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Intravital microscopy: Imaging host-parasite interactions in lymphoid organs

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Abstract

Intravital microscopy allows imaging of biological phenomena within living animals, including host-parasite interactions. This has advanced our understanding of both, the function of lymphoid organs during parasitic infections, and the effect of parasites on such organs to allow their survival. In parasitic research, recent developments in this technique have been crucial for the direct study of host –parasite interactions within organs at depths, speeds, and resolution previously difficult to achieve. Lymphoid organs have gained more attention as we start to understand their function during parasitic infections and the effect of parasites on them. In this review, we summarize technical and biological findings achieved by intravital microscopy with respect to the interaction of various parasites with host lymphoid organs, namely the bone marrow, thymus, lymph nodes, spleen, and the mucosa-associated lymphoid tissue, and present a view into possible future applications.

Introduction

Intravital microscopy (IVM) allows visualization of organs in living animals, down to sub-cellular resolution, to study cellular interactions, cell dynamics, motility, adhesion, rheology, and anatomical changes in different tissue compartments through time. Major advances in imaging have allowed more organs, and a wider range of physiological phenomena, to be visualized *in vivo* (reviewed by De Niz et al., 2019). Amongst the most studied systems by IVM is the immune system (reviewed by Secklehner et al., 2017). Altogether, various methods have been developed to study lymphoid organs, and the voyage of immune cell populations across the body of living animals upon challenges including tumours, infection, and inflammation (Chtanova et al., 2014; Chtanova et al., 2009, 2008; Hampton et al., 2015; Ladi et al., 2008; Torcellan et al., 2017).

Immune cells are organized within primary and secondary lymphoid organs, which are vital for innate and adaptive immunity. Primary lymphoid organs (PLOs) include two anatomically complex tissues: the bone marrow and the thymus, where lymphocyte differentiation and maturation occurs. Secondary lymphoid organs (SLOs) include the lymph nodes, spleen, and mucosa associated lymphoid tissues (MALT). These organs host diverse populations of functionally mature, naïve lymphocytes, and are optimally localized across the body to enable efficient surveillance, detection of, and response against foreign antigens (reviewed by Ruddle and Akirav, 2009). In parasitology, primary and secondary lymphoid organs have been the focus of interest in three main contexts: immune response generation upon parasitic infection; organ invasion and remodelling by parasites; and the advantages of tropism for parasite biology. In this review, we will focus on key findings on parasite and host-immune cell interactions, visualized within primary and secondary lymphoid organs using IVM. Equally, we will discuss the advances on surgical procedures, optical windows, and imaging platforms to visualize these organs.

Primary lymphoid organs in parasitic infections

The bone marrow and thymus are the PLOs where the largest part of lymphocyte development occurs. Hosting complex interactions between bone and immune compartments, the bone marrow is critical for haematopoiesis, immunological memory and bone regeneration. Critical for the development of T cells, the thymus is vital for the generation of strong, yet self-tolerant, adaptive immune responses. Below, we discuss the relevance of performing IVM in

these organs, available techniques, and key biological findings obtained by IVM in the context of parasitology (summarized in **Figure 1**).

Biological relevance of the bone marrow

The bone marrow is amongst the organs where IVM has permeated the least, perhaps because of the challenges it represents for imaging. However, histology techniques combined with human and animal autopsies have shown that the bone marrow can influence pathology related to parasitic infections and in some cases, is even a key niche for parasite development. Direct and indirect bone marrow involvement in pathology has been suggested for parasites including *Plasmodium* spp., (Baro et al., 2017; De Niz et al., 2018; Duffier et al., 2016; Farfour et al., 2012; Joice et al., 2014; Lee et al., 2017; Lee et al., 2018; Mayor and Alano, 2015; Messina et al., 2018; Neveu et al., 2018; Alano, 2017; Rogers et al., 2000; Smalley et al., 1981; Waseem et al., 2016; Wickramasinghe et al., 1987), *Toxoplasma gondii* (Brouland et al., 1996), *Leishmania* spp., (Ali and Hussain, 2014; Hellal and Kundu, 2013; Kumar et al., 2007), *Schistosoma* spp., (Azevedo et al., 2015; Jones and Leday, 2014; Kamal, KA et al., 1989), and *Trypanosoma* spp. (Baena Terán et al., 2012; Bockstal et al., 2011a, 2011b; Carbajosa et al., 2017; De Diego et al., 1998; Felizardo et al., 2018; Habila et al., 2014; Mabbott and Sternberg, 1995; Müller et al., 2018; Obishakin et al., 2014; Souza et al., 2014; Stijlemans et al., 2016).

The bone marrow is a heterogeneous environment. Within this organ, the stroma provides the structural and chemical niche that supports survival, differentiation and proliferation of pluripotent hematopoietic stem cells (Calvi et al., 2003; Erslev, 1971; Hackney et al., 2002; Lemischka and Moore, 2003; Trentin, 1971; Zhang et al., 2003). In addition, the bone marrow possesses a dense vascular network, which occupies about 30% of this tissue's volume. This vascular network is largely heterogeneous, with sinusoidal blood vessels being the most prominent vessel type (Itkin et al., 2016; Spencer et al., 2014). This is relevant in the context of parasite colonization, as the sinusoids are characterized by specific haemodynamics, including slow flow rate and high permeability (Itkin et al., 2016; Jung et al., 2018), which affect whether and how parasites cross the vascular endothelium and establish in the bone marrow.

Bone marrow studies envisaging the use of IVM must consider three key aspects: a) the heterogeneity of the bone marrow niche across the body; b) the suitability and comparability of animal models with human bone marrow composition; and c) accessibility for IVM, which varies across bone locations, making some sites more accessible for imaging.

Methods for IVM-based visualization of the bone marrow

A commonly preferred site for bone marrow IVM is the calvarium, between the sagittal suture bifurcation and the intersection of sagittal and coronal sutures (**Figure 1A,i**). Historically, bone marrow imaging was first performed in the calvarium (Mazo et al., 1998). This location is preferred mostly because it provides easy surgical access, and the calvarial bone marrow is sufficiently thin and transparent to allow light penetration for high resolution image acquisition. To perform IVM in the calvarial bone marrow, one of the possible surgical procedures is to open the scalp skin to access the calvarial bone, and then suture after imaging. This method represents a challenge for longitudinal imaging because the scalp must be opened and sutured for each imaging session, causing significant scarring and tissue damage that often induce inflammation, degrade image quality, and limit the number of imaging sessions that can be performed at the same site (Lo Celso et al., 2011). Therefore, it is preferable to pursue methods that, consistent with the 3R perspective relative to work with living animals, diminish discomfort and damage to the animal. To refine the procedure for improved animal welfare, the mouse calvarial window model was developed. Here, a small section of the scalp is removed, a coverglass is attached to the frontoparietal region of the calvarium, and the exposed skull area is covered with a mixture of dental cement powder and cyanoacrylate glue to prevent re-growth of the membrane layer (Le et al., 2017). Alternatively, the cortical bone can be removed, and only a thin layer of bone left. This removal can be done mechanically, or by laser ablation (Lo Celso et al., 2011, 2009, 2009(1); Turcotte et al., 2014).

In 2009, Köhler *et al* presented IVM methodology for imaging bone marrow in long bones (Köhler et al., 2009). In their work, the authors argued that although the calvarium had been consistently imaged by IVM, it was unclear whether this area was representative of events taking place in long bones (e.g. the tibia). They described a procedure for imaging the tibia, consisting on removing the skin and muscle on top of the bone with a scalpel and/or an electric drill to obtain a very thin (30-50 μm) layer of bone tissue covering the bone marrow (**Figure 1A,ii**). In this technique, it is important not to damage this thin bone layer, to prevent vascular collapse in the bone marrow cavity. In 2017, an advance for this procedure was published, whereby in addition to the surgical procedure, a medium-to-long-term imaging window could be incorporated (Kim et al., 2017). Conversely, an alternative non-destructive method has been explored to investigate the bone marrow niche, and allows visualization of the intact tibia by two-photon microscopy (Lawson, M.A., et al., 2015).

A more recent method involves the development of a microendoscopic multi-photon imaging approach to perform longitudinal imaging deep within the bone marrow (LIMB) at various anatomical locations, including the calvarium, the tibia, and the femur (Reismann et al., 2017) (**Figure 1A,iii**). This approach consists on surgically implanting into the mouse femur, a biocompatible fixation plate containing a gradient refractive index (GRIN) lens. This setup is based on a fixation plate originally developed to stabilize the femur after an osteotomy (Matthys and Perren, 2009). Two types of setups were designed to account for tissue heterogeneity in different areas, allowing access to diaphyseal (mid-section of the long bone) or metaphyseal (narrow portion of the long bone) regions. Moreover, a varying length of the endoscopic tubing allows imaging at different depths of the bone marrow. The lenses are permanently glued to titanium tubes mounted on the fixation plate. Window types, and their advantages and limitations, are summarized in **Table 1**.

Biological findings in parasitology by IVM: the bone marrow

Various parasites home to the bone marrow including *Leishmania* spp., *Trypanosoma* spp., *Schistosoma* spp., and *Plasmodium* spp. Histology studies on autopsies from humans, cattle and rodent models, have shown that either by their presence alone, or indirectly due to systemic effects, some of these parasites induce drastic changes in various bone marrow compartments. Reported changes include hypercellular marrow, granulomas, lymphoid nodules, fibrosis, and even necrosis in *Leishmania* infections, all of which correlate with poor prognosis (Haque et al., 2018; Hellal and Kundu, 2013; Kumar et al., 2007). More recently, a murine model of visceral leishmaniasis explored for the first time how CD4+T cell-mediated immunopathology contributes to repression of medullary erythropoiesis in the bone marrow niche, resulting in affected hematopoietic competence and anaemia – one important sequela of *L. donovani* infections (Preham et al., 2018). *T. cruzi, T. brucei, T. vivax,* and *T. congolense* also affect the bone marrow, and induce both acute and chronic effects throughout infection. These include anaemia, thrombocytopenia, leukocytosis, reticulocytosis, and bone marrow hypoplasia (Bockstal et al., 2011b, 2011a; Marcondes et al., 2000; Ojok et al., 2001; reviewed by Silva Pereira et al., 2019). Among other parasites, *Schistosoma* eggs and *Toxoplasma* have been detected directly on human bone marrow (Brouland et al., 1996; Jones and Leday, 2014). In rodent models, the presence of *Schistosoma* influences differentiation at the progenitor level, resulting in induced granulopoietic activity (Joshi et al., 2008). This is thought to be highly linked with maturation of *Schistosoma* worms, and oviposition (Azevedo et al., 2015; Elkhafif

et al., 2010; Kamal et al., 1989). To our knowledge, neither *Leishmania* spp., *Trypanosoma* spp., *Toxoplasma gondii*, or *Schistosoma* spp. have been visualized in the bone marrow using IVM.

In *Plasmodium*-infected humans, non-human primates, and mice, both asexual stages and gametocytes of *Plasmodium* have been found in the bone marrow during blood stages of infection. The presence of parasites and parasite products has been associated with pathology including bone loss (Lee et al., 2017), anaemia, and immune dysregulation (Waseem et al., 2016).

However, the main interest in the bone marrow, particularly in recent years, has been in the context of *Plasmodium* gametocyte maturation. *P. falciparum* has five morphologically distinct stages of maturation (I-V), of which only the last stage (V) is present in peripheral circulation. For some time, the anatomic site allowing homing of immature gametocytes, remained unknown. In the early 1980s, various studies performed in either bone marrow aspirates or autopsies from human patients showed enrichment of immature *Plasmodium* gametocytes in the bone marrow extravascular space (Smalley et al., 1981), and *Plasmodium* sequestration in bone marrow sinusoids (Wickramasinghe et al., 1987).

Only in the last decade has *Plasmodium* transmission biology research re-gained great momentum, leading to a large number of questions regarding *Plasmodium* interactions in the bone marrow, including mechanisms of extravasation, development, and egress (Aguilar et al., 2014; Aingaran et al., 2012; Farfour et al., 2012; Joice et al., 2014; Lavazec, 2017; Lavazec et al., 2013; Lavazec and Alano, 2014; Messina et al., 2018; Naissant et al., 2016; Neveu et al., 2018; Ramdani et al., 2015; Rogers et al., 2000). Beyond *P. falciparum*, the bone marrow has been shown to be an important niche for *P. vivax* development, including gametocyte maturation and asexual development responsible for chronicity (Baro et al., 2017; Markus, 2018; Mayor and Alano, 2015; Obaldia 3rd et al., 2018).

While *Plasmodium* gametocyte maturation dynamics had previously not been addressed in detail using animal models, three important advances were achieved over the last 3 years: a) the use of humanized mouse models to study *P. falciparum* gametocyte dynamics (Duffier et al., 2016); b) the demonstration that organs involved in erythropoiesis, including the bone marrow are an important reservoir for rodent malaria gametocytes (De Niz et al., 2018; Lee et al., 2018), and c) the use of IVM to study the dynamics of gametocyte maturation including extravasation, maturation, and egress (De Niz et al., 2018). The IVM study showed that bone locations enriched with red marrow were preferentially colonized by *P. berghei*, and that bone marrow sinusoids are key sites for sequestration and invasion. Within the bone

marrow parenchyma, *P. berghei* gametocytes mature in the proximity of erythroblastic islands, consistent with observations in human autopsies (Joice et al., 2014). IVM showed that as infection progressed, vascular leakage contributes to the entry of sexual and asexual parasite stages to the extravascular space. Finally, mature gametocyte re-entry to the peripheral blood was observed, and was shown to require great deformability (De Niz et al., 2018) (findings are summarized in **Figure 1B**).

Altogether, the bone marrow represents a puzzling environment so far greatly understudied in parasitology, for which IVM could contribute significantly in the context of immune responses, host-pathogen interactions, chronic and acute BM remodelling, parasite latency, and drug delivery.

Biological relevance of the thymus

The thymus is a primary lymphoid organ which supports the 'education' of T lymphocytes, resulting in a T cell pool that is self-restricted and self-tolerant. Anatomically, the thymus is located in front of the heart, and behind the sternum (**Figure 1C,i**). It consists of two merged lobes surrounded by a capsule. The lobes have a dense outer cortex, and an inner medulla. Both the cortex and the medulla are populated by cells of hematopoietic origin, and thymic stromal cells, including epithelial cells. These epithelial cells are essential for the successful selection of a T cell repertoire that is capable of antigen recognition, and of distinguishing self from foreign. The cortex supports the early events of T cell development following their migration out of the bone marrow, including T cell receptor gene rearrangement and positive selection. The medulla supports the late steps of T cell development, including negative selection rounds necessary to eliminate autoreactive T cells. Mostly in the medullary region, thymic dendritic cells play a key role in the establishment of central tolerance by presenting a broad range of self-antigens. A high affinity interaction with self-antigen-specific thymocytes results in thymocyte death, avoiding self-reacting thymocytes from differentiating into mature T cells (reviewed in Oh and Shin, 2015). Additional to specific cell interactions, hormones and cytokines present in the thymic environment regulate T cell maturation, while strict chemokine expression directs cell migration within the thymus, and egress of mature T cells to the periphery once their development is complete (Bunting et al., 2011).

Methods for IVM-based visualization of the thymus

Although T cell responses during infection are a relevant topic for various fields of parasitology, the thymus has not directly been imaged by IVM *in situ*. Due to its anatomical location, the thymus presents important challenges for direct and continuous visualization. To circumvent such challenges, a frequently used alternative has been the transplantation of the thymus to the kidney capsule (Caetano et al., 2012). After acceptance of the thymus, the transplanted kidney is exposed, fixed in a stereotactic organ holder, and kept under physiological conditions for *in vivo* imaging (**Figure 1C,ii)**. To prevent the kidney from returning to its original position, improved methods suggest closing the lateral sides of the skin incision with stitches (Caetano et al., 2012). Although the transplanted thymus approach has been successfully used in various studies, limitations of this approach include the complexity of the surgery required for transplantation, and the fact that different corporal conditions exist in the surrounding environment of a thymus transplanted in the kidney as opposed to a thymus located in the thoracic cavity (Aghaallaei and Bajoghli, 2018). *Ex vivo* alternatives to the complex transplantation procedure include extraction of individual thymic lobes perfused with oxygenated medium, followed by imaging by two photon microscopy (Ladi, E., et al 2008), and imaging thymic slices (Bousso et al., 2002; Bousso and Robey, 2004; Ehrlich et al., 2009; Ross et al., 2016). Both methods have been used to observe thymocyte dynamics shortly after extraction. A limitation reported by the various groups who have used this method is that the cytokine and chemokine millieu of the slices might not be representative of the intact thymic environment *in vivo*. Finally, another alternative for imaging the thymus, is the use of teleost fish models including zebrafish and medaka. These models have allowed performing time-lapse imaging of the entire thymus, and visualization of T cell dynamics at different stages of development (Bajoghli et al., 2015; Hess and Boehm, 2012). Window types, and their advantages and limitations, are summarized in **Table 1**.

Biological findings in parasitology by IVM: the thymus

To our knowledge, IVM has not been used to study the thymus in the context of parasitology. However, various parasites invade the thymus, causing important disruptions in this organ's delicate architecture, and infection has important implications for T cell mediated responses. This suggests that IVM implementation to study this organ might be highly relevant, and worth pursuing. Parasites known to involve the thymus include *T. cruzi* (Cotta-de-Almeida et al., 2003; Farias-de-Oliveira et al., 2013b, 2013a; Leite-de-Moraes et al., 1992; Mendes-da-

Cruz et al., 2006, 2003; Pérez et al., 2007; Savino et al., 1989), *Plasmodium* spp., (de Meis et al., 2012; Francelin et al., 2011; Gameiro et al., 2010) and *Toxoplasma gondii* (Huldt et al., 1973; Kugler et al., 2016; reviewed by Nunes-Alves et al., 2013; Savino, 2006) (**Figure 1D**).

Infections with *T. cruzi, T. gondii,* and *P. berghei* cause thymic atrophy by different processes (de Meis et al., 2012; Farias-de-Oliveira et al., 2013a, 2013b; Francelin et al., 2011; Nunes-Alves et al., 2013). Infection of the thymus by *T. cruzi* or *P. berghei* results in significant changes to the extracellular matrix, as well as increased fibronectin, laminin deposition, and chemokine ligand expression (Cotta-de-Almeida et al., 2003; Gameiro et al., 2010; Mendes-da-Cruz et al., 2006; Savino et al., 1989) (**Figure 1E**). Altogether, both infections result in accelerated intrathymic T cell migration, and premature release of T cell populations to the periphery (Cotta-de-Almeida et al., 2003; Mendes-da-Cruz et al., 2003), some of them being potentially autoimmune (Morrot et al., 2012). *T. gondii* also contributes to thymic atrophy, by inducing destruction of the thymic epithelium, and of the overall architecture of the thymus. This has mostly been associated with a profound and persistent destruction of the CD4+ T cell pool, which contributes to an immunocompromised state and to the maintenance of chronic *T. gondii* infection (Kugler et al., 2016) (**Figure 1E**).

None of the studies performed so far have studied parasite interactions with the thymic resident cells, nor the effects of parasite presence on events such as lymphocyte migration and development *in vivo*. IVM would enable a better understanding on T cell-mediated immunity, and alterations during infections due to thymic compromise.

Secondary lymphoid organs in parasitic infections

Following their development in the primary lymphoid organs, competent lymphocytes populate secondary lymphoid organs such as the lymph nodes, the spleen, and the MALT. These organs have a key distribution across the body to allow surveillance and efficient responses upon challenges including infections.

Biological relevance of the lymph nodes

Lymph nodes (LNs) are secondary lymphoid organs distributed throughout the body that serve as critical hubs for the induction of cellular and humoral immune responses. LNs are divided into discrete regions: the capsule, made of connective tissue; the cortex (divided into an outer and inner region) which harbours T and B cells, as well as dendritic cells and macrophages; and the inner medulla, which harbours plasma cells. LNs form an interface

between the blood and lymphatic systems. Cells enter the LNs through two main routes: the lymphatic vessels or specialized blood vessels called high endothelial venules. The capacity of lymph nodes to trap and filter particulate antigens serves not only to concentrate antigen for B and T cell presentation but also to contain pathogens' systemic spread (Moran et al., 2019).

Methods for IVM-based visualization of the lymph nodes

Murine LNs have been extensively studied by IVM, and successfully imaged in the context of parasitology. Previous work has identified 22 LN sets in mice (**Figure 2A**): 4 in the head and neck region (mandibular, accessory mandibular, superficial parotid, and cranial deep cervical); 2 in the forelimb (proper axillary, accessory axillary); 3 in the hind-limb (subilliac, sciatic, popliteal); 3 at the intrathoracic region (cranial mediastinal, tracheobronchial and caudal mediastinal); and 10 in the abdomen (gastric, pancreaticoduodenal, jejunal, colic, caudal mesenteric, renal, lumbar aortic, lateral iliac, medial iliac, and external iliac) (Van den Broeck et al., 2006). One of the main drawbacks for IVM is that while enlarged LNs can be readily identified, the LNs in mice under homeostatic conditions are difficult to distinguish from surrounding adipose and connective tissues. Furthermore, not all LNs are equally accessible, and some have been preferred for intravital studies in various fields – in particular the popliteal LN. *Plasmodium* and *Leishmania* IVM imaging have been mostly done in the popliteal LN (Bajénoff et al., 2006; Radtke et al., 2015); *ex vivo* imaging of *T. brucei* infected was done in the cervical and mandibular LN (Caljon et al., 2016), while *Toxoplasma gondii* was imaged by IVM in the mesenteric LN (Chtanova et al., 2008). Due to the vast possibilities for imaging LNs *in situ* by IVM, this section will focus on the technical details of visualization of the popliteal LN (**Figure 2Bi, Bii (grey circle))** while briefly discussing novel and relevant techniques to investigate alternative locations. Regarding the mesenteric LNs, the surgical procedure and optical windows are equal to those described below for spleen and GALT IVM.

As for other organs, successful LN IVM consists on the immobilization of the tissue within a living, anaesthetized mouse without disruption of vascular and lymph flow. This requires good surgical skills, and for some organs, including the LNs, dedicated microscope stages. To overcome potential problems regarding tissue motion, a recent technical development is real-time drift offset correction, which adjusts the positioning of the stage based on pattern matching, allowing certain anatomical landmarks to remain in the same position despite tissue shifts during image acquisition (Vladymyrov et al., 2016).

The popliteal LNs are located within the popliteal fossa, or knee joint. Various protocols have been developed for surgical exposure and IVM of this LN (Liou et al., 2012; Mempel et al., 2004; Stein and F. Gonzalez, 2017). Three explanations for the preference of this LN over most others for IVM, are a) that detailed methods for surgical exposure and imaging of this site were the earliest developed (Mempel et al., 2004); b) that there is significant lymphatic drainage from the footpad to this LN, making this a good location to visualize antigen and pathogen arrival (reviewed by Nitschke et al., 2008; Stein and F. Gonzalez, 2017); and c) that the popliteal LN is small enough to allow visualization of cell-cell interactions in all 3 compartments (reviewed by Stein and F. Gonzalez, 2017). Following careful hair removal in the area, surgery requires a 2-3mm incision at the knee, to expose the extensor tendon. This involves a midline vertical incision through the skin at the mid-calf, and two horizontal incisions at the top of the vertical incision line, to generate skin flaps. To secure different layers of the tissue prior to exposure of the LN, tissue adhesive can be used to glue these layers to the holder, and provide stabilization to the leg for imaging. For stabilization, the knee tendon can be secured between the skin flap holders. The LN then lies within the popliteal fossa, and must be extremely carefully separated from surrounding muscles and adipose tissues. Once exposed, it is crucial to maintain proper body temperature and prevent dehydration. For the latter point, the LN can be submerged in saline prior to coverage with the glass coverslip (Liou et al., 2012; Mempel et al., 2004). Once the mouse is secure in the customized stage, it is advised that the tail is carefully secured, as it will provide stability to the leg while imaging (**Figure 2B,i**). The mouse can then be imaged using an upright microscope directly above the exposed popliteal LN. Various protocols have been suggested to generate the customized stage that best supports mice for IVM (Liou et al., 2012).

Although not yet used in a context of parasitology, a recent development which could be useful to the field is the chronic lymph node window (CLNW) model, which allows longitudinal imaging of the inguinal LNs (Jeong et al., 2015; Meijer et al., 2017). In their work, Meijer *et al* designed a stage specifically to allow access to this LN, while preventing rotational changes and respiration or heart-beat induced artefacts during imaging (**Figure 2B,ii**) (Meijer et al., 2017). The CLNW allows for longitudinal imaging over 14 days, with uncompromised blood flow and/or vessel integrity. This window model was first modified from a chronic mammary fat pad window model (Chauhan et al., 2012; Jeong et al., 2015; Kedrin et al., 2008). It involves the surgical implantation of symmetrical titanium frames that hold the LN 'sandwiched' in-between. These frames are secured via sutures and bolts. The ventral skin covering the LN is removed, and the LN separated from the surrounding muscle and fat. A

coverslip can then be secured in one of the ventral titanium frame, to allow visualization of the LN. It is suggested that mice used for CLNW implantation weigh over 25g, so that the window does not prevent leg movement. Following CLNW implantation, the mice recover for 48h. Similarly novel as the CLNW, is a model that circumvents the need for invasive surgery, and allows longitudinal imaging. This novel protocol involves the transplantation of LNs to the ear pinna (Gibson et al., 2012) for direct visualization using multiphoton microscopy. These transplanted LNs maintain the structure, function, and organization of LNs *in situ,* and have functional lymphatic and vascular supplies thus allowing longitudinal studies of lymphocyte dynamics and antigen presentation, among other phenomena (**Figure 2B,iii**). Window types, and their advantages and limitations, are summarized in **Table 1**.

Biological findings in parasitology: the lymph nodes

Vector-borne protozoans inoculated in the skin of the mammalian host, such as *Leishmania*, *Trypanosoma* and *Plasmodium* reach the draining LNs undergoing distinct fates. The first quantitative IVM study of *P. berghei* transmission from mosquito to mammals has shown that in mice only a proportion (~50%) of sporozoites, the highly motile parasite stage inoculated by the mosquito, leave the skin (Amino et al., 2006; reviewed in Graewe, S., et al., 2012). Moreover, only sporozoites that invade skin blood vessels find their way to the liver. Sporozoites entering skin lymph vessels stop their journey at the proximal LN, where most are degraded inside CD11c+ dendritic cells (DCs). However, a few parasites invade cells expressing podoplanin (a membrane protein found in lymphatic endothelial cells) where they partially differentiate into exoerythrocytic forms (EEF) (Amino et al., 2006). The skin was also shown to support the development of sporozoites till complete maturation into red blood cells infective forms, a process that was thought to occur exclusively in the liver (Coppi et al., 2011; Gueirard et al., 2010). This implies that the LN draining the inoculation site will receive parasite antigens not just from sporozoites (sporozoites actively reaching the LN or dead sporozoites left in the skin) but also from differentiating parasites (skin EEF aborting at various stages of their development). Regarding the immunological implications of these findings, so far it is known that after an infectious mosquito bite the first cohort of protective CD8+ T cells is primed by DCs in cutaneous LNs (Chakravarty et al., 2007; Obeid et al., 2013). Dynamic *in vivo* and static imaging has shown the uptake of parasites by LN DC followed by the DC cluster with $CD8^+$T cells. Indeed, $CD8^+$ T cells are primed by resident $CD8\alpha^+$ DCs with no apparent role for skin-derived DCs. This study established a critical role for LN resident $CD8\alpha^+$ DCs in

$CD8^+$ T cell priming to sporozoite antigens while emphasizing a requirement for motile sporozoites in the induction of $CD8^+$ T cell-mediated immunity (Radtke et al., 2015) (**Figure 2C, i and 2D, i**). Altogether. IVM discoveries have changed our view of the malaria pre-erythrocytic phase and the previously unanticipated role of the skin and draining LN in this phase.

While *Plasmodium* sporozoites reach the draining LN in the first hour after inoculation in the skin (Amino et al., 2006) African trypanosomes take longer as the first parasites are detected in LN 18 hours after infection with tsetse flies (Caljon et al., 2016). IVM studies revealed that engulfment of trypanosomes by neutrophils at the inoculation site was rarely observed and was restricted to parasites with reduced motility/viability whereas live parasites escape phagocytosis (Caljon et al., 2018) (**Figure 2C, ii and 2D, ii**). When and how trypanosomes invade lymph vessels remains to be elucidated as well as the cell types with which parasites interact within the LN. Flagellated *Leishmania* promastigotes are on the other hand rapidly taken up by neutrophils after inoculation in the skin. This was seen for *Leishmania* species that cause either cutaneous or visceral infection (Peters et al., 2008; Ribeiro-Gomes et al., 2012; Thalhofer et al., 2011, 2010). However, few days after infection the parasites persist in the form of intracellular amastigotes mostly inside monocytes, macrophages and DCs (De Trez et al., 2009; Hurrell et al., 2015; León et al., 2007; Peters et al., 2008). The role of neutrophils in both promoting or supressing host immunity remains controversial and a clear demonstration on whether these cells act as Trojan horse for the dissemination of *Leishmania* parasites to distant sites remains to be demonstrated in vivo (Ribeiro-Gomes et al., 2012; Ribeiro-Gomes and Sacks, 2012). IVM studies combined with flow cytometry showed that *Leishmania mexicana* use neutrophils as a safe transient shelter and that this impacts parasite-specific immune response in mice. Indeed, an increase in monocyte-derived DCs in the draining LNs of neutropenic mice was seen and correlated with the subsequent increased frequency of IFNγ-secreting T helper cells and better parasite control (Hurrell et al., 2015). IVM also elucidated the dynamics of NK cells following *Leishmania major* infection. NK cells, which reside in both the LN medulla and paracortex in steady state conditions, were shown to be recruited from the blood to the paracortex where they produce IFN-y that activates parasite-specific $CD4^+$T cells (Bajénoff et al., 2006) (**Figure 2C,iii and 2D, iii**).

IVM imaging of *Toxoplasma gondii*-infected mice has shown that neutrophils are able to migrate in a coordinated manner within the LNs (Chtanova et al., 2008). Cooperative action of neutrophils and parasites egressing from host cells triggers neutrophil swarm formation leading to the removal of macrophages that line the subscapular sinus of the lymph node. These

results provide insight into the cellular mechanisms that lead to neutrophil swarms and suggest new potential functions for neutrophils in LNs (Chtanova et al., 2008; Coombes and Robey, 2010) (**Figure 2C,iv and Figure 2D, iv**).

Biological relevance of the spleen

The spleen is a specialized organ that combines the innate and adaptive immune system within a complex architecture. The structure of the spleen enables it to remove older erythrocytes from circulation, thus constantly maintaining a pool of red blood cells best adapted to transport oxygen and iron across tissues. Equally, the spleen's architecture allows efficient removal of blood-borne microorganisms, including parasites. The spleen is organized into three main compartments: the red pulp, where pathogens and senescent red blood cells are removed from the blood by specialized macrophages; the white pulp, a highly organized lymphoid region composed of B and T cell zones; and the marginal zone, which constitutes a bridge between innate and adaptive immune responses due to its specialized macrophage and B cell subsets. The specialized architecture of the spleen is coordinated by the expression of lipid mediators, adhesion molecules, and chemokines, which direct the migration and retention of specific lymphoid subsets across the splenic compartments (Reviewed by (Mebius and Kraal, 2005)).

Methods for IVM-based visualization of the spleen

The spleen presents important challenges for IVM. Two significant microscopy-based limitations imposed by the spleen are a) that the optically dense capsule contributes to significant light scattering, making some anatomical sections of the spleen difficult to access for imaging; and b) that the spleen is highly vascularized and highly complex in its cellular composition, with multiple regions of very fast circulation (Discussed in (Grayson et al., 2001)). Many confocal systems lack the capability of scanning or recording data at a speed rapid enough to allow distinguishing cellular interactions or sub-cellular events at high speed. In other organs, poor penetration depth has been largely addressed by the use of two-photon microscopes, while speed limitations have been overcome to a certain extent, by spinning disc methods, or opto-acoustic deflectors achieving a minimum of 30 frames per second (Discussed in (Grayson et al., 2001)). IVM techniques adapted for spleen imaging not based on fluorescence, but which overcome the above challenges, include optical frequency domain imaging (OFDI) (Kubo et al., 2017; Otake et al., 2018; Yun et al., 2003), and coherent anti-

Stokes Raman spectroscopy (CARS) (Vogler et al., 2015). The former allows detection of scattering properties of tissues at multiple depths to detect angiogenesis and tissue viability, while the latter uses multiple photons to detect intrinsic molecular vibrations, allowing imaging of chemical structures. These techniques have been used in other areas of research, but remain to be introduced in parasitology. An alternative to these methods, is the use of time- and polarization-resolved fluorescence detection, such as fluorescence lifetime imaging (FLIM), whereby the combination of high-speed acquisition and novel methods of image processing, allow for visualization almost in real time (Niesner et al., 2008).

In terms of imaging windows, various types of setups have been designed to image the spleen. One includes the abdominal imaging window (AIW) for imaging with either an upright or an inverted microscope (**Figure 3A,i**). In 2012, Ritsma *et al* developed the abdominal imaging window (AIW), which consists of a titanium ring with a 1mm groove on the side, and a coverslip which can be fixed on the top with glue, and exchanged as required (Ritsma et al., 2013, 2012). Following this, the AIW is implanted in the skin and abdominal wall, and held in position by a purse-string suture, which prevents the mice from biting or removing the sutures. The AIW was reported to be used over a maximum of 28 days, without considerable changes in the anatomical position of the window and without disturbing physiological processes. Although the AIW offers major advantages for long term imaging, less complex, temporary windows which simply consist on surgically exposing the organ of interest, hydrating it, and attaching it to a coverslip using glue, can also be used for short-term (4-8 h) imaging. Such setup has been used for *Plasmodium* imaging, and involves the exposure of the spleen through a small incision on the mouse flank, followed by attachment to a glass coverslip for imaging on an inverted microscope (De Niz et al., 2016; Ferrer et al., 2012) (**Figure 3A,ii**). While using glue for attaching the abdominal organs is possible for short-term imaging (i.e. no more than 12 hours, continuously), it is not suitable for imaging over a period of days. Alternatively, a vacuum-coupled window can be implemented for imaging using an upright microscope (**Figure 3A,iii**); or a a glass-bottom cell culture dish, whereby the spleen is exposed and immobilized over sterile saline, allowing use of an inverted microscope (Grayson et al., 2001).

Specific challenges during splenic surgery and exposure include a) that care should be taken to avoid the vasculature while performing the incisions on cutaneous and muscle layers to expose the organ, to prevent bleeding over the spleen during imaging; b) that extreme care should be taken upon pulling the spleen through the muscle, peritoneal, and skin incisions in order to neither compress the spleen vasculature (which would hinder circulation and all chances of visualizing cells in motion), nor to cause bleeding or incisions in the spleen (which

could be lethal due to rapid exanguination in extreme cases). Moreover, the intercostal incision should be relatively small, because with larger incisions, the risk that the spleen retracts into the abdominal cavity and away from the window is higher. IVM of the spleen upon even minor splenomegaly (often observed in parasitic infections) should be performed with extreme caution, as the tissue is very fragile and could easily rupture.

In addition to the surgical considerations for imaging, an equally important issue is the biological relevance of the mouse spleen relative to the human spleen. Three key differences between the mouse and human spleens include a) that the marginal zone of the human spleen lacks a delimited marginal sinus and is surrounded by an additional perifollicular zone; b) that the human spleen is sinusoidal, while the mouse spleen is less so; and c) that while the mouse spleen is a key organ for erythropoiesis, the human spleen is less so. This has been relevant in the study of malaria, and might equally be so in the study of other parasitic diseases for which animal models exist. Although pathogens including *Plasmodium, T. brucei, T. cruzi, Leishmania, Schistosoma, Babesia, Echinococus* and *Paragonimus* cause spleen involvement and pathology, to our knowledge only *Plasmodium* has been imaged by IVM. Window types, and their advantages and limitations, are summarized in **Table 1**.

Biological findings in parasitology by IVM: the spleen

As part of its functions, the spleen destroys senescent red blood cells in health, and aids in the detection and immune response formation against blood-borne pathogens. The spleen's involvement in malaria is significant. Splenomegaly is a hallmark of the disease in endemic areas; furthermore, splenectomy both in rodents and humans has been consistently associated with more frequent fever, and higher parasitemias. In 2009, Buffet PA *et al* discussed a paradigm, arising from studies of the spleen of infected children, whereby the spleen is regarded as a double-edged sword. While retention of *Plasmodium* rings and uninfected RBCs aids in reducing the risk of cerebral malaria, it also increases the risk of severe malarial anaemia (Buffet et al., 2009). Moreover, because of its role as a blood surveillance organ, it is believed that the presence of the spleen has driven the evolution of a plethora of evasion mechanisms in *Plasmodium* to avoid destruction. Perhaps the most significant is the parasite's ability to sequester in the peripheral vasculature of multiple organs – a phenomenon responsible for pathology and complications associated with malaria (reviewed by (del Portillo et al., 2012; Engwerda et al., 2016)). Previous work to study *Plasmodium* in the spleen, *ex vivo*, has included an *ex vivo* perfusion system whereby the spleen retained its clearing and processing functions

and allowed visualization of *P. falciparum* (Buffet et al., 2006), and a spleen-on-a-chip approach which reproduced splenic basic units (splenon) and areas of fast and slow circulation, where *Plasmodium* dynamics could be investigated (Rigat-Brugarolas et al., 2014).

To our knowledge, the malaria-infected spleen has been imaged by IVM in four separate studies over the last 6 years. The first spleen IVM study in rodent malaria models focused on the differential remodelling of this organ induced by lethal and non-lethal GFP-expressing *P. yoelii* strains (Py17XL and Py17X, respectively). A significantly higher number of parasites of the non-lethal strain were detected in the spleen, than those of the lethal strain. This was proven to neither be the result of different blood flow, not differential macrophage activity. The non-lethal strain, however, displayed an adhesive rolling-circle behaviour, while the lethal strain did not. Conversely, FITC-labelled uninfected RBCs and fluorescent beads displayed equal flow patterns in mice infected with either strain (Martin-Jaular et al., 2011) (**Figure 3B,i**). This differential motion specific to the parasites, together with further MRI, EM and other *ex vivo* data, led to the conclusion that the non-lethal strain induces a spleen blood barrier of fibroblastic origin, to which Py17X-infected reticulocytes can adhere to, to escape from macrophage clearance. The image-processing pipeline developed to track parasite directionality, residence time, mean velocity, and volumetric blood flow allowing for normalization of erythrocyte and lumen vessel diameters, was made available in parallel to the original study (Ferrer et al., 2012). In line with the aim of investigating *Plasmodium* interactions with splenic populations, a later study went on to explore the role of DCs in the control of blood stage infections with *P. yoelii* and the chronic malaria model *P. chabaudi*, at different phases of acute and chronic malaria infections. IVM in this study for the first time showed interactions between DCs and CD4+T cells at different phases of acute malaria, and it was the first work suggesting that aside of their role in antigen presentation, DCs also directly participate in the elimination of iRBCs during acute infection (da Silva et al., 2013). Finally, given that sequestration in the vascular endothelium is believed to be a mechanism developed by *Plasmodium* to avoid passage through the spleen, a recent study used IVM to demonstrate that non-sequestering *P. berghei* lines lacking export-mediating Maurer's cleft resident proteins MAHRP1a and SBP1 do not sequester, and extensively accumulate in the spleens, inducing exacerbated splenomegaly (De Niz et al., 2016) **(Figure 3B,ii)**.

The spleen has recently gained great interest in the context of malaria transmission. Studies in humanized mice infected with *P. falciparum* (Duffier et al., 2016), and rodent models of malaria infected with *P. berghei* (Lee et al., 2018), have consistently shown increased numbers of immature gametocytes in the spleen. The relevance of this organ for host-

to-vector transmission, as well as its study in the context of transmission-blocking drugs, remains to be fully explored, and IVM could be an extremely relevant tool for this purpose.

Although various technological improvements now allow for greater penetration depths and faster imaging to record cellular interactions, the greatest improvements likely to impact spleen IVM are image-processing algorithms for events occurring at high speed. In terms of biology, spleen involvement in the context of immunomodulation and immunopathology have received increased interest, and IVM is a key tool to potentially shed light on parasite-mediated responses. Given its relatively easy use, the implementation of this technique to study other parasites in the spleen, might provide interesting insights into other host-pathogen interactions.

Other immune organs: the Mucosa-associated lymphoid tissue (MALT)

The mucosa-associated lymphoid tissue (MALT) is situated along the surface of all mucosal tissues, and is responsible for initiating immune responses to antigens encountered at these sites. About half of the lymphocytes of the immune system are in the MALT, with the most studied of such locations being the gut-associated lymphoid tissue (GALT) (reviewed by (Jung et al., 2010)), the nasopharynx-associated lymphoid tissue (NALT), and the bronchus-associated lymphoid tissue (BALT) (reviewed by (Bienenstock and McDermott, 2005)). These anatomical locations can be divided into two key sites: effector sites and inductive sites. Inductive sites contain secondary lymphoid tissues which support IgA class switching and clonal expansion of B cells in response to T cell activation. Following activation and IgA class switching, T and B cells migrate from inductive sites to effector sites. Effector sites are present as diffuse lymphoid tissue along mucosal surfaces, and allow IgA transport across the mucosal epithelium. The functional compartments of the GALT, NALT, and BALT are lymphoid follicles, the interfollicular region, the subepithelial dome region, and the follicle-associated epithelium (reviewed by (Cesta, 2006)).

Various parasites of clinical relevance enter the body via the gastrointestinal route, and interact with the GALT. These include *Giardia* spp., *Toxoplasma gondii*, *Entamoeba* spp., *Ascaris* spp., *Ancylostoma* spp., *Necator* spp., *Strongyloides* spp., *Paragonimus* spp., and *Cryptosporidium* spp. Vector-borne parasites compromising the GALT include *Schistosoma* spp. Equally, many of these same parasites have an obligatory passage through the lungs, and interact with the NALT and BALT. To our knowledge, only *Plasmodium* spp. have been visualized by IVM in the lungs. However the findings are outside the scope of this review, and are discussed in a separate review of this mini-series (unpublished).

Methods for IVM-based visualization of the GALT

The intestines are a crucial site in the life cycle of most orally-transmitted parasites, as well as a main site of pathology for the host. Despite the huge relevance of this site, intestine IVM has not been extensively reported. Aside from the abdominal imaging window (**Figure 3A,i**), recent work by (Kolesnikov et al., 2015; Xu et al., 2012) described two alternative methods for imaging the intestines and Peyer's Patches. The surgical procedure involves performing a small incision through the skin along the abdominal midline to expose the peritoneal wall, after which an incision to the peritoneal wall itself follows. Next, a section of the intestine was externalized, and an incision made, further exposing the luminal surface. Care should be taken in particular to a) avoid disrupting the mucosal layer and b) avoid damaging the mesentery or mesenteric vasculature. For this purpose, the use of an electrocautery is recommended (Kolesnikov et al., 2015), rather than a scalpel, to seal off vessels damaged during the surgical procedure. Aside of preventing bleeding, controlling intestinal peristaltic movements and the associated motion artefacts is key. Imaging methods to overcome this issue have been discussed (Kolesnikov et al., 2015; Xu et al., 2012). If using an inverted microscope, upon completion of the surgery, the externalized intestine can be sandwiched between a coverslip and sterile gauze at the animal's abdomen. In this setup, the animal's weight stabilizes the tissues and prevents dehydration (Xu et al., 2012). Conversely, an imaging chamber suitable for imaging using an upright microscope can be used, which is arguably better suited for physiological preparations (Kolesnikov et al., 2015). Two key considerations for intestine IVM which differ from other tissues are on one hand, that autofluorescence areas in the mucosal layer exist which may represent challenges for imaging (although such autofluorescence is absent from the epithelia, and the lamina propria). On the other hand, live intestinal tissue is more sensitive to imaging than the skin, spleen, or other tissues which allow long imaging periods. The intestinal tissue reportedly begins to deteriorate within a 3h window following surgery and exposure to air. Internal controls to monitor membrane integrity, vascular permeability, and cellular motility are therefore highly recommended during intestinal IVM (Kolesnikov et al., 2015). Similar to *ex vivo* alternatives for other organs, imaging can be performed in intestinal sections cultured in aerated media (Chieppa et al., 2006; Coombes et al., 2013). The downside of this method is that the vascular, lymphatic, and nervous connections of the tissue are severed, thus preventing a full reproduction of physiological conditions of the intestines. Window types, and their advantages and limitations, are summarized in **Table 1**.

To our knowledge, only *T. gondii* has been imaged in the intestines by IVM (Coombes et al., 2013; Watanabe et al., 2018).

Biological findings in parasitology by IVM: the GALT

T. gondii infection occurs via the oral route. Although the most important clinical complications involve the brain and the developing foetus in pregnant women, *T. gondii* first establishes infection in the small intestine after ingestion of cyst-contaminated meat, or oocyst-contaminated water. Following entry to the intestine, the parasite must cross the intestinal epithelium, before it can establish a chronic infection in the brain. The study by Coombes *et al* aimed to investigate the role of motile immune cells in acting as transport vessels for *T. gondii* across tissues (Coombes et al., 2013). Using IVM, they found that neutrophils are recruited to the villi and lumen of the small intestine upon *T. gondii* infection, and are motile reservoirs of live *T. gondii*, contributing to spreading the infection (**Figure 3C**). RFP-expressing *T. gondii* parasites were used to infect reporter mice including CD11c-YFP, and LysM-GFP mice to investigate the role of dendritic cells, macrophages, and neutrophils in the spread of *T. gondii* away from the intestine. Complementary *ex vivo* experiments showed that parasites within neutrophils remained infection-competent. Important additional observations included the existence of hot spots of parasite replication across the small intestine, and an increased parasite density at the tips of the villi (Coombes et al., 2013).

Altogether, this IVM-based finding is fascinating in that it enabled elucidating a Trojan-horse-like mechanism of pathogen spread which might be highly relevant for other parasites also able to cross tissue barriers. Like-wise, besides of the possibility of imaging host-pathogen interactions, reporter animals could be of use to study immunological responses in the intestines. These might be particularly relevant in the context of infection with parasites including hookworms and nematodes.

A more recent study used IVM to investigate the leukocyte's behaviour in the mesenteric circulation, as well as leukocyte-endothelium interactions following infection with *T. gondii* (Watanabe et al., 2018). The study's key findings were that haemodynamic changes already happen at the beginning of infection, in which molecules such as ICAM1, PECAM1 and P-selectin are upregulated on the vascular endothelium. These not only contribute to the parasite's adhesion and transmigration, but also to leukocyte mobilization within the mesenteric vasculature, resulting in a mild inflammatory response.

Given the involvement of the gastrointestinal tract and the lungs by a large number of parasites, the relevance of the MALT for pathology and immunity, and the little understanding of the dynamics we have of parasites in these organs, the implementation of IVM has vast potential in the years to come.

Outlook

In this review we have discussed the relevance of primary and secondary lymphoid organs in parasitic infections, and the knowledge we have gained as a community through the use of IVM to investigate dynamic interactions in organs including the bone marrow, spleen, lymph nodes, and GALT.

Interestingly, an alternative technology that has been explored for two parasites (*T. gondii* and *Plasmodium*), in the context of lymphoid organs, is the use of organs-on-chip (OOCs), and of organoids. Organoids consist of organ-specific adult mammalian stem cells grown in a three-dimensional structure whilst OOCs are based on a similar principal as organoids, but with the addition of microfluidic systems to allow for the distribution of nutrients and soluble mediators throughout the constructs. The cells in both systems self-organise into morphologically distinct layers that are capable of successfully mimicking organs. One of the major benefits of OOCs is that they can mimic blood flow, allowing for the assessment of shear stress and deformability of circulating cells. The use of a spleen on a chip model demonstrated the poor deformability of *P. falciparum* pRBCs and their ability to occlude narrow vessels (Picot et al., 2015; Rigat-Brugarolas et al., 2014). Another important advance has been the generation of an organoid for the intestinal epithelium to study *T. gondii* interactions with the epithelium and immune cell populations (Derricott et al., 2019). While both organoids and OOCs are still in their infancy, they have great potential for assessing parasite behaviour in a consistent and reproducible manner.

A complementary advantage of IVM, relative to organoids and OOCs is the possibility to study parasite interactions with and effects upon lymphoid organs within the complexity of a full organism. With better imaging platforms allowing faster speed acquisition, better resolution, and increased penetration depth, IVM holds great potential for the study of parasites and their interaction with cells of the immune system across all immune organs. Of great interest is the relevance of the parasites' presence within the bone marrow and thymus, and as

this may impact immunity, immunopathology, infection chronicity, tolerance, relapses, transmission and virulence among other phenomena involving the immune system of the host, and the relevance of its recognition of parasites. Equally, although various parasites interact with the BALT, NALT, GALT, LNs and spleen, relatively very little has been studied in these organs in a dynamic manner, particularly with respect to parasite homing, cell-cell interactions, organ remodelling, and impact on immune responses and immunopathology. IVM therefore holds great potential for questions on a plethora of parasites including those food-, water-, and soil-borne, entering via the gastrointestinal tract like *Giardia, Toxoplasma, Entamoeba, Ascaris, Ancylostoma, Necator,* and *Cryptosporidium,* and vector-borne with involvement of the blood, lungs or gastrointestinal tract, including *Leishmania, T. brucei, T. cruzi, Schistosoma,* and *Plasmodium.*

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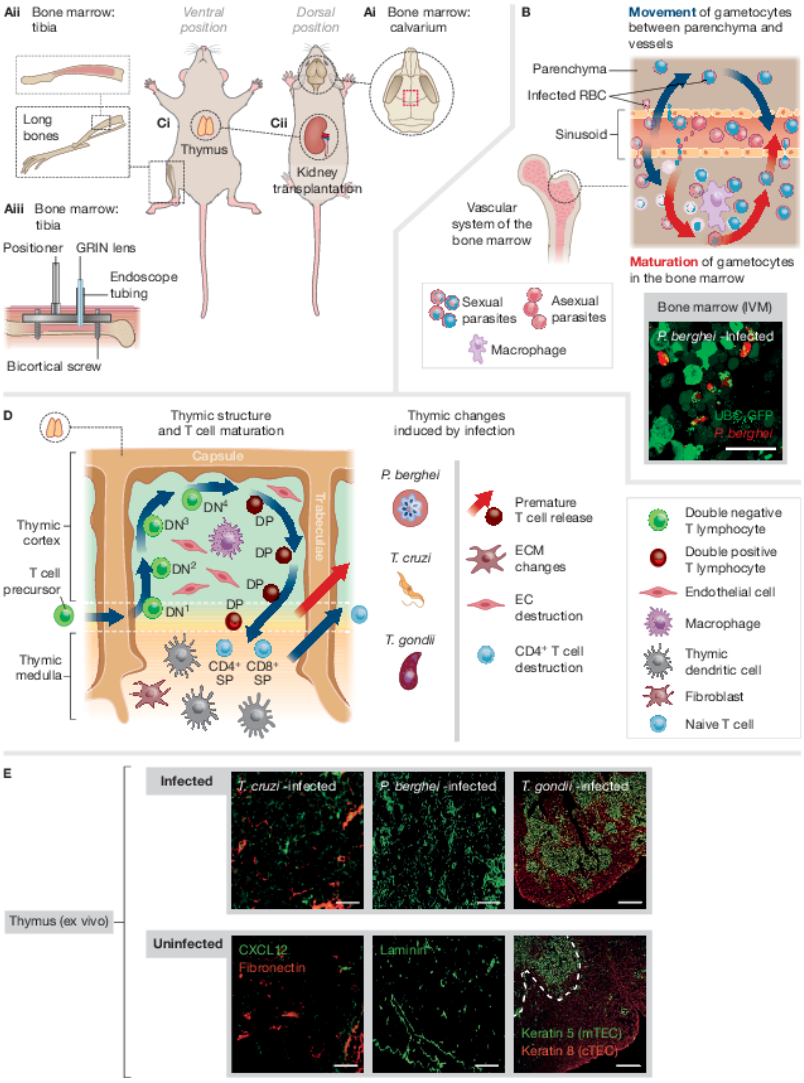

Figure 1. Windows for IVM and biological findings on primary lymphoid organs (PLOs): bone marrow (A-B) and thymus (C-D). Ai) The calvarial BM is located close to the intersection of the cortical and sagittal sutures, and the bifurcation of the sagittal suture. Access requires an incision of the scalp to expose the cranium, and either direct access with an objective, or the implantation of a glass window. Methods to access long bones include Aii) ventral exposure of the tibia and femur, with careful separation of tendons and muscles to allow access to the BM close to the bone head. This can allow for IVM imaging with or without a chronic window; and Aiii) surgical implantation of a device to allow entry of an endoscope tubing and a GRIN lens for long term imaging and access to deeper sites of the BM of various bones. B) Key findings in parasitology by IVM include the observation of *Plasmodium berghei* gametocyte homing to and development within the extravascular space of the BM of long bones. Primary figure shows a still frame from an IVM visualizing mCherry-tagged *P. berghei* gametocytes in the BM of a UBC-GFP reporter mouse (Adapted from De Niz et al., 2018). The other lymphoid organ is the thymus (C), which is located in the thorax, above the heart. Direct access to the thymus is challenging. A technique used for IVM is the transplantation of the thymus to the kidney capsule, and visualization of the kidney capsule using a dorso-lateral window. Imaging can be done in the dorsal position. D) Although no IVM has been reported for the thymus in the context of parasitology, various studies have shown that parasites including *T. cruzi*, *T. gondii* and *Plasmodium* alter the thymic architecture, inducing phenomena such as thymic atrophy, accelerated T cell release

(*T. cruzi* and *Plasmodium*), and increased endothelial cell and CD4+ T cell destruction (*T. gondii*). E) *Ex vivo* images of the thymus of Top panel: control (left) and *T. cruzi*-infected mice (right) showing increased deposition of CXCL12 (green) and fibronectin (red) in infected mice, consistent with altered thymocyte migration (Adapted from Mendes-da-Cruz, et al., 2006). Middle panel: control (left) and *P. berghei* –infected mice (right) showing increased laminin deposition (green) in the thymus of infected mice, consistent with changes in extracellular matrix components upon infection (Adapted from Gameiro et al., 2009). Bottom panel: control (left) and *T. gondii*-infected mice (right) showing increased convolution of the cortico-medullary border upon infection with *T. gondii*. Labelling shows keratin 5 and 8, markers of medullary and cortical thymic endothelial cells respectively (Adapted from Kugler et al., 2016). (Abbreviations. Referring to T cell receptor: DN: double negative; DP: double positive; SP: single positive. Other abbreviations: ECM: extracellular matrix; EC: endothelial cell.) All original images were published under a Creative Commons Attribution (CCA) license, and/or reproduced with permission.

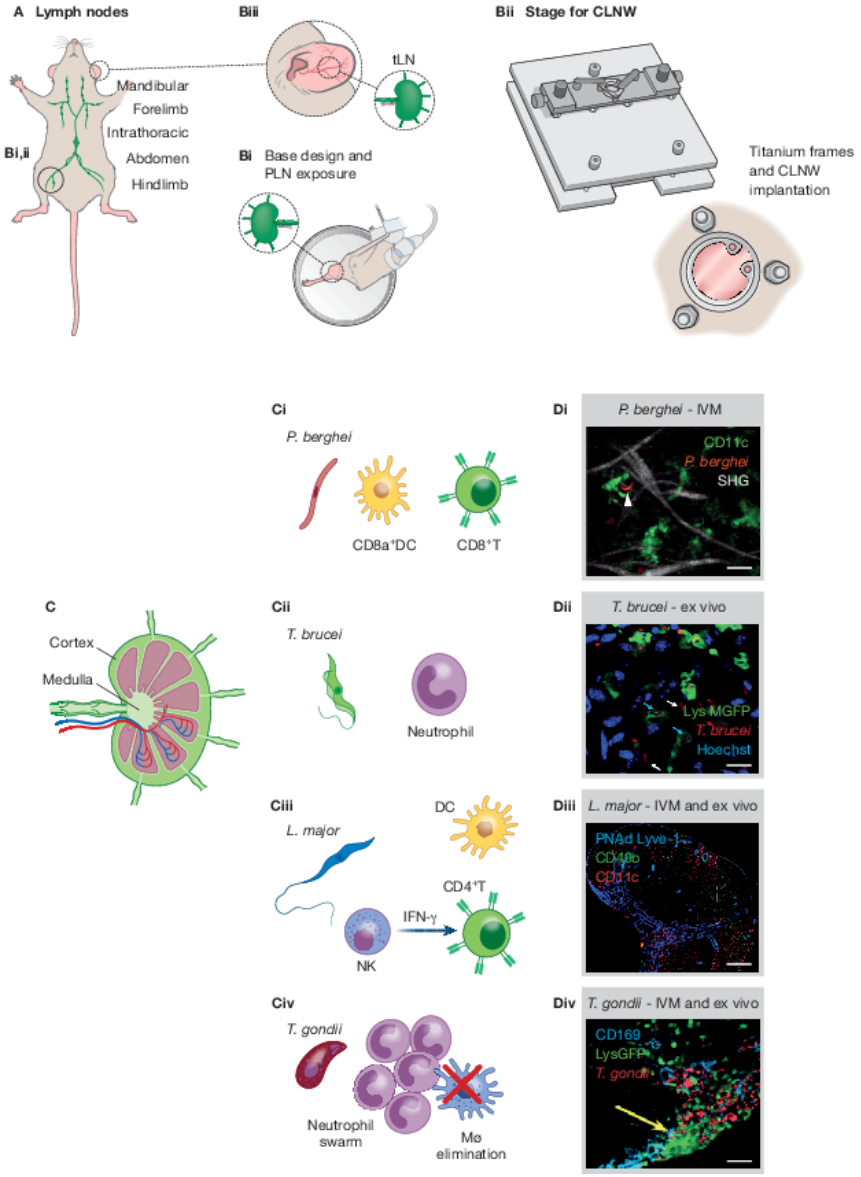

Figure 2. Windows for IVM, and biological findings on secondary lymphoid organs (SLOs): lymph nodes (2A-D). A) 22 LNs have been identified in mice in the head and neck, the forelimb, the hindlimb, the intrathoracic region, and the abdomen. One of the preferred sites for LN imaging is the popliteal lymph node (PLN), close to the knee joint. Various methods exist to image the PLN: Bi) direct visualization following stabilization for which a customized stage is recommended; Bii) the generation of a chronic LN window (CLNW) with a customized stage that prevents rotational changes and motion-induced artefacts, and relies on the surgical implantation of titanium frames holding the LN sandwiched in-between. An alternative method is Biii) transplantation of the LN to the ear pinna, followed by direct imaging. Biological findings by IVM in the lymph nodes include Ci) sporozoite antigen presentation by LN resident CD8α^+ dendritic cells necessary for CD8$^+$ T cell priming; Cii) that monocyte-derived DCs increase in neutropenic mice, and are correlated with better T-cell-mediated *Leishmania mexicana* control, and that NK cells from the LN cortex aid in the activation of parasite-specific CD4+T cells against *Leishmania major*; and Ciii) neutrophil swarm formation in *T. gondii* infections, resulting in macrophage elimination in the subscapular sinus of the LN. Primary images show Di) Maximum intensity projection of *P. berghei* sporozoites (red) and CD11c+ antigen-presenting cells (green) at the draining lymph

node of CD11c-EYFP reporter mice, 5 hours post-intradermal inoculation – imaged by IVM (Adapted from Radtke et al., 2015). Dii) Confocal image of *T. brucei* AnTat 1.1 (red) uptake by Lys-MGFP+ neutrophils (green) in the dermis of infected mice. (Adapted from Caljon et al., 2018). Diii) Confocal image of ear draining lymph node of mouse 12 hours following infection with *L. major*. Staining shows peripheral node addressin (PNAd) and Lyve1 (blue), CD49b (highly expressed in NK cells, (green)), and CD11c+ antigen presenting cells (DCs). In chosen region, numerous NK cells and DCs are in physical contact. (Adapted from Bajenoff et al., 2006). Div) Confocal image of a lymph node 3 hours after infection of a mouse with *T. gondii* parasites. Neutrophils (Lys-GFP) are shown swarming in areas adjacent to *T. gondii* (red), and macrophages (CD169, blue). (Adapted from Chtanova et al., 2008). All original images were published under a Creative Commons Attribution (CCA) license, and/or reproduced with permission.

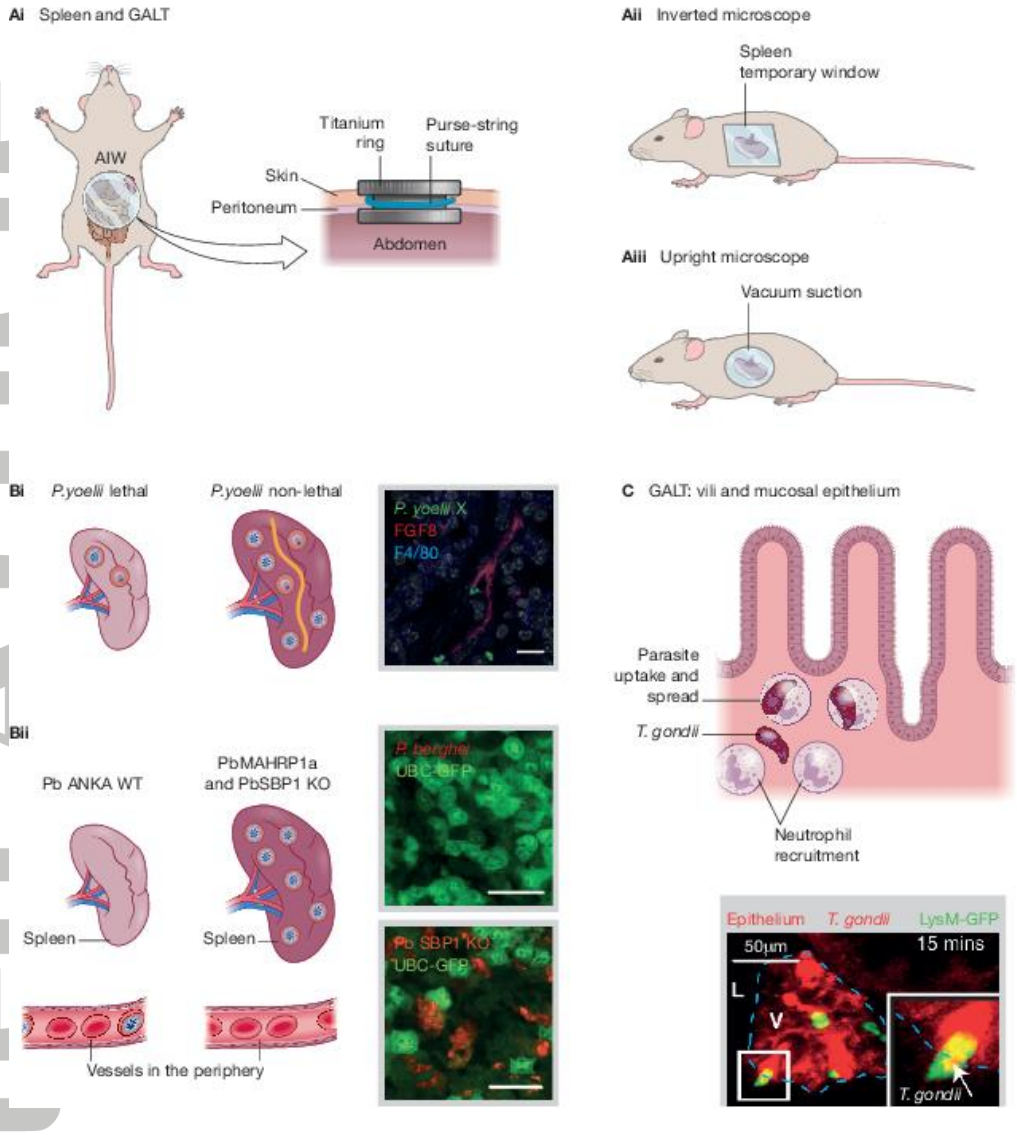

Figure 3. Windows for IVM, and biological findings on secondary lymphoid organs (SLOs): spleen (2A-D) and GALT (E). **Spleen.** Ai) shows the abdominal intravital window, which gives access to the abdominal organs including the gut and the spleen (on the dorso-ventral side). The chronic window consists on the implantation of titanium rings to enable imaging. Aii) shows a temporary window consisting on the exposure of the spleen, and visualization through a coverslip using an inverted microscope. Aiii) is an alternative whereby the mouse can be imaged using an upright microscope. For stabilization, the coverslip and organ are immobilized using a vacuum. Main biological findings in the spleen using IVM involve *Plasmodium* infections, namely Bi) that lethal and non-lethal strains of *P. yoelii* differentially remodel the spleen. The non-lethal strain induces the formation of a barrier of fibroblastic origin which protects the parasites from phagocytosis, causing vast splenomegaly. Primary image shows the spleen of a mouse infected with non-lethal *P. yoelii* 17X (green) at day 3 post-infection, showing FGF8 as a marker of a barrier of fibroblastic origin (red) and F4/80 macrophages (blue). (Adapted from Martin-Jaular et al., 2011). Bii) Shows that at early synchronized infections, *P. berghei* ANKA schizonts sequester in the vascular periphery, avoiding the spleen, while parasites unable to sequester (PbΔMAHRP1a and PbΔSBP1) are absent from the vasculature in the periphery, but are present in vast numbers in the spleen, where they are eliminated. Primary images (middle and bottom panels) show spleens of reporter mice (UBC-GFP, green) infected with *P. berghei* WT and PbΔSBP1 (red), 16-18

hours following intravenous injection of synchronized schizonts. (Adapted from De Niz et al., 2016). **GALT.** The GALT can be observed using various windows including the AIW (Ai). Biological findings in parasitology by IVM are restricted to observation of *T. gondii*, whereby C) neutrophil recruitment was observed to the vili and mucosal epithelium. Within neutrophils, *T. gondii* survives and disseminates to other organs. Primary image shows *T. gondii*-containing LysM-GFP cells (yellow) migrating across the intestinal epithelium (red). Images were obtained by two-photon microscopy. (Adapted from Coombes et al., 2013). All original images were published under a Creative Commons Attribution (CCA) license, and/or reproduced with permission.

TABLE 1 Optical windows for accessing primary and secondary lymphoid organs for intravital microscopy.

Organ	Technique	Key points	Complexity	Advantages	Limitations
Bone marrow	Direct imaging on the calvarium	Easy surgical access.	Low	Easy access. Low risk. Fast surgical procedure.	Sutures required after each imaging session. This can cause scarring, and is a challenge to longitudinal imaging.
	Calvarial window	Prevents re-growth of the membrane layer. Involves implantation of a window. Cortical bone can be removed.	Low	Easy to perform. Allows longitudinal imaging.	None if limited to the calvarium.
	Direct imaging of the tibia, or incorporation of a window.	Allows access to long bones.	Very high. Surgery difficult to perform.	Allows imaging marrow of long bones.	Requires a long and complex surgical procedure.
	Window for LIMB	Micro-endoscopic multi-photon imaging method using GRIN lenses.	High.	Allows imaging sites deep within the calvarium, tibia, and femur.	Difficult to perform.
Thymus	Dorso-lateral window for kidney capsule.	Requires transplantation of thymus to kidney capsule.	Very high.	Allows imaging of the thymus.	Different milieu at kidney that may not represent the thymic environment.
	Teleost fish models	Allow imaging the entire thymus.	Low.	Allows longitudinal imaging of the entire thymus and visualization of T cell dynamics throughout all developmental stages.	Require the use (and suitability) of fish models.
Lymph nodes	Direct imaging of popliteal lymph node	Allow imaging of cell-cell interactions in the three compartments of the lymph node	High	Extensive use and detailed protocols available. Significant lymphatic drainage from the footpad thus a good location to image pathogens and cells arrival	Require good surgical skills to expose the LNs Customized stage to support mice in the microscope Temporary
	Chronic lymph node window	Allow imaging of the inguinal LNs	Very high. Surgery difficult to perform.	Allows longitudinal imaging without uncompromised	The setup is complex. Requires significant

				blood flow and/or vessel integrity Minimal local inflammation and vascular remodelling	surgical experience.
	Transplantation on ear pinna	Allows easy access due to transplantation at the ear pinna.	High.	Allows non-invasive longitudinal imaging. Preserves lymphatic and vascular supply.	Transplantation can be unsuccessful. Requires good surgical skills.
Spleen and GALT	Abdominal imaging window (AIW)	Requires surgical implantation in the abdomen.	Very high.	Allows for longitudinal imaging without disturbing physiological processes.	The setup is complex. Requires significant surgical experience.
	Temporary splenic window	Consists on adhering a window directly to the spleen.	Medium.	Fast procedure.	Requires good surgical skills. Extreme care needed for the exposure of the spleen to avoid vascular collapse or exsanguination.
	Temporary intestinal window	Allows observation of the intestines and Peyers Patches.	Medium.	Straightforward surgery.	Care should be taken to avoid damaging vasculature and intestinal epithelium, as well as dehydration. Requires control of peristaltic motion.