fluorescent stains are a versatile tool for quick and easy access staining of any parasite cell line you might have at your disposal. They exist over a wide range of emission and excitation spectra and can be tailored to desired cell biological application. Their small size further eliminates the so-called linkage error, where the fluorophore is at a significant distance from the target molecule thereby limiting localisation precision (see chapter on superresolution microscopy). Nevertheless, fluorescent stains have some caveats and a few considerations alongside empirical testing are required. Firstly, cell permeability must be taken into account as, for example, only certain Hoechst stains are cell permeable for living parasites.[123] Further, the stain might display some cytotoxicity or alter cell behaviour. Hoechst, for example, will prevent mitotic progression above certain concentrations.[124] Taxol-based dyes such as TubulinTracker can influence microtubule dynamics and have led to an atypical distribution of microtubule signal in blood stage parasites.[121] Conversely cell fixation and permeabilisation might alter or inhibit the binding of the stain as is the case for Bodipy C5-ceramide.[116] With its divergent biology *Plasmodium* might be less specifically stained than expected as its specific organelles like the food vacuole might have differential sensitivity to lyosomal stains. A more striking example is observation that *Plasmodium* actin is divergent enough that any phalloidin-based stain will nicely stain host cell actin but fail to label the parasite.[125] Further, general biochemical properties of the dye can also alter their localisation as highly hydrophobic groups might induce unspecific membrane association. The fluorescent properties of the stains, such as brightness and photostability should also be taken into account. Even though DNA stains like DRAQ5 are initially very bright, they will quickly fade upon excitation making them suitable for flow cytometry but less for imaging. Depending on the overall brightness in the staining solution careful titration of the working concentration and washing protocols must be established, while respecting the shelf life of the fluorophore.

3.5 | Next generation fluorescent stains

Several of those caveats are addressed by a newer generation of so-called fluorogenic stains. They have the property of only becoming strongly fluorescent upon target binding and display great photostability. While strictly speaking the fluorescence of Hoechst and other DNA dyes is also increased upon target binding, the fluorescence intensity ratio between bound and unbound state is orders of magnitude higher with fluorogenic stains. This class of fluorophores was pioneered by coupling silicon-rhodamine (SiR) derivatives to tubulin and actin targeting moieties.[126] SiR derivatives can switch between an on and an off state, that is, a fluorescent zwitterion and a nonfluorescent spirolactone conformation, respectively. They display exceptional fluorescent properties and allow the use at very low concentrations that would not significantly interfere with cellular dynamics. SiR-Tubulin and SiR-Actin were quickly followed by the design of fluorogenic DNA stains, different colour versions, and stains improved molecular properties such as brightness and permeability,[127–130] ultimately leading to the commercialisation of those class of stains. In *Plasmodium* SiR-Tubulin was first used to visualise the microtubule skeleton of oocyst stages, which surprisingly were still stained after fixation, suggesting divergent microtubule stability in *Plasmodium*.[131] This allowed to circumvent the issue of oocyst being largely refractive to antibody penetration required for immunofluorescence. The advantageous properties of fluorogenic SPY555-Tubulin were critical to allow for the first time the visualisation of mitotic microtubule dynamics in living blood stage parasites.[112,132] Shortly thereafter an improved version of SiR-Hoechst was used to reveal DNA-replication dynamics at the single cell level without perturbation of cell cycle progression.[133–135] By now even different biophysical characteristics of fluorogenic stains than their spectral properties can be exploited. Depending on the microenvironment the fluorescence lifetime, that is, the delay between excitation and emission can vary. A recent study leveraged this phenomena in *Plasmodium* by quantifying the membrane tension in live parasites using fluorescence lifetime imaging of the Flipper-TR probe and found that the membrane tension of gametocyte and infected host cells decreased upon different activation cues.[136]

3.6 | Outlook and conclusion

The advancements in fluorogenic stains and fluorescent protein design have significantly improved imaging contrast and easy access labelling in malaria research. Next generation stains, which fluoresce only upon binding to their target, address limitations of earlier fluorophores, providing better photostability and reducing background noise. New labelling strategies combining protein tagging with functional proteins, like HaloTag,[137] together with HaloTag-binding fluorogenic stains, like MaP-SiR-Halo,[138] promise to merge optimal labelling specificity with

excellent brightness in living parasites.[139] Those abilities to label specific subcellular structures dynamically offer promise for studying intricate parasite processes like differentiation, proliferation, and host-pathogen interactions. As the field evolves, innovative fluorophores will open new opportunities to facilitate more detailed exploration of *Plasmodium* biology.

4 | 'EXPLORING THE THIRD DIMENSION' – VOLUME MICROSCOPY AND SAMPLE PRESERVATION

Life unfolds in a, at least, three-dimensional space. Capturing the spatial organisation of a cell in three dimensions using imaging comes with its own set of challenges. Fundamentally, the medium on which we are capturing image information, just like the one I am using to communicate with you right now, is intrinsically two-dimensional. Yet, there are also some concrete optical limitations associated with imaging along the third dimension or the *z*-axis, as it is frequently referred to. Due to the arrangement of the objective and the light path the achievable resolution in the *z*-direction, is only half of what can be achieved in *x* and *y*, as defined by Ernst Abbe.[140] Secondly, in classical imaging approaches all fluorophores are excited along the entire *z*-axis causing so called out of focus emission light that reduces the signal to background ratio and therefore image quality. The later aspect becomes increasingly important as the thickness of the sample increases. It might be a smaller problem for, for example, free merozoites, sporozoites, and infected erythrocytes. The thickness of mature infected hepatocytes, the oocyst stage parasite, tissue samples, or even whole organisms, however, presents a significant hurdle for proper optical *z*-sectioning.

4.1 | Confocal microscopy

An important milestone mitigating the issue of out of focus fluorescence light was the invention of confocal microscopy originally patented by Marvin Minsky in 1957.[141] The concept revolved around inserting a small pinhole into the light path to block the light coming from fluorophores outside the focal plane. A lot of technology development, including the synthesis of new fluorophores, had to occur to yield the first commercially available beam scanning confocal laser microscopes at the end of the 1980s.[142] These early models were already available to some malaria researchers, aiding studies on liver-stage parasite development and membrane reorganisation, suggesting the presence of parasite-derived vesicles within liver cell cytoplasm.[143,144] They could reveal with some clarity structures such as myosin, microtubules, and stress fibres within mature exoerythrocytic parasite forms, highlighting already then an atypical cytoskeletal organisation that will preoccupy malaria researchers until today.[17,145] Some of recent discoveries on liver stage that were enabled by this imaging technique were the interplay between host autophagy and the parasite.[146,147] Also, the investigation of blood stage benefited from confocal microscopy early on as improved *z*-sectioning yielded a better understanding and quantification of vesicles within host cells.[107] Since then, confocal microscopy has become a standard method on which many other technologies have been built.

4.2 | Sample preservation

Sample preservation is fundamental for any successful imaging protocol, particularly when one aims to investigate the three-dimensional architecture of the cells. In earlier years parasite preparation was often achieved by air-dried blood smears, which are still useful in the context of diagnostics as well as monitoring parasite growth during culturing. The complete dehydration of cells, however, will lead to flattening and therefore loss of most of the three-dimensional organisation, for example, nuclear architecture of the parasite.[148] Air drying can further increase unspecific background staining and is therefore suboptimal for the use in immunofluorescence assays and similar imaging protocols (personal observation). Chemical fixation is to be favoured and careful consideration about the fixative is essential to preserve structural integrity and maintain epitope availability for immunodetection.[149] Suboptimal fixation protocols are shown to cause flattening of mammalian nuclei and can also impact organisation of the parasite nucleus.[148,150] Methanol precipitates proteins by displacing water molecules and can successfully preserve microtubules networks but will lead to dehydration of the cells and may disrupt membrane-associated structures.[151] Formaldehyde is often preferred due to its fast penetration and cross-linking capabilities. However, in protocols involving *Plasmodium*-infected erythrocytes the lysis of the host cell has presented an issue. To mitigate this Tonkin et al. established a widely used protocol fixing infected erythrocytes in suspension with 4% paraformaldehyde, while adding a small amount of 0.0075% glutaraldehyde.[152] The protocol aims to balance good structural preservation and decent antibody labelling. Yet, even those small quantities of glutaraldehyde have been shown to have a strong effect on the binding affinity and labelling density of several antibodies.[125] As alternative infected erythrocytes can be fixed on coverslips using only paraformaldehyde prior to staining, allowing for partial host cell lysis while fully

preserving parasite structure and epitopes binding, which yields highly efficient antibody staining.[125] The choice of fixative should be aligned with the specific needs of the immunodetection reagents, with paraformaldehyde often being the fixative of choice for preserving both antigenicity and cell morphology.[151] The use of 4% paraformaldehyde from a methanol-free 16% paraformaldehyde (w/v) stock solution followed by quenching free aldehyde group (e.g., with sodium borohydride) should provide a good starting point. Even prior to fixation, samples must be handled with care since, for example, washing infected erythrocytes in PBS rather than RPMI medium can cause depolymerisation of dynamic spindle microtubules.[125] Therefore, if the cellular structure is important for your scientific question the integrity of the parasite should be carefully monitored by measurement of reference structure and, if possible, using live parasite labelling as a benchmark.

4.3 | Light sheet microscopy

A fundamentally different way to capture light emitted by fluorophores that are deeply embedded within the *z*-axis of biological samples was developed in the 1990s and opened the way for modern light sheet fluorescent microscopy.[153] Initially named Selected Plane Illumination Microscopy (SPIM) scientist devised a setup that excited the fluorophores in very large biological samples, such as Medaka embryos, via a thin sheet of excitation light that was positioned orthogonally to the actual imaging objective.[154] This microscope configuration had several key advantages by (i) all but eliminating out of focus light for arbitrarily large samples, (ii) producing very low phototoxicity in living samples, (iii) allowing high acquisition speeds and would change developmental cell biology forever.

In malaria research light sheet microscopy has not yet found wide-spread application but imaging of entire parasite-infected mosquitoes was achieved.[155] However, finding optimal clearing protocols to reduce light scattering by the cuticle remains challenging. Integration of electron or X-ray based volume microscopy techniques might assist the study of entire infected mosquito midguts in the future.[156] CUBIC, a more recent tissue clearing method, has yielded exciting results showing that parasite accumulates preferentially in the olfactory bulb of entire nonsectioned brains in infected mice models.[157] This might provide interesting insights into how the deadly cerebral malaria, of which the molecular pathogenesis is not fully resolved, unfolds in vivo. It is to hope that light sheet microscopy will further develop to study malaria in organs and organisms whilst the many methodological challenges of this technology are systematically addressed.[158]

4.4 | Outlook and conclusion

Confocal microscopy and sample preservation methods have been instrumental in advancing malaria research by enabling detailed 3D imaging of *Plasmodium* stages, overcoming challenges associated with imaging along the *z*-axis. A recent technological jump was the invention of lattice light sheet microscopy, which produces a light sheet whose size is below the diffraction limit opening unprecedented insights into the dynamics of merozoite invasion, which will be discussed in detail below.[159–162] Although chemical fixation is state-of-the-art it will never be instantaneous, with some regions of the cell being affected more quickly than others while the cell dies in the process. This makes cryofixation a superior preservation technique and can under some circumstances be considered preceding cell labelling and awaits it application for light microscopy in the malaria field.[163–165] Optimal sample preservation is critical for any imaging experiment to succeed, as only then can the intricate and unconventional cell biology of the malaria parasite be revealed with increasing detail.

5 | 'CLOSER, EVEN CLOSER!' – SUPERRESOLUTION MICROSCOPY

To the despair cell biologist, Abbe mathematically defined in 1873, 7 years prior to the discovery of the malaria parasite, the maximal resolution achievable by diffraction-limited optics.[140] This limit was already reached by Carl Zeiss in collaboration with Otto Schott and other colleagues around 1883 and was to shackle classical microscopy for more than a century.[166,167] At the turn of the millennium, however, different strategies were developed that would allow subdiffraction imaging culminating in the awarding of the 2014 Nobel Prizes to Eric Betzig, Stefan W. Hell, and William E. Moerner. Thus, the age of superresolution microscopy (SRM) was born revealing the intricate organisation of cells at ever finer detail.[168,169] Here, I will only provide a brief introduction to the major superresolution microscopy technologies, which have been reviewed by countless articles over the last decades and from which a plethora of variants have emerged since.[170]

5.1 | Structured illumination microscopy

Structured Illumination Microscopy (SIM), which appeared alongside the Nobel Prize-winning technologies, is based on excitation of the sample with sinusoidal patterns at different angles and translations.[171,172] By combining the information about spatial patterns contained

within those different images a new superresolved image is generated in a postprocessing step. Classically this technique is limited to a twofold improvement of resolution, which is, however, equally achieved in all three dimensions of space (3D-SIM). The volume that can be imaged therefore is eightfold smaller than in classical microscopy providing significant more detail especially relevant for cells as small as malaria parasites. Despite being limited to a twofold resolution increase SIM retains some importance alongside other SRM techniques. The requirements for sample preparation don't significantly differ from the principles for generating a good sample for classical microscopy although a specialised microscope is needed. Yet, SIM requires only little image processing, allows multicolour imaging, uses comparatively moderate illumination intensity, and can reach significant acquisitions speeds making it well compatible with live cell imaging of parasites (see chapter on time-lapse microscopy).[173]

3D-SIM was the first SRM technique implemented in malaria research.[174] It revealed the detailed organisation of the exomembrane system which the parasite deploys within the infected red blood cell.[175] The improved resolution helped clarify that the membrane structures labelled by Bodipy C5-ceramide are discontinuous and revealed for the first time substructures within the Maurer's Clefts. The study further highlights the potential of SRM to bridge the significant resolution gap that had persisted between light and electron microscopy. A subsequent study investigated the formation of the inner membrane complex, which underlies the cell cortex and is an integral of the machinery driving motility and invasion.[176] Localising the GAP50 protein during late stages of schizogony revealed flat ellipsoid structures containing subdiffraction sized 'pores'. Simultaneously the power of 3D-SIM was harnessed to investigated the by far smallest parasite stage and its main function, the merozoite as it invades the red blood cell.[177] The formation of the tight junction between the RBC and the merozoite and its associated protein, could only be localised with sufficient precision by the deployment of 3D SRM. Timed sampling in combination with the exploration of several invasion-associated protein revealed several molecular steps required for this rapid and small-scale process.

5.2 | Potential alternatives to SIM

Several alternative technologies approaching although not quite reaching 3D-SIM resolution have been developed as proprietary technologies by different microscopy manufacturers. Those include the Yokogawa SoRa spinning disk, the Nikon NSPARC detector, and the Leica Lightning module.[178,179] They usually increase the resolution by a factor of up to 1.7× while maintaining low illumination. The technology that has, however, been the most extensively used in the malaria field is the Zeiss Airyscan-detector based microscopy.[180,181] It has improved the impact of expansion microscopy (see below) and allowed superresolved long-term time-lapse of blood stage parasite allowing counting of individual nuclei in live parasites despite them being tightly packed.[132,182]

5.3 | Stimulated emission depletion microscopy

STimulated Emission Depletion Microscopy (STED) relies on a second red-shifted depletion laser that forms a 'donut' shape preventing fluorophore emission anywhere but at the central zero intensity minimum.[183–185] This zero intensity minimum is then used to scan the sample producing a superresolved picture. Thereby, in theory, arbitrarily small resolutions can be achieved as the laser power is increased. However, there are practical limits to the laser powers that can be used and, more critically, the photostability of the used fluorophore making axial resolutions of about 40 nm realistic in malaria parasites. From this emerges an important criterion for a successful STED experiment, which is the choice of the right fluorophore. It should be bright, highly photostable, and 'STEDable' that is, efficiently interact with the depletion laser thereby being prevented from emission. Depending on the wavelength of the excitation laser, for example, Atto594 or Abberior Orange dyes have been shown to work. Dual colour STED applications are possible by combination with, for example, Atto647 or Aberrior STAR RED dyes. Three colour STED is significantly more difficult to achieve and relies on long stokes shift dyes. Classical STED laser configurations only improve the resolution in 2D, but 3D-STED is possible albeit resulting in compromises for the axial resolution. Being a high laser intensity imaging technique, the number of slices or time frames is, however, limited making it much less suitable for live cell imaging than, for example, SIM. A key advantage of STED is that there is no additional requirement for sample preparation. Further a STED microscope can largely be operated like a standard confocal microscope requiring some adjustment of the STED laser power.

Initial studies introducing STED applications to malaria research looked at proteins secreted by invading merozoites.[186] STED allowed to confirm co-localisation of certain proteins whereas at those increased resolutions it became clear that other proteins form distinct pools. But even larger cells, such as the parasite-infected hepatocytes could be studied in more detail using STED.[187] Contact sites between parasite plasma membrane and the ER that were surmised by electron microscopy could be confirmed. Within this small parasite the

investigation of the subnuclear organisation of mitotic structures within those parasites has required a more recent STED application.[112,125] In combination with electron microscopy those studies revealed the intricate organisation of intranuclear microtubules at the centriolar plaque, the centrosome equivalent in malaria parasites. This also exposed a very specific challenge of STED microscopy in parasite stages containing hemozoin as its illumination with the STED laser can induce vaporisation of the cell.[32] Three separate strategies to mitigate this issue have therefore been developed.[32,112,188]

More recently STED, despite its high laser intensity has been made compatible with live cell imaging of blood stage parasites.[139] Tagging of the centrosomal protein PfCen1 with the HaloTag allowed live cell labelling with a fluorogenic fluorophore, MaP-SiR-Halo.[138] Time-lapse STED imaging revealed the rapid dynamics of centrin proteins at the centriolar plaque within a subdiffraction compartment.[139]

5.4 | Single molecule localisation microscopy

A different class of SRM techniques now collectively called Single Molecule Localisation Microscopy (SMLM) is based upon the concept of serial detection of individual fluorophores.[189] Using the mathematical description of the diffraction pattern created by a single fluorophore, the so-called point spread function (PSF) as described by Abbe, one can calculate its precise position. By combining thousands of images, the spatial distribution of fluorophores within a cell can be determined with precisions of less than 20 nm.[190] This stochastic sampling employed in one of the most applicable variants of the technique i.e. direct STochastic Optical Reconstruction Microscopy (dSTORM), also requires fluorophores with specific optical properties most notably 'blinking'.[191] This blinking, which is displayed by, for example, Alexa647 ensures that in every image frame each fluorescent spot is statistically only emitted by a single fluorophore. Although achieving the highest resolution (see conclusions and outlook) establishing SMLM comes with a certain set of challenges including, the use of special imaging buffers, requiring acquisition and registration of thousands of images, postprocessing, risk of undersampling, limited use of optical sectioning over large stacks, and limitations in simultaneous multicolour acquisition. Even though many successful efforts have been made to mitigate these challenges this might have limited more widespread use in malaria research.

Nevertheless a few studies investigating remodelling of the host red blood cell by the parasite in the context of antigenic variation have leveraged SMLM. The first study applying STORM exploited its exquisite sensitivity to detect very small amounts of protein and colocalise them precisely with the ER demonstrating changes in protein behaviour depended on an upstream open reading frame (uORF).[192] Due to its small size nuclear biology can significantly benefit from SRM. In a study linking redox sensing to regulation of antigenically variant genes dSTORM revealed two distinct localisation patterns of thioredoxin peroxidase I that partly overlapped with the nucleolus.[193] In an analysis of the organisation of adhesive complexes the parasite builds at the surface of the infected red blood cell, the so called knobs, quantitative Photo Activated Localisation Microscopy (PALM) was employed.[194] The authors used mEos, a SMLM compatible fluorescent protein, to tag a specific surface adhesin PfEMP1 and determined cluster sizes and revealed the number of proteins in different strains and in blood cells from haemoglobinopathic donors. A subsequent analysis of the knob structure determined they contain about 60 KAHRP proteins further showcasing the capacity of SMLM to count individual proteins within cellular complexes.[195]

5.5 | Expansion microscopy

Just 1 year after the Nobel Prizes for SRM were awarded an entirely novel concept, which is not based upon instrumentation or image reconstruction burst onto the scene. Somewhat unconventionally the technique of expansion microscopy (ExM), rather than improving the resolution of the image, revolves around increasing the dimensions of the imaged cell.[196] This is achieved by embedding the sample in a swellable polymer network that is then isotropically expanded in all three dimensions increasing the cell size by a factor of at least four.[197] Imaging those expanded and stained samples on a conventional microscope effectively creates a superresolved image. This technique offers several advantages such as the ability to produce multicolour, 3D superresolution images without additional technological or informatic enhancements. A more nuanced benefit is the fact that the linkage error is reduced as the size of a primary antibody is smaller in relation to an expanded cell. Most strikingly, however, this technique requires only affordable chemicals and can be performed using conventional microscopes thereby eliminating the hurdle of purchasing dedicated imaging setups and requiring special expertise in image acquisition and processing. Despite the prolonged sample preparation time this has reduced the entry barrier for SRM and contributed to the rapid adaptation within the apicomplexan field resulting in already more than fifty studies using this technique.[198] Since its inception several

iterations of expansion microscopy that modify polymer chemistry, denaturation, the labelling strategy, as well as the expansion factor, now reaching around 20×, have emerged.[199,200] These iterations addressed some of the potential caveats of ExM such as nonisotropic expansion and labelling efficiency. The variant that made ExM most accessible to the malaria field was Ultrastructure-ExM providing excellent sample preservation and labelling.[201] Yet, ExM retains some intrinsic limitations as it only works in fixed cells, requires imaging significantly bigger volumes, some structures such as the hemozoin within the digestive vacuole being less prone to expansion, and 'dilution' of the epitope of interest sometimes challenging detectability. Since ExM in apicomplexan has been thoroughly reviewed recently I will only briefly highlight the pioneering studies in malaria.[198]

Using U-ExM the cytoskeletal structure at different stages of the parasite life cycle was revealed with unprecedented detail. This analysis revealed the presence of a conserved tubulin-based conoid structure in *Plasmodium ookinetes*.[202–204] Previously, this structure was believed to be exclusive to the evolutionary branch of Apicomplexa that includes *Toxoplasma gondii*. The authors further used staining with fluorescent NHS-Ester which unspecifically binds to proteins. This reveals, not unlike electron microscopy, a highly resolved protein density distribution within the parasite, giving highly valuable contextual information for the localisation of the protein of interest. Quickly thereafter several studies investigated the organisation if the microtubule organising centre and the mitotic apparatus of dividing parasites using U-ExM.[112,116,205] They showed the bipartite organisation of the centriolar plaque and the peculiar structure of the microtubule hemispindle in asexual and sexual blood stages as well as allowing a more detail analysis of chromosome segregation phenotypes. In a more systematic approach U-ExM was leveraged to visualise the distribution and structure of all major cellular compartments throughout the asexual blood stage development of the parasite showing that this SRM techniques is not limited to the cytoskeleton and can provide a highly valuable complement to whole-cell 3D electron microscopy.[206]

5.6 | Outlook and conclusion

All the aforementioned SRM techniques are constantly evolving and should be reevaluated regularly regarding their efficacy and applicability for the research question at hand. In ExM microscopy there have been two notable advancements that remain to be explored in the context of malaria research. Cryo-ExM achieves significantly better preservation of cellular compartment such as the ER, the cytoskeleton and other membrane associated structures by initiating sample preparation with a cryofixation step.[164] Iterative U-ExM (iU-ExM) has successfully been implemented in *Toxoplasma* and combines two rounds of expansion microscopy yielding a about 16-fold increase of the sample, effectively reaching resolutions equivalent to about 10–20 nm.[207] Further ExM can be readily compounded with other SRM technique as has been done with Airyscan-detector-based imaging in parasites but also with STED and SMLM yielding even better resolution.[130,206,208]

The most recent breakthrough in SRM was the invention of MINFLUX.[209] It is an SMLM technique that uses a donut-shaped excitation beam with a precisely defined centre where the photon flux is minimal. This allows single digit nanometre localisation precision of fluorophores. Thereby, more than a hundred years after August Köhler built a fluorescent microscope prototype, we have effectively reached the maximal achievable resolution in fluorescence microscopy as the only remaining limiting factor is the size of the fluorophore itself and its distance for the target.[98] Even though MINFLUX is still highly challenging and time-consuming to implement it is a rapidly expanding field now allowing 3D and multicolour imaging.[210] In two groundbreaking studies MINFLUX has allowed the quantification of individual kinesin motor steps in living cells.[211,212]

This leaves the questions which SRM application to use for your specific research question. There are too many considerations to be discussed here exhaustively, but a most critical one is the question whether the biological phenomenon of interest actually unfolds at the scale which is bridged by the respective technology (Figure 3) or whether a simpler approach is sufficient to answer the question.[10] Yet one should remain open to the idea that unforeseen discoveries can be made by just looking at higher resolution. Other considerations are whether live cells are required, how many colours need to be imaged, and whether 3D imaging is of importance. As highlighted by work done on cryo-ExM a key consideration about SRM, as the resolution improves further and further, is at which point are we still resolving accurate cell organisation or rather artefacts resulting from sample preparation. This consideration emphasises the importance of quantitative cross-validation of your findings using different microscopy techniques.

6 | 'SO WHAT HAPPENS AFTER WHAT?' – TIME-LAPSE IMAGING

Time-lapse imaging is a subcategory of live cell imaging, where the same cell is imaged sequentially over a certain time while undergoing a biological process. This offers

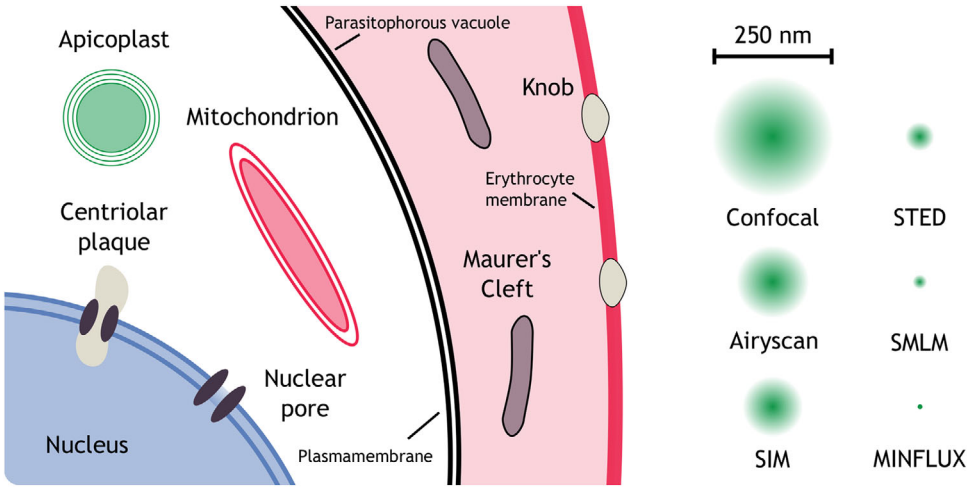

FIGURE 3 Depiction of the 2D Point Spread Functions (PSF) of different imaging modalities alongside approximate parasite organelle dimensions. The drawings are rough approximation for a trophozoite stage parasite not taking into account the often-elongated shape of organelles and significant changes in their morphology throughout parasite development. Drawings are to scale.

many key advantages for our understanding of biology. Importantly witnessing the biological process unfolding in an unperturbed and productive manner gives us some confidence that we are witnessing a reasonable version of reality. Secondly, biological systems are highly dynamic and only temporal data can reveal with certainty the correct sequence of biological events as they unfold. Live cell imaging has been used since the beginning of malaria parasite research as it was the movement of the beating microgamete flagella that convinced Laveran about the parasitic nature of the 'elements' he surmised in blood.[1] Also moving sporozoites and ookinetes were described as early as the late 19th century [213–215] and the circular motions of sporozoites was noted mid-20th century.[14] Yet they had no means to record and quantify the phenomenon and the implementation of fluorescent time-lapse microscopy was still far away.

6.1 | Time-lapse microscopy

Time-lapse 'cinephotomicrography', that is, the recording of time-lapse images, was carried out on malaria parasites in 1960 capturing a few time frames of a macrophage attempting phagocytosis of a schizont using film strips.[216] Even though this and earlier publications wrote about a potential invasion events of the red blood cell by merozoites they were yet unable to provide conclusive time-lapse data thereof.[217] The '…old controversy as to whether malarial parasites are within or upon their host erythrocytes…' posed in 1956 was resolved by subsequent electron microscopy studies,[218] but the short-lived nature of the merozoite invasion event was only fully captured first in 1975 using an optic-electrical recording device.[219]

Two decades later the detailed molecular study of the sporozoite gliding machinery motivated more quantitative time-lapse analysis,[220] which also led to the implementation of fluorescent time-lapse microscopy using parasites expressing cytoplasmic GFP.[221] Around the same time the formation of Maurer's Clefts and protein trafficking in the infected red blood cell had also proven to be a prime subject for study by time-lapse imaging.[222] Already then Fluorescence Recovery After Photobleaching (FRAP), which can reveal protein turnover kinetics in subcellular locations, aided the finding that Maurer's clefts are rather stable and show little to no exchange with the other membrane bound compartments. Fluorescent time-lapse microscopy of parasites was further pioneered in the context of intravital imaging,[10] which by default creates optimal conditions for live cells and strongly benefits from temporal information (see chapter on intravital microscopy).

Subsequent work on the cultured parasite stages revealed the importance of spikes in calcium signalling during the very short lived invasion process.[223] Further pushing the acquisition speed below a 1 ms time interval allowed analysis of the waveform of the beating microgamete flagella with holographic imaging.[224] But also the limits of fluorescent time-lapse imaging have recently been pushed by imaging microtubule, DNA, and host cell dynamics during microgametogenesis at the microsecond scale using multiple fluorescent stains and widefield microscopy.[225]

On the opposite spectrum of the 'time scale' infected red blood cells have only been imaged over almost the entire blood cell cycle of 48 h in 2011 to investigate morphological changes and protein trafficking in the parasite and host cell.[96] Long-term time-lapse imaging was further used to reveal changes of mitotic microtubule

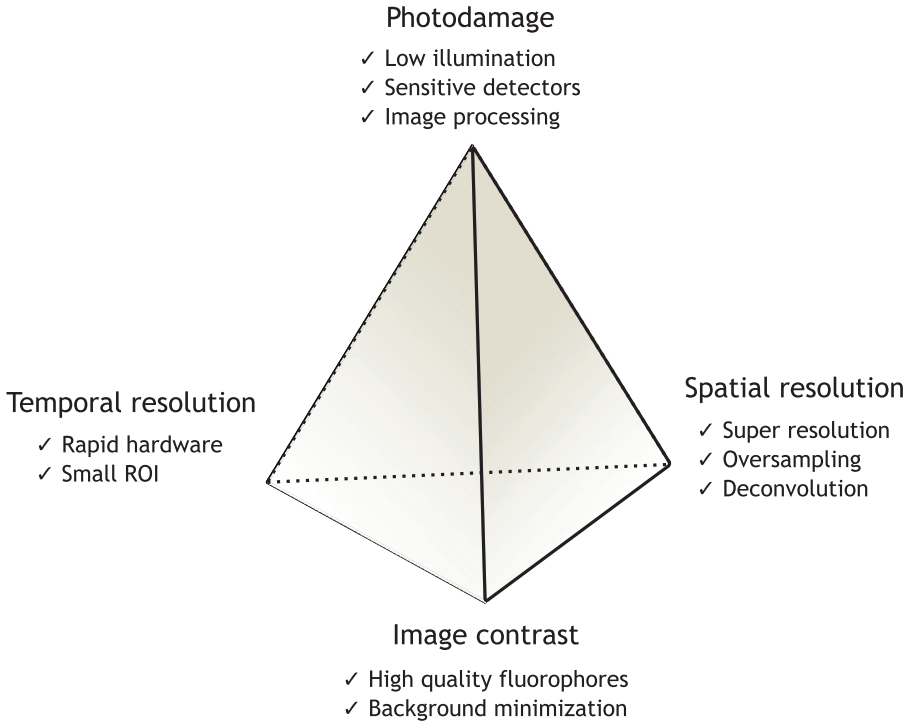

FIGURE 4 'Pyramid of frustration' for live cell time-lapse image acquisition. The four corners of the pyramid depict the measures of image data quality which are challenging to reconcile when imaging live cells over prolonged times. From this results the necessity to find, wherever possible, a suitable balance in the microscopy settings to achieve the desired image data properties. A few suggestions to improve image data quality that might mitigate the necessity to compromise are given.

dynamics and the regulation of genome replication during asynchronous nuclear multiplication events.[112,134] Super-resolving confocal imaging of the blood stage development was implemented to allow counting the individual nuclei resulting from schizogony.[182,226] Yet, data reproducing the entire blood cell cycle from invasion until egress still needs to be explored in more depth.[227]

6.2 | Challenges and practical considerations

A reason why long-term time-lapse imaging is less explored in malaria might relate to the significant challenges that need to be addressed to complete a successful and meaningful protocol. Aside the availability of acquisition time on a dedicated microscope, there are a number of technical and biological considerations to be taken into account. I will not address those in depth but there have been excellent reviews on the subject that detail all the critical steps along the time-lapse microscopy pipeline.[228,229]

The core challenge of time-lapse imaging revolves around the so called 'Pyramid of Frustration' (Figure 4). It entails balancing the four mutually exclusive corners of the pyramid, that is, photodamage, spatial resolution, temporal resolution, and image contrast. Photodamage also called phototoxicity is the effect of high energy light irradiation harming the cells thereby potentially causing morphological changes or even cell death.[230] The molecular causes of photodamage are ill-defined but propositions have been collected to mitigate the effect.[231] A related issue is the one of photobleaching, were fluorophores, usually FPs, switch into a dark state during excitation, effectively causing a loss of signal. Photobleaching will significantly vary between fluorophores and depend on the cellular context. Hence, one must take care to reduce the photodamage, that is, duration, number, and intensity of illuminations to the point where the parasite development is largely unperturbed while still collecting sufficient signal. This will require validation experiments that show that cellular functions, for example, speed of motion, progeny number and cell cycle duration are not significantly altered by the observation. Whether the experimenter wants to compromise on spatial resolution, temporal resolution, image contrast, or the number of channels will ultimately depend on the specific research question. The guiding principle for time-lapse microscopy therefore should be to aim for the minimal resolution and contrast necessary to answer the respective research questions.

Pioneering work has developed a suitable imaging protocol for in vitro malaria parasite cultures.[96] It addressed

the challenge of immobilising red blood cells, which do not naturally adhere, by using Concanavalin A.[232] Further this study has advocated the use of phenol red free cell culture medium to improve signal, but other imaging media formulations, for example, lacking riboflavin or containing antioxidants still need to be explored. Experience suggests that blood stage malaria parasites, and particularly the host red blood cell itself, are sensitive to fluorescent excitation light. Even brief observations of cells with epifluorescence illumination by eye prior to the start of imaging experiments can cause red blood cell damage and in extreme cases lysis making parasite development impossible (personal observation). Hence fluorescence exposure prior to acquisition should be avoided and UV-filters on broad spectrum illumination sources are mandatory. Obviously host stage parasites should always be maintained at 37° requiring a temperature-controlled incubation chamber. Additionally, parasites are, contrary to most other cell types, cultivated in a hypoxic environment. To reproduce this environment during imaging two approaches have been taken. Either one provides the parasite with the imaging dish with copious amounts of equilibrated medium and sealing the dish airtight.[96,232] Alternatively, imaging setups that provide hypoxic conditions themselves have been successfully used[132,182] and can support parasite imaging beyond three days.[233]

6.3 | Lattice light sheet microscopy

A new microscopy technique that addressed multiple challenges around time-lapse imaging at once was invented in 2014.[159] Lattice light sheet microscopy (LLSM) integrates the low photodamage and acquisition speed of light sheet microscopy with excellent spatial resolution. Contrary to classical light sheet microscopy it generates an ultrathin nondiffracting light sheet with lattice shaped illumination using an intricate two objective setup to produce SIM-like (see chapter on SRM) superresolved 3D images at superior speeds and with very little photodamage in living cells.

First use of LLSM in malaria produced outstanding footage of the membrane rearrangements and calcium signalling occurring during the red blood cell invasion process.[160,161] Their exquisite temporal resolution allowed them to document the sequential occurrence of membrane deformations, calcium influx, parasite internalisation, vacuole sealing, and echinocytosis in a process taking less than 2 min in total. They could then investigate changes in those dynamics after alterations in membrane cholesterol content. In a subsequent study, the same instrument was used to characterise invasion phenotypes that would likely not have been visible in classical time-lapse microscopy.[162] Despite some good progress in providing off the shelf commercial solutions it remains a technical challenge to exploit the full potential of lattice light sheet microscopy outside a highly specialised imaging platform. Nevertheless, it might in some cases be the only viable solution to resolve, without significantly harming the parasite, small scale and highly dynamic processes with stunning detail.

6.4 | Outlook and conclusion

Malaria research can still benefit from a more widespread application of time-lapse microscopy technologies. As the availability of live cell compatible markers (see chapters on fluorescent stains) and the list of proteins amenable to tagging increases, the potential of live cell imaging is raised. Further new strategies to reduce the impact of photodamage on your samples that implicate postprocessing of image have emerged. Image deconvolution is a long-standing method to improve image contrast and in some cases even resolution. More recently many new computational enhancement techniques such as image denoising have been developed and can be used further improve image quality when illumination is limiting[234,235] (see also chapter on image processing). Leveraging these techniques also for malaria research and further improving time-lapse imaging protocols will provide unique opportunities to better understand the dynamic and drastic morphological changes the parasite is undergoing throughout its life cycle.

7 | 'PHYSIOLOGICAL, MORE PHYSIOLOGICAL!' – IN VIVO IMAGING

The fate of the malaria parasite is intimately linked to its host and vector and their biology has been profoundly shaped by a reciprocal adaptation. From this naturally emerges the desire to study parasites in their in vivo context. Intravital microscopy surmounts substantial technical hurdles to offer insight into parasite biology of most physiological relevance. Particularly the transitional stages as the parasite move from one life cycle stage to the next have intrigued malaria researchers and necessitated investigations in their natural environment.

7.1 | Intravital microscopy

Intravital microscopy is an imaging technique used to visualise and study biological processes in live organisms in real time, which comes with several key challenges. (i) Due to the scarcity of events within a whole organism, one must image exactly at the right place and at the right time. (ii)

The thickness of the sample usually requires deep penetration of the light into the tissue, which might affect image quality by, for example, absorption, diffraction, or autofluorescence. (iii) The movement of the animal and its organs due to vital functions such as heartbeat and breathing need to be accounted for to achieve stable imaging conditions.[236] A microscopy technique that is frequently associated with intravital imaging and has significantly advanced the field of neurobiology is two-photon microscopy.[237] It uses long wavelength excitation that requires two-photons to simultaneously excite a given fluorophore, which is statistically more likely at the focal point. This allows much deeper penetration of the sample with less optical interference and further reduces issues with signal coming from beyond the focal plane. Yet two-photon microscopy is less widespread in malaria research, although it could have applications in imaging parasites inside living organs as well as in thick tissue samples.

The first imaging of malaria parasites in living organisms was achieved in the early 2000s by widefield and spinning disk confocal microscopy.[238,239] They allowed direct observation of the motile stages within the mosquito organs giving us a much better understanding about the gliding locomotion required to travel through the midgut, haemolymph, salivary gland, and finally the proboscis of the insect.[240,241] Around the same time the first observation of parasites at the injection site on the host put an end to the initial misconception that parasites are injected directly into the blood upon mosquito feeding.[242] Importantly, this supported the concept the skin is the first immunological barrier to parasite development. Usage of improved imaging setups and quantitative analysis then led to the seminal discoveries of how sporozoite stages actually migrate through the skin to reach the blood vessels.[243,244] Taken together this critical body of work provided the rationale for the parasite motility already observed in the 19th century[245] (see chapter on time-lapse microscopy). Also, the liver stages that were refractory to direct observation before, were analysed in the wake of the emerging intravital imaging technologies. It was revealed that parasites are released from the infected hepatocytes not as individual merozoites, but by budding of parasite-filled vesicles, which were called merosomes, which might contribute to protection from host immunity.[246,247]

Imaging of the brain of infected animals has been applied more recently to understand the mechanism underpinning the most lethal cerebral malaria caused by the asexually replicating blood stage parasites.[3] This requires careful consideration concerning the method by which the skull is made transmissible for light and has benefitted from two-photon microscopy as well as from miniaturised fluorescent microscopes implanted within the mouse cranium.

7.2 | Bioluminescence microscopy

Bioluminescence microscopy is a nonfluorescence based imaging technique that detects light produced by a biochemical reaction rather than by re-emitted excitation light. Early work on bioluminescence was tightly linked to the investigation of the fluorescence in marine jellyfish (see chapter on genetically encoded fluorophores). The molecule that enables bioluminescence when catalysed by a dedicated enzyme often called Luciferase is Luciferin, which was purified as crystal in 1962.[248] In the case of *Aequorea victoria* the oxidation of coelenterazine, a luciferin-like molecule, via the enzyme Aequorin produces a blue light.[249] This blue light in turn has the capacity to excite the green fluorescent protein without the jellyfish being exposed to any external excitation light.

In the context of malaria research bioluminescence microscopy has enabled minimally invasive intravital imaging of malaria parasite infections in mice using Luciferase-expressing parasite lines.[250,251] The luminescence signal emitted by the parasite can then be repeatedly detected at any point of the infection without surgical intervention on the mouse. This allows quantification of total parasite mass previously being limited to the circulating parasite population. Further it enables a more detailed analysis of the infectious state of individual organs and tissues. Since its inception bioluminescence microscopy has been consistently improved in terms of sensitivity and quantification.[252,253] This allowed studying parasite clearance by drugs or the immune system as well as the specific tissue tropism of parasites in a highly physiological infection model. Critically, bioluminescence helped the study of gametocyte sequestration within the bone marrow of infected hosts revealing a new parasitic niche, which was further supported by other intravital imaging modalities.[254,255] Despite some controversy concerning the transferability of rodent malaria models towards human malaria bioluminescence can provide a view into the systemic infection process that would otherwise remain entirely obscure.[256]

7.3 | Outlook and conclusion

Intravital microscopy can still benefit from a broader application using, for example, two-photon microscopy and could still reveal many insights about in vivo parasite biology. The uptake of gametocytes by the mosquito, as an example, remains completely unexplored to date and many open questions about parasite sequestration throughout the body remain. Yet the options to achieve intravital imaging in humans remain limited. Here, bioengineering of the malaria infection models combining multiple cell types

with 3D-printing platforms will provide an attractive middle ground bridging mouse model systems and the human infection model.[257]

8 | 'MAKING SENSE OF PIXELS' – COMPUTATIONAL IMAGE PROCESSING AND ANALYSIS

With the exponential growth of imaging technologies, the volume of image data is increasing at an unprecedented rate, making computational assistance essential for effective processing and analysis. In this chapter, I provide a brief primer on the fundamental techniques of image processing and analysis, which given the vastness of the field is by no means exhaustive. However, I want to encourage readers to delve deeper into the subject before establishing their own imaging pipelines. Most data generated by the aforementioned imaging modalities can be further enhanced by computational processing. With the advent of machine learning the range of possibilities has significantly expanded in this area. Broadly speaking, image processing can be divided into two categories, that is, image enhancement and image analysis. Importantly, these computational techniques are not a substitute for adequate sample preparation and imaging protocols. They rather work complementary or as data scientists eloquently put it 'garbage in, garbage out'.

8.1 | Image enhancement

The most prominent examples of image enhancement techniques are deconvolution and denoising. Deconvolution is applicable on 2D and 3D images and can significantly improve image contrast, that is, the ratio between signal and background, by reassigning pixel intensities based on the point spread function.[258] Although it might not affect intensity quantification it can significantly enhance the appreciation of cell morphology, co-localisation, and will also significantly benefit data representation. Yet, it might not always be required if image quality is already sufficient, and 'too aggressive' deconvolution can cause artefacts that should not be mistaken for real signal.[259] Despite having been used already in the 90s to improve imaging of proteins in the lumen of the tubovesicular vacuole networks within infected erythrocytes,[260] deconvolution is not consistently used in malaria imaging. Although it is an integral part of some imaging modalities like STED or Airyscan-detector based microscopy (see chapter on SRM).

Denoising, as the name suggests, makes inferences about the noise that might stochastically appear in a microscopy image, caused by, for example, fluctuations in detector sensitivity due to temperature, and attempts to remove it.[261] Multiple denoising approaches exist but fundamentally they work either (i) by using a high contrast/quality image of the same sample type, which will serve as a ground truth to then denoise low contrast/quality images of the same sample[235] or (ii) making a priori assumption about what the noise might look like to remove it from the same image.[262] The second approach being much less labour intensive is readily available as, for example, Fiji plugins such as Noise2Void.[263] Denoising has to my knowledge never been implemented malaria research.

Deconvolution and denoising are compatible and in my opinion should excel in the domain of parasite live cell imaging. They allow the acquisition of suboptimal images using very low illumination thereby limiting photodamage (see also chapter on time-lapse imaging). Through image enhancement those noisy images can then a posteriori be restored into images with sufficient quality for further analysis.

8.2 | Image analysis

Computationally driven image analysis offers many advantages. Due to the many open source solutions it can be highly cost effective if some basic computational resources are available.[264,265] As the amount of imaging data increases, automated image analysis can alleviate time-consuming manual image annotation. In some cases such as large 4D image data sets or in the context of high content screening manual annotation might simply not be feasible. Importantly, however, automated image analysis, even if not error-free, can deliver robust and reproducible data devoid of annotator bias. Core steps are (i) preprocessing, to normalise the input images; (ii) segmentation, which divides the image into meaningful objects; (iii) feature extraction, which quantifies characteristics of the objects; and (iv) classification, which groups the objects in classes according to specific rules. All these steps can be carried out across the multiple dimensions of an image, that is, space, time, and channels to deliver high content data. Important applications are the extraction of quantitative geometrical features, as well as approximation of changes in protein amounts throughout different cell compartments or time. These types of quantitative assessments further form the basis for mathematical modelling of imaging data. Yet, Image analysis is a field of its own with an ever-growing toolbox that is far from converging to a unified consensus. Although some software solutions are highly valuable it would still be challenging to objectively highlight specific ones including the ones

implemented within Fiji. As I will not attempt an detailed discussion here, the recently published community-driven AI assistant BioImage.IO can be a highly valuable tool to start navigating the intricacies of computational image analysis.[266]

Among many different applications automated image analysis in malaria occurred in the context of identifying *Plasmodium* species and counting parasitemia on Giemsa-stained blood smears.[267,268] The idea being to facilitate and abbreviate this routine and time-consuming procedure in research laboratories as well as in the field. Since then many efforts have been made to implement automated malaria diagnosis.[53] Reliable detection of infected and uninfected red blood cells remains challenging. Many sources of variability such as quality of the smear, staining efficiency, sample illumination, presence of nonred blood cells, microscope types, alongside the inherent lack of contrast of white light microscopy (see chapter white light microscopy) make it difficult to implement a robust and cost-effective automated quantification platform. The fact that these methods are not wide spread throughout the malaria labs of the world is a testament to the significant challenges to rival visual sample inspection by a skilled observer.[53] Nevertheless, significant progress has been made in this area through the implementation of convolutional neural networks, and development low cost microscope solutions that can even leverage smart phone application and 3D printing.[51,269,270] Automated counting of blood stages parasites using fluorescence less challenging, although it is not yet used to determine parasitemia [271] Using fluorescent parasite lines automated counting of oocysts in the mosquito using ImageJ based tools has been achieved and helped the assessment of potential transmission blocking drugs.[272]

Other types of data that lend themselves to automated image analysis are time-lapse movies and high content screening as the large number of images can make manual annotation prohibitively time-consuming. This was leveraged early on to track the 2D motility of sporozoites using custom made ImageJ plugin to reveal their different movement speeds and motility patterns, which helped understand the role of adhesion in motility.[273,274] Using a specific matrix another group could automatically track ookinete movement in 3D with Imaris and quantified their helical motion precisely for the first time.[275] More recently automated image analysis with machine learning was applied on intracellular feature showing how ectopic centrin accumulations with condensate-like properties increase and decrease throughout the blood stage parasite development.[139] To test phenotypic consequences of a large number of different conditions high content screening has been combined with machine learning and automated annotation and found various applications in quantifying the effect of drugs on parasite proliferation as well as sexual differentiation in the blood.[276–278]

8.3 | Outlook and conclusion

The integration of AI-driven image processing will continue to advance the field of malaria research, requiring some interdisciplinary collaborations. By standardising automated image readouts and feeding this data into computational models, we can unlock new insights into parasite biology. For instance, modelling time-lapse imaging data has already revealed unexpected predictions about parasite behaviour during proliferation.[134] Automated image processing and analysis, when applied consciously and transparently, is a powerful tool to maximise the value of imaging data. As these technologies evolve, they will play a key role in revealing deeper mechanistic insights, driving malaria research forward.

9 | FINAL CONCLUSION

Since the foundational work of Laveran and Ross, the malaria imaging community has made significant strides. Over the decades, the time gap between the invention of novel imaging technologies and their adaptation to malaria research has steadily decreased (Figure 2). In fact, with techniques like expansion microscopy, parasitologists have even emerged as early adopters. The trend of rapid adoption of new imaging technologies will hopefully continue, with researchers selecting the most appropriate techniques for their specific questions, potentially guided by this article. Maintaining the pioneering spirit that drives the use of cutting-edge methods can lead to new, unexpected research questions and further breakthroughs in malaria research. As visual beings, humans naturally benefit from microscopy's unique ability to deepen our understanding of life making it an invaluable tool in research but also for science communication. To gain the most meaningful insights, I advocate for an integrated approach that simultaneously considers sample preparation, fluorophore selection, imaging modality, and data analysis – always with the core scientific question at the forefront. This integral strategy ensures that each step enhances the clarity and relevance of the results that help us understand and combat malaria.

ACKNOWLEDGEMENTS

I thank Florence Le Corre from the 'Musée du Service de santé des armées, Val-de-Grâce' in Paris for providing pictures of Laveran's microscope and complementary information. I further thank Vicky Ingham for support. Every

effort has been made to ensure the accuracy and completeness of the literature reviewed. I sincerely apologise for any omissions or chronological inaccuracies, which may result from limited journal access or inadvertent oversight.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

FUNDING INFORMATION

J.G. received funding from the Chica and Heinz Schaller Foundation.

DECLARATION OF USE OF AI IN THE WRITING PROCESS

Large Language Model (ChatGPT) has been used to revise certain segments of the text regarding grammar, clarity, and readability.

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How to cite this article: Guizetti, J. (2025). Imaging malaria parasites across scales and time. *Journal of Microscopy*, 1–29. https://doi.org/10.1111/jmi.13384