Achieving *Superb* Resolution – Imaging Malaria Parasite Invasion of the Human Red Blood Cell

Jake Baum¹, Dave Richard² and David T. Riglar³

¹ Department of Life Sciences, Imperial College London, London, UK.
² Department of Microbiology, Infectious Diseases and Immunology, Laval University, Quebec, Canada.
³ Department of Systems Biology, Harvard Medical School, Boston MA, USA.

For correspondence:

Dr. Jake Baum

Sir Alexander Fleming Building, Department of Life Sciences, Imperial College London, Exhibition Road, South Kensington, London, SW7 2AZ. Tel: +44 (0)207 5945420; Email: jake.baum@imperial.ac.uk
It is only in the last decade that sub-cellular resolution of red cell invasion by the malaria parasite, *Plasmodium falciparum*, has been possible. Here we look back on the development of methodologies that led to this possibility, and the subsequent advancements made in understanding this key event in malaria disease.

Malaria pathogenesis resides with the blood stages of infection, where the cycles of parasite entry, development, rupture and re-entry lead either directly or indirectly to all the symptoms of the disease. This has long-focused attention on the mechanisms that underlie parasite entry into the red blood cell; a refuge away from immune detection in which the parasite can grow and divide. Beginning in the late 1960s through to the 1970s, early advances in electron and light microscopy gave scientists the first detailed insights into the invasion event, laying the foundations for our understanding of the process. However, even in these heady days at the beginning of cellular parasitology, capturing invasion was limited in resolution and the frequency with which it was seen. Furthermore, most insights gained came from species of *Plasmodium*, the genus responsible for malaria across vertebrates, that were non-pathogenic to humans. Specifically, to the best of our knowledge, only a single invasion electron micrograph for *P. falciparum*, the most virulent species affecting humans, was published in the 20th century, a chance encounter of the blood stage merozoite caught in the act of red cell penetration (Langreth et al., 1978).

Thus, in 2009, with one of us (JB) just starting out as an independent investigator, another in the final year of his postdoc (DR) and the third (DTR) a student at the beginning of a PhD, understanding of *P. falciparum* erythrocyte invasion was a case of so close and yet so far. The intervening years had been far from quiet of course. Major advances had been made in the identification of key antigens required for invasion leading to an emerging model of invasion
mechanics driven by a parasite actin-myosin motor. The newly published malaria parasite genomes had also identified a burgeoning list of candidates of interest that were undergoing systematic gene deletion, tagging and localization by immunofluorescence or immuno-electron microscopy (EM) within the parasite (reviewed in (Cowman et al., 2012)). And yet, despite these advances and an expanding tool kit of molecular genetics, the inability to isolate the invasion event in *P. falciparum* left many ambitious projects hamstrung. As such, imaging of invasion and biochemical understanding of the steps involved rested with snapshots before host cell entry (late in the developing cycle with mature schizonts or released merozoites) or after entry (early ring stages) to infer how the complex interactions involved were coordinated in a process that lasts just a few 10s of seconds. When invasion was captured by fluorescence microscopy, it relied on leaving a parasite-infected culture to rupture over-night at high parasitemia, often in the presence of an inhibitor (such as the actin binding drug cytochalasin) with the hope of chancing upon an invasion event in the morning, inferred by finding a merozoite in close proximity to an erythrocyte. The size of the merozoite, at around 1 micron, meant that many images were simply an estimate of whether green (for example a label of one parasite protein) and red channels (perhaps labelling another parasite or red cell protein) overlapped enough to give a yellow signal (for co-localization studies) or whether they were apical, basal or diffuse for sub-cellular localization estimation. Whether such events gave a true picture of where proteins resided and their potential functions remained hard to judge.

With this backdrop, the key question perpetually niggling our understanding was what were the core components of the merozoite entry machinery doing *during* invasion. One technique that had advanced significantly was live imaging of *P. falciparum* invasion. By painstakingly synchronizing cultures, learning what to look for in a late stage, ready-to-rupture late stage
parasites (called schizonts) and waiting, Glushakova and colleagues (Glushakova et al., 2005) and later Gilson and Crabb (Gilson and Crabb, 2009) were able to capture invasion events reliably and begin to dissect the kinetics of invasion, mirroring work carried out by the great Jim Dvorak using *P. knowlesi* (a parasite normally associated with macaques in South East Asia) in the 1970s (Dvorak et al., 1975). These studies were added to by others using clever combinations of genetics and inhibitors with video microscopy to begin to lay a foundation of molecular and cellular understanding of mid-invasion events. Nevertheless, for each of these studies, capturing invasion was synonymous with long days, or often long nights, sitting at the microscope and gambling on which schizont stage parasite was close enough to bursting, avoiding light damage so it would actually rupture and waiting for the chance moment when the parasite might then invade an awaiting red cell. And, of course, this could not be just any red cell but one separated from its neighbors enough so as to give some level of resolution! The challenges involved is testimony to the herculean efforts of the few who published in this area in the first decade of the 21st century. As for our own efforts in this area, on one memorable afternoon, after many hours of non-stop imaging of schizonts seemingly ready to burst but not yet seeing any merozoite invade, we gave up and left the microscope running. On return an hour or so later, looking over the footage we found perhaps our most successful video of invasion at the time – it seems a watched parasite never invades (see Supplementary Movie File S1). We were not alone in thinking there must be a better way.

As Pasteur’s idiom reminds us *chance favors only the prepared mind*. In a neighboring lab to ours at the Walter and Eliza Hall Institute (WEHI), the laboratory of James Beeson was experimenting with use of heparin-like molecules to synchronize and inhibit *Plasmodium* invasion. Through their painstaking efforts exploring combinations of synchronous cultures,
inhibitors of egress and various filter types, Michelle Boyle, Danny Wilson and Jack Richards along with others in the Beeson lab finally succeeded in generating purified *P. falciparum* merozoites that were competent for invasion – something that could be routinely achieved for *P. knowlesi* but not, until this point, for *P. falciparum*. From our perspective, and many in the field, their combined efforts, published in a key paper in 2010 (Boyle et al., 2010), was nothing short of a revolution and very much the answer to what we’d been looking for. With the generous collaboration of the Beeson laboratory and colleagues at the University of Melbourne under the guidance of Stuart Ralph, we then asked the question whether taking these merozoites and mixing them with blood cells for varying degrees of time might yield parasites reliably caught during entry for immunofluorescence and electron microscopy dissection – finally seeing molecules and cellular structures in action midway through entry. Our initial characterization included both light microscopy and electron microscopy and we were very much encouraged by the exciting results, hinting strongly that we could capture merozoites in the process of invasion on demand (Boyle et al., 2010). However, we were still struggling to differentiate an invasion event from proximity to the red cell without ambiguity.

At the same time, a flurry of interest had developed around a complex of rhoptry neck proteins (the RONs) of *Toxoplasma gondii* (a distant cousin of *Plasmodium*). These RON proteins were identified by their interactions with the micronemal Apical membrane antigen protein 1 (AMA1) and found to co-localize during invasion by tachyzoites, the replicative intracellular stage of *T. gondii*. (Alexander et al., 2005). Prior to this point, little was known about the rhoptry neck proteins and AMA1. The latter, whilst long implicated in invasion, had no defined mechanistic role in the process. The two protein classes, released from very different compartments, came together at a key structure of invasion, the tight junction. This structure, seen as an electron
dense interface between parasite and target cell was first identified by Aikawa and Bannister in the 1970s and was seen by many to be the key structure indicative of an invasion event, representing the fulcrum where the molecular and cellular processes of entry concentrated. During our earlier studies, we had generated antibodies against the *P. falciparum* orthologues of the RON proteins (Richard et al., 2010) and were thus poised to test these with our imaging of invading merozoites. Using an anti-PfRON4, we captured our first immuno-fluorescence images of merozoites mid-way into an erythrocyte using z-stacks and deconvolution of fluorescence images. On inspection, each invasion event was encircled with a beautiful ring of green PfRON4, tracking the merozoite tight junction. We were awe struck. Having tried in vain for so many years to define invasion, through the combined efforts and collaborative generosity of several key groups, we now had a reliable platform to study merozoite invasion events in detail.

The subsequent months became a whirlwind of activity. Electron microscopy of invasion events combined with immuno-EM gave us further confidence linking immuno-fluorescence observations with cellular structures underlying entry. Yet we still struggled with resolution for immunofluorescence – a critical goal if we were to be able to place invasion proteins in space and time during parasite entry. Another chance encounter, this time with Cynthia Whitchurch from the University of Technology in Sydney (UTS), introduced us to the new revolutions of super-resolution imaging, specifically structured illumination microscopy (3D-SIM), an ideal platform for three dimensional reconstruction of sub-micron cellular events. Using the super-resolution microscope at UTS, involving many trips forwards and backwards from Melbourne, we started to build a portfolio of images detailing the precise events of invasion. In combining these images, electron microscopy from the Ralph lab along with the use of mutant lines and
inhibitor experiments, we were able to piece together a step-wise model for the molecular events underlying malaria parasite invasion of the human erythrocyte – a paper we were thrilled to have published in *Cell Host & Microbe* (Riglar et al., 2011). Central to the impact of the paper was the demonstration of the ordering of events that led to the establishment of the tight junction, the release of rhoptries, the formation of the parasitophorous vacuole or PV (in which the parasite eventually develops) and the coordination of these events with merozoite surface protein shedding and motor activity. Super resolution imaging was able to provide striking images of some of these events, for example, the merozoite climbing through the junction directly into the nascent PV (Figure 1).

Arresting merozoite invasion and subsequent imaging became a routine approach for us in the lab and helped estimate or resolve the localization of many proteins predicted to play a role, debunk others as playing no role, or dissect the cellular basis of invasion using other techniques, for example cryo-electron tomography. Using the same approach, we were also able to address questions about the events immediately subsequent to invasion, as the early ring stage parasite establishes infection within the erythrocyte. However, a final key challenge remained, that of quantification. Whilst we could reliably see invasion, comparing between invasion events was always somewhat subjective – and though we placed a high bar on objectivity we frequently faced criticism of selectivity with image choices. To resolve this, we developed a simple computational pipeline to enable comparative imaging between invasion events, a process we termed *longitudinal intensity profiling* (Riglar et al., 2016). Using this approach, we could reliably follow a protein through invasion events (sorted in order of progression through the RON4 junction) to explore the dynamic localization of proteins from static images. It was important to us that this didn't require access to novel technology, but
instead could be undertaken on a good light microscope, using deconvolution and of course having access to fixed merozoite invasion events to image. Using this approach, we were then able to validate some of our prior observations, giving them quantitative rigor, such as the localization of actin at the junction, and raise hypotheses about the role some proteins, predicted to be present but glaringly absent from invasion events (such as the merozoite thrombospondin related anonymous protein, MTRAP), might actually play (Riglar et al., 2016). The prediction with MTRAP, i.e. that it plays no role in invasion (given absence of a fluorescence signal for the ecto-domain of the protein during invasion), has proven to be particularly noteworthy with subsequent work now showing it can be knocked out in blood stages using the Crispr/Cas9 system with no observable growth defect but a major phenotypic effect in the transmission stages of development (Bargieri et al., 2016).

In the intervening years since our study, the field has seen incredible active with seminal studies adding more detail to the step-wise events of invasion (Weiss et al., 2015), inclusion of biophysical forces into understanding merozoite surface adhesion using optical tweezers (an amazing achievement in parasite imaging) (Crick et al., 2014), use of complex genetics to combine imaging with functional analysis of essential genes (Volz et al., 2016), and early attempts to bring some resolution to the role of the often forgotten erythrocyte in the process (Zuccala et al., 2016). As microscopy advances in the three dimensions of space with added resolution in time, further clarity is likely to be gained into the precise mechanics of how the malaria parasite establishes infection within the erythrocyte.

Importantly for us, the journey taught us many key lessons. Foremost, although the gap in the literature suggested merozoite imaging in *P. falciparum* would be next to impossible, such a seemingly insurmountable question or problem should not deter the tenacious student from
continuing to try. Secondly, in what became very quickly apparent to us in this work, collaboration, often with those in neighboring labs, is often the deal clincher when it comes to answering these same problematic questions. And finally, that superb resolution (not to be confused with super), although we certainly initially sought it out using the most powerful and expensive state-of-the-art platforms we could get our hands on, often came easiest from a good wide-field microscope, used well with some quantitative rigor. It is our opinion that understanding of merozoite invasion is really only just beginning, and that there are still many insights and discoveries, especially those relating to mechanism (what does each protein do?), still to be made.

Acknowledgements

We would like to thank all our colleagues in the field for creating such a vibrant community of researchers. We recognize that in presenting a singular account of the evolution of the field we may have overlooked substantial contributions by others. In particular, we’d like to acknowledge all authors whose key contributions to the field we have not cited due to limitations of space. JB is supported by the Wellcome Trust, through an Investigator Award (100993/Z/13/Z). DR is supported by a Fonds de la Recherche du Québec-Santé Junior 1 career award. DTR is supported by a Human Frontier Science Program Long Term Fellowship and an NHMRC/RG Menzies Postdoctoral Fellowship.

References


Figure 1. Three dimensional, structured illumination microscopy (SIM) of merozoite invasion. The image shows a time course of parasite invasion with different parasites caught at different stages of entry into the host cell (not shown). The parasite is labelled with three markers: the nucleus labelled blue (DAPI), the tight junction labelled green (RON4) and the nascent parasitophorous vacuole (PV) labelled red (rhoptry associated protein, RAP1) deriving from secreted rhoptries that release concomitantly with invasion. The parasite is seen to progress from early in invasion (with a small apical tight junction), through mid-way with the junction encircling the parasite at the halfway point, to full entry, with the parasite now enclosed within the PV with the junction now virtually sealed at the rear of the parasite. Scale par is as indicated. Image reproduced from (Riglar et al., 2011).

Supplementary Movie S1.

Bright-field video microscopy of *P. falciparum* invasion, chanced upon as the camera was left on during a coffee break.