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Intravital microscopy in historic and contemporary immunology

Judith Secklehner^{1,2}, Cristina Lo Celso^{3,4,*}, and Leo M. Carlin^{1,2,*}

¹Cancer Research UK Beatson Institute, Garscube Campus, Switchback Road, Bearsden, Glasgow G61 1BD, UK

²Inflammation, Repair & Development, National Heart & Lung Institute, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, UK

³Department of Life Sciences, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, UK

⁴The Francis Crick Institute, 1 Midland Road, London NW1A 1AT, UK

Abstract

In this review, we discuss intravital microscopy of immune cells, starting from its historic origins to current applications in diverse organs. It is clear from a quantitative review of the literature that intravital microscopy is a key tool in both historic and contemporary immunological research, providing unique advances in our understanding of immune responses. We have chosen to focus this review on how intravital microscopy methodologies are used to image specific organs or systems and we present recent descriptions of fundamental immunological processes that could not have been achieved by other methods. The following target organs / systems are discussed in more detail: cremaster muscle, skin (ear and dorsal skin fold chamber), lymph node, liver, lung, mesenteric vessels, carotid artery, bone marrow, brain, spleen, foetus and lastly vessels of the knee-joint.

Introduction

The use of intravital microscopy (IVM) to describe physiology started soon after the first recorded compound microscopes were developed in approximately 1595. Marcello Malpighi, who described the alveoli of the lung, performed some of the earliest IVM studies attempting to image the lungs of mammals and amphibians documented in letters to his mentor¹. Towards the end of the 17th century, Antonie van Leeuwenhoek improved the optics used in microscopes. Although he is best known for his discovery of bacteria, or, as he called them, ‘animalcules’, he also described his observations of blood ‘particles’ in frogs². Almost 200 years later, Rudolf Wagner used the same animal model to describe leukocyte rolling in the vasculature³. Around the same time, Elie Metchnikoff used IVM to shape his pioneering work on two processes central to our understanding of immunology,

*Correspondence to: L Carlin, CRUK Beatson Institute, Garscube Campus, Switchback Road, Bearsden, Glasgow G61 1BD, UK. l.carlin@beatson.gla.ac.uk, C Lo Celso, Department of Life Sciences, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, UK c.lo-celso@imperial.ac.uk.

Conflict of interest

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phagocytosis and diapedesis⁴. These initial findings, although remarkable, could only be publicised through detailed drawings of the events observed. Photography, and in the case of live-cell imaging and IVM, cinematography, enabled researchers to share exactly what they observed with a wider audience. Ries and Vles independently created the first movies of proliferating cells of the developing sea urchin (1909) reviewed in 5.

Wagner and Metchnikoff employed bright field microscopy (BFM) for their intravital studies, an approach limited to transparent structures^{3, 4}. The first fluorescence microscopes were developed in 1911, but it was not until injectable fluorophores became available that IVM could be applied to more complex tissue imaging⁶. With the addition of a pinhole to reject out-of-focus light, and laser excitation, Confocal and Laser Scanning Confocal Microscopy (LSCM) was born^{7, 8}. This permitted direct recording of high resolution images in four dimensions. Initially theorised in 1931, multiphoton (MP) microscopy cleverly removes the problem of 'out-of-focus' light by only exciting fluorophores at the focus plane. However, MP microscopy only became technically possible almost 50 years later when the necessary lasers became available. The use of MP and LSCM for IVM has recently been reviewed in detail⁹. Using MP excitation, deeper tissue penetration can be achieved due to reduced scattering of longer wavelengths, however, single photon confocal microscopy should not be dismissed for IVM. Particularly in tissues where deep imaging is not required, single-photon LSCM has the advantage of an adjustable pinhole to gain or sacrifice resolution depending on the brightness of the object being observed. In fact, brightness and photostability of the fluorescent markers used for IVM are of key importance and the use of fluorescent labelling techniques and label-free MP imaging for IVM have been reviewed elsewhere^{9–11}. At least in some instances, the combination of the two (e.g. fluorescent proteins and dyes with second harmonic generation) allows for the simultaneous acquisition of the widest palette of signals¹².

Since the 1950s, the number of studies using IVM as a research tool has increased from one publication per year to more than 100/year in the 2000s and there have already been more than 1200 IVM papers this decade alone (Fig. 1A.). High impact factor (IF) journals are well represented amongst papers that use IVM with a quarter of the IVM papers in the last 5 years being published in journals with an IF of 4.5-10 and over 10% in journals with an IF of over 10 (Fig. 1B.). IVM has been instrumental in immunological research and around 50% of all IVM papers are in immunology however this is still a relatively small margin of the entirety of immunological research (Fig. 1C.). The two top-cited IVM publications (Scopus) focus on leukocyte-vasculature interactions^{13, 14}.

IVM is currently the only method that attains direct and immediate spatiotemporal information at a cellular / subcellular level from the intact mammalian immune system with functional circulation. It has brought particularly significant advances in understanding the biology of neutrophils, tissue resident immune cells and cells of the haematopoietic niche as these cannot easily be studied *ex vivo* due to short life spans, or the difficulty in replicating a complex microenvironment that is essential for functions but is not yet well understood. In fact, IVM studies are essential for the development of better reductionist *in vitro* systems to test particular hypotheses. By imaging the immune response in several different tissues, it has become evident that paradigms established in some organs may not apply in others. For

example, the leukocyte adhesion cascade, originally studied in postcapillary venules, follows different mechanisms in specialised systems such as the lung or liver (Reviewed in^{15, 16}). Here we review IVM in rodents specifically, however IVM in invertebrates such as *Drosophila* and *Caenorhabditis elegans* or in zebrafish has also been instrumental in shaping our knowledge of immunology.

Recent advances in IVM methodology are apparent when IVM imaging sites are analysed using papers from the last 5 years in the Scopus (SJR) top ten ranked immunology journals (Fig. 2). Although the cremaster, skin and lymph nodes account for roughly two thirds of IVM studies, diverse organs occupy the last third including liver, lung, arteries, brain, joints, etc. (Fig. 2A.). Leukocytes are all well represented, with a clear majority of studies focusing on neutrophils, perhaps reflecting continued enthusiasm for studying phagocytes by IVM that dates back 200 years, or, as noted earlier, the difficulty involved with studying these cells *in vitro* (Fig. 2B.). Whereas early IVM relied heavily on the transparency of the tissues studied, recent advances have made imaging of less favourable tissue possible, perhaps reflected in the fact that around three-quarters of the studies analysed used either confocal or multiphoton microscopy (Fig. 2C.)

Here we focus on state-of-the-art methods for IVM in different organ systems and how they were developed. To put these in context we also give examples of how this technique has impacted the field of immunology and our understanding of immune cell behaviour.

Cremaster muscle

The cremaster is a thin muscular layer surrounding the testes of male mice or rats that is easily accessible with minor surgery. Due to its transparency, the blood vessels in the cremaster can be imaged using transmitted light. Although the technique in rats was established in the early 1960s by R. T. Grant (and later modified for mice)¹⁷, imaging the vessels in the cremaster is still the most widely used application of immunological IVM (Fig. 2A.). An extensive description of cremaster muscle IVM is provided in¹⁸.

Limited to the SJR top journals in immunology of the last five years (Fig. 2), 15 studies used IVM in the cremaster muscle. Leukocyte adhesion and extravasation (diapedesis) is a critical step in protective immunity and inflammation as it delivers immune cells to the site of insult or injury. It is established that initiation of neutrophil extravasation is dependent on β_2 -integrin- (LFA-1/Mac-1-) mediated firm adhesion to the endothelium¹⁵. However, the molecular basis of the process is incompletely understood and a number of studies have focused on revealing these mechanisms. High-resolution visualization of neutrophil trans-endothelial migration in post-capillary venules in the cremaster has made several seminal contributions to immunological research in the last few years describing a multistep process with distinct molecular requirements at each stage¹⁵. Although the leukocyte adhesion cascade in post-capillary venules has now been established in molecular detail, recent high-resolution IVM also helped make the unexpected observation that neutrophils can pass back from the tissue into the blood vessel lumen, termed 'reverse trans-endothelial migration'¹⁹.

Skin: Ear pinna and Dorsal Skin Fold Chamber

Perhaps regarded as the simplest intravital methodology due to its non-surgical approach, imaging the dermis in the ear pinna of mice was first described in 1980²⁰ and the method has been refined over the last 30 years²¹. The inner skin of the rodent ear is naturally hairless, making it an ideal site to investigate immune cells in the skin (e.g. Langerhans Cells, Dendritic Cell (DC) subsets, Dendritic Epidermal T cells and circulating leukocytes in the dermal blood vessels)²² or to implant other lymphoid tissue for ease of access²³. However, in cases where a larger area or deeper skin tissue is required, surgery is necessary. The dorsal skin fold chamber (DSFC) was adapted for studies in mice in 1943²⁴ and is commonly used in cancer research as tumour cells can be injected into the exposed area and imaged continuously or intermittently over long periods as the tumours develop²⁵.

Leukotriene B4 was found to play a crucial role in regulating directional neutrophil swarming at the sites of skin sterile injury in a series of elegant experiments where experimentally manipulated cells were directly compared by IVM in the same tissue with differently fluorescently-labelled control cells²⁶. Skin IVM was also used to demonstrate the close interaction of perivascular macrophages specifically with the skin vasculature, which permits them to mediate neutrophil arrest in *Staphylococcus aureus* infection²⁷. Interestingly, it has also been shown that pericytes can ‘instruct’ innate leukocytes during interactions just outside the blood vessels during tissue infiltration²⁸.

Lymph nodes

As the major sites orchestrating the adaptive immune response, it is important to be able to probe the spatiotemporal basis of immune cell interaction in the lymph nodes (LNs). IVM of the peripheral LNs was reported a little over 20 years ago²⁹. The relatively superficial location of inguinal, cervical and popliteal LNs provides access for imaging with minor surgical preparation and direct IVM of these LNs has been used in a number of studies³⁰. However, as noted above, LNs may be implanted in the skin to allow ease of access and longitudinal imaging without further surgery²³.

The microanatomical organization of the LN has been shown to play a crucial role in the acute response to pathogens that drain into LNs³¹. Natural Killer (NK), NK T cells and $\gamma\delta$ -T cells are positioned within close proximity to pathogen-sensing macrophages, and IVM has shown that all these cells can respond quickly to macrophage cytokine signals. This helps resident macrophages in limiting or preventing dissemination of pathogens into the bloodstream, describing a complementary role for LNs in promoting innate immunity to pathogens alongside their traditional role in adaptive immunity³¹. Moderation of the adaptive immune response to *Staphylococcus aureus* in LNs has been directly probed by IVM³². Neutrophils infiltrate the draining LNs via high endothelial venules in response to the arrival of *S. aureus* bioparticles and interact with local B cells where TGF- β production by neutrophils suppresses B cell maturation and impairs the humoral response. Additionally, neutrophils robustly phagocytose *S. aureus*, limiting antigen presentation in the LN and hindering the development of a stronger adaptive immune response³².

Liver

The liver's critical metabolic functions, highly-specialised vasculature and mononuclear phagocyte system make it an important target for investigation by IVM. Even though the liver has impressive repair properties, chronic or severe acute damage is often fatal and characterised by inflammation and scarring / fibrosis mechanisms that involve cells of the immune system. Liver IVM has been achieved by multiple approaches including relatively simple non-recovery surgical exposure and more complex window implantation for repeat longitudinal imaging. The liver has been imaged by conventional confocal, multiphoton and spinning disc microscopy to investigate processes with different speeds / depths and optical properties and thus specialised imaging demands, see³³ for an example protocol.

IVM helped to provide evidence that, in addition to their role in haemostasis, platelets are important regulators in intravascular immune surveillance³⁴. Platelets actively scan the vasculature and interact with Kupffer cells (KCs) in liver sinusoids to form firm adhesions in response to bacterial infection. This scanning behaviour is reduced outside the liver or when KCs are depleted. IVM revealed that platelet:KC interaction precedes neutrophil recruitment to the liver, a crucial factor for microbial clearance. Failure of platelet adhesion leads to liver cell death and increased host mortality³⁴. Liver IVM has also been used to determine the mechanisms of neutrophil recruitment in sterile liver injury and defined that Bruton's tyrosine kinase signalling is required for neutrophil Mac-1 activation at focal hepatic necrosis³⁵.

Lung

Another highly-specialised vascular bed with potentially unique and critically important immune homeostatic mechanisms, the lung is a valuable target for IVM. However, there are obvious challenges in imaging a structure that oscillates millimetres at approximately 2.5hz in mice. Modern lung IVM (L-IVM) dates to the early 1930s when lung movement and the pulmonary vasculature were studied in dogs, cats and rabbits^{36–38}. Initial attempts at imaging the lungs of mice were conducted in 2000 to study altered flow of erythrocytes in lung metastasis using confocal microscopy³⁹. In the past decade, the number of studies using L-IVM has increased rapidly as technical challenges are overcome in both acquisition and analysis of imaging data^{40–44}. Based on lung imaging in larger animals, small custom-built circular suction chambers were developed to be surgically inserted into the thorax of terminally anaesthetised mice (Fig.3a) for imaging on upright microscopes^{42, 44}. An alternative approach is to glue a transparent membrane into the thorax after resecting ribs and achieve mechanical stability with a separate device⁴¹. All of these methods cause an initial pneumothorax, however, positive end-expiratory pressure and gentle suction stabilises the tissue or a chest drain installed at the time of surgery restores normal pressure. Fluorescent antibodies to label the endothelium and different blood cells are widely available and can be introduced intravenously. In the peripheral lung, L-IVM images compare favourably with control images of precision cut lung slices of agarose inflated tissue. (Fig 3. B-D).

Deep tissue penetration is hampered in the lung by refraction of light at the air: tissue interface of the alveoli, although L-IVM has been successfully performed using both single- and multi-photon excitation, only the peripheral alveoli can be visualized. Recent refinements have concentrated on achieving better image stability using either inverted microscope based approaches⁴⁵ or using a flanged apparatus that sits between ribs⁴⁶.

Changes in the behaviour of lung CD11c⁺-DCs after allergen challenge has been directly visualised by a combination of L-IVM and *ex vivo* imaging of live precision cut lung slices⁴⁷. DCs were found to be important in physiological surveillance of alveoli, demonstrating direct phagocytosis of allergen particles. In an allergen challenge, localised accumulation of antigen-rich DCs and T cells were revealed, suggesting direct, local pathogenesis in the development of asthma⁴⁷. Metastasis to the lung is of major importance in end stage cancer, but the mechanisms of immune antagonism and tumour promoting inflammation are poorly understood. The initial immune response to 'pioneer' metastatic cells has been recently observed by L-IVM⁴⁶ as well as the direct interaction of Ly6C^{low} monocytes with cancer cells⁴⁸.

Mesenteric vessels

Like the cremaster muscle, the mesenteric vessels that supply the gut are relatively transparent and straightforward to access surgically. Here, vessels up to a few hundred microns in diameter can be imaged and their stereotypical arrangement makes it easy to identify arteries, veins and lymphatics. To image the mesenteric vasculature, mice are anaesthetised and the small intestine is exteriorised via laparotomy, the mesentery is gently spread out over a large coverslip or slide, gently held in position and kept moist with saline soaked gauze or tissue⁴⁹. However, a significant caveat to this approach is that mesenteric vessel IVM is only possible in young mice (up to 8-9 weeks in C57BL/6 for example) as in older mice a layer of highly-scattering autofluorescent adipocytes obscure the vessels almost entirely.

A combination of IVM in the dermal blood vessels of the ear pinna and mesenteric blood vessels revealed a population of CX3CR1^{high}, Ly6C^{low} CCR2⁻ monocytes that 'patrol' the luminal side of blood vessels insensitive to the direction of blood flow⁵⁰. IVM of the mesentery made it possible to observe and quantify many more cells than would be possible in the dermal vessels alone⁵⁰. Another advantage of mesenteric IVM is similar ease-of-manipulation to the cremaster muscle, and more recently this technique was used to show reduced leukocyte adhesion when endothelial BMP (bone morphogenetic protein) was antagonised, demonstrating the role of BMPER (BMP endothelial precursor cell-derived regulator), proposed as a novel biomarker in vascular inflammation⁵¹.

Carotid Artery

Atherosclerosis and related diseases are a major cause of mortality and morbidity worldwide (eg.⁵²). Due to location and pulsatile movement, the major arteries are a challenging target for IVM, and studies using isolated arteries *ex vivo* have been crucial in examining the pathology of atherosclerosis. However, these reductionist approaches need to be informed by

models that address the multiple potential origins of the immune cells involved. An improved method for IVM in the carotid artery is described in detail in 53.

IVM in the carotid artery helped to determine the role of TLR-4 on platelets in thrombus formation. A pro-thrombotic role was already established for the cellular fibronectin containing extra domain A (Fn-EDA+) located in the extracellular matrix but it was unclear how this was mediated. Live imaging of ferric chloride induced injury was used to assess thrombus formation in real-time and to image platelet aggregation to highlight the role of platelet TLR4 interaction with Fn-EDA⁵⁴. Carotid imaging was also used to show Nestin +stromal cells direct inflammatory cell migration in a Monocyte chemoattractant protein 1-dependent manner⁵⁵.

Bone Marrow

IVM of the bone marrow (BM) was pioneered by the von Andrian group, and driven by the need to understand the dynamic processes allowing transplanted haematopoietic stem and progenitor cells to extravasate from bone marrow vasculature and home within the parenchyma⁵⁶. BM contained within the mouse calvarium (top of the skull) remains the most utilised BM model for IVM studies because of its accessibility, allowing for longitudinal studies that follow haematopoietic processes developing over the course of days or weeks. More invasive techniques allow for imaging of long bones^{57, 58}.

BM IVM allowed the first visualization of haematopoietic stem cells (HSCs) interacting with the BM microenvironment¹² (Fig. 4.) and has uncovered that these cells are unexpectedly dynamic upon exposure to physiological infection⁵⁹. Most recently it highlighted the otherwise unrecognised migratory behaviour of T-cell acute leukaemia cells at all stages of disease including following chemotherapy, and revealed that leukaemia drives rapid and dramatic remodelling of endosteal niches, including widespread apoptosis of osteoblastic cells⁶⁰. Very few immune cells in the BM have been studied with IVM, however regulatory T cells were shown to patrol HSC niches, making them a site of immune privilege⁶¹.

Brain

Due to the blood-brain barrier (BBB), the immune system in the central nervous system must overcome unique challenges. The optical density of brain matter makes it particularly amenable to multiphoton microscopy with imaging depths of up to 1.6mm recorded⁶². Two main methods are used for brain IVM, open-skull versus thinned-skull imaging windows and these approaches have been compared and described in detail⁶³. Both methods can be performed longitudinally as mice can be recovered after implantation of the window and are not impeded in their normal behaviour.

Invasion of parasites and fungi through the BBB is a challenge for the immune system due to the shortage of circulating leukocytes. Cerebral malaria can be a life-threatening complication when parasite-infected red blood cells (iRBC) are sequestered in brain vasculature. Consequential vessel blockages result in neurological pathology. IVM revealed worsened disease outcome after accumulation of Ly6C⁺ monocytes in response to T cell

signalling in a model of cerebral malaria⁶⁴. Another pathogen, *Cryptococcus neoformans* is a highly prevalent cause of meningitis in immunocompromised individuals. In a recent study brain IVM revealed direct removal and engulfment of vessel-adherent cocci by neutrophils, limiting the transmigration of fungus through the BBB⁶⁵.

Spleen

Although splenic IVM had previously been performed in larger animals, the first murine examples were published in the early 1990s using transillumination microscopy to visualize the flow of red blood cells through the spleen⁶⁶. The first confocal IVM in the spleen was published much later in 2003⁶⁷.

IVM in the spleen is technically less challenging compared to other internal organs because the spleen is relatively superficial and its distal end is easily manipulated. A small laparotomy (1cm) is sufficient to exteriorize the spleen without restricting blood flow. Imaging can be performed either on an upright or on an inverted microscope by carefully positioning the mouse on its left lateral side with the exposed spleen resting on a cover slip.

Intravital imaging in infectious disease studies is a major contemporary challenge and will reveal important dynamic information on microbial infiltration and direct immune responses to pathogens. In addition to the brain, the spleen is a focus in malaria research. Phagocytosis of iRBC by splenic DCs and the subsequent displacement and interaction of DCs with CD4⁺-T cells was visualized by IVM, revealing a previously unappreciated function for DCs in direct clearance of infected cells⁶⁸.

Foetus

Our understanding of how the immune system operates *in utero* is severely limited by access to relevant tissue. One of the most recent developments in IVM is imaging leukocyte adhesion in foetal blood vessels⁶⁹. In anaesthetised mice, the foetus and the yolk-sac within the placenta can be exteriorized and imaged under a coverslip while submerged in buffer. Although this method allows direct access to foetal leukocyte behaviour, it is important to consider the viability of the foetus during imaging. This can be severely compromised under anaesthesia particularly when using α 2-agonists (e.g. Xylazine, Medetomidine) as these rapidly pass through the placenta and can also restrict blood flow to the foetus⁷⁰, therefore etomidate or inhalation anaesthesia may be preferable.

The risk of bacterial sepsis is particularly increased in early premature infants but the molecular basis of this impairment is unclear. Therefore, foetal IVM was developed to characterise differences between leukocyte adhesion in foetal and adult vessels⁶⁹. IVM was essential for this work as *in vitro* studies of leukocyte adhesion cannot represent the foetal microenvironment. It is suggested that neutrophils in the developing foetus lack essential adhesion molecules to enable interaction with the endothelium and consequential extravasation into inflamed tissues⁶⁹.

Knee-joint

The molecular mechanisms underlying arthritis are not fully understood, therefore knee-joint IVM was developed as a tool to gain better information in mouse models of arthritic development in joints⁷¹. For imaging, mice are anaesthetised and carefully positioned on a customised stage. Subsequently the skin over the flexed knee-joint is resected and the patellar tendon is cut transversally to expose the Hoffa fat pad that contains synovial cells. A coverslip can be directly applied to the tissue and the microvasculature imaged *in situ*⁷¹.

Outside of investigating the mechanisms of autoimmune rheumatoid arthritis, this approach was recently used to analyse the transmigration of *Borrelia burgdorferi* spirochetes, a cause of Lyme disease in humans, revealing two spirochete adhesins important for bacterial dissemination⁷².

Concluding Remarks and Future Perspectives

Taken together, the quantitative literature review (Fig. 1 and 2) and the site-specific information portray an evolution in immunological intravital microscopy from generalised mechanisms to site-specific information in each major organ. State of the art IVM now allows access to most areas of the host to some extent, which is reflected by recent studies that even address immune responses in the heart⁷³. Major future advances will shift data from purely spatial to functional information at a microscopic level. For example, the spatiotemporal organisation of signalling activity in immune cells can now be directly probed using genetically encoded Förster Resonance Energy Transfer biosensor reporter transgenic mice at a subcellular level *in vivo*⁷⁴. However, a continued challenge in IVM is to maintain resolution deep into optically unfavourable tissue. The adoption of adaptive optics in many fields of microscopy including IVM holds real promise here⁷⁵. Most importantly, IVM has been, and will continue to be instrumental in uncovering the dynamics of immune cell function and especially the differences in the immune reactions taking place in different tissues and organs.

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Figure 1. Overview of the application and importance of Intravital Microscopy.

A. Using the search terms ‘intravital imaging’ or ‘intravital microscopy’ and not ‘*in vitro* maturation’ in the title or abstract on Pubmed a total of 3766 articles were found. **B.** To assess the contribution of studies using this technique, the journal impact factor (IF) of IVM articles from the last 5 years is shown. The 5 year IF of journals as published by Reuters Web of Science website was used for analysis. Newer journals (e.g. Intravital, eLife) that do not have a published IF were excluded from the analysis. The proportion of immunological studies using IVM is shown in **C.** The search terms ‘immunology’, ‘immunity’, ‘immune’, ‘myeloid’, ‘leukocyte’ or ‘lymphoid’ were used to distinguish immunological studies.

Figure 2. Immunological studies using IVM from the last 5 years.

Ranking based on the top 10 journals in Immunology as stated in the Scimago Journal and Country Rank (Scopus). The percentage of IVM studies from 2010-2016 (review articles were excluded from the analysis) in different organs is shown in **A**. The main cell type relevant to each study is plotted in **B**. Which imaging method was applied in the various immunological studies is shown in **C**. In articles where IVM was performed in more than one organ or cell type, each was counted individually. When more than one imaging method was applied each was counted individually.

Figure 3. Lung Intravital microscopy

A. Imaging set-up for IVM on the murine lung as described by Looney et al (2012). Gentle suction is applied via the vacuum port to stabilize the tissue. The anaesthetised and mechanically ventilated mouse is placed on a heat-mat in a right lateral position. Images are acquired using a long working distance water immersion objective. Comparison between precision cut lung slices (**B.**) and live lung imaging (**C.**). Live lung imaging can detect distinct changes in cell morphology *in vivo* (**D.1-3**). **B.-D.** PE conjugated Ly6G-Ab (1A8; red, D. 1) and Isolectin B4-Alexa488 (cyan, D. 2) were injected intravenously to label neutrophils and the lung vasculature respectively. D. 3 = pseudocolour merged image.

Figure 4. IVM of hematopoietic cells in the bone marrow.

A. Maximum projection of a z-stack showing two HSCs labelled with DiD (red) observed in the proximity of osteoblastic cells (green) one day after transplantation into irradiated Col2.3GFP transgenic osteoblast reporter recipient mice. **B.** 3D rendering of a 4D time-lapse image of a T-ALL cell (represented by the red dot) migrating within the bone marrow space. Grey: vasculature highlighted by Cy5-labelled dextran; green: col 2.3GFP+ osteoblastic cells. The colored line shows the track of the cell during the three hour-long imaging period. The white arrow points at the position where the cell underwent mitosis and the two

asterisks indicate the position of the two daughter cells at the end of the recording (reproduced from Hawkins et al. 2016 under Creative Commons).