Review

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Understanding liver immunology using intravital microscopy

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Summary

The liver has come a long way since it was considered only a metabolic organ attached to the gastrointestinal tract. The simultaneous ascension of immunology and intravital microscopy evidenced the liver as a central axis in the immune system, controlling immune responses to local and systemic agents as well as disease tolerance. The multiple hepatic cell populations are organized in a vascular environment that promotes intimate cellular interactions, including initiation of innate and adaptive immune responses, rapid leukocyte recruitment, pathogen clearance and production of a variety of immune mediators. In this review, we focus on the advances in liver immunology supported by intravital microscopy in diseases such as isquemia/reperfusion, acute liver injury and infections.
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Historical perspective of intravital microscopy

Humans always wanted to see farther and better. The oldest recognized magnifying device is the "Nimrud lens", which dates back more than 2700 years and it was supposedly used as a magnifying glass and to produce heat by concentrating the sunlight. In this context, these first lenses might have been also used to cauterize small wounds, linking them immediately to medical and biological sciences [1]. These lenses were further adapted in many ways, culminating in the development of first telescopes and microscopes. The invention of the microscope (ca.1600) and its improvements in the last half-century were essential to breakthrough discoveries in many fields of scientific investigation. Such findings in microvascular morphology and physiology built the basis for elucidation of the leukocyte recruitment cascade [2]. Apparently, the conventional histopathology techniques using fixed tissues were insufficient to describe the dynamics of biological processes, which generated a demand for *in vivo* imaging strategies. Thus, the terminology "intravital microscopy" (IVM) was coined for the adaptation and use of a microscope to image cells and tissues in live animals. Due to inefficient light sources and limitations in imaging solid organs at that time, the first live tissues visualized were from thin and transparent organs such as frog's interdigital webbing or tongue. The first report of intravital microscopy dates back from 1846 in Marshall Hall (1790–1857) and August Waller (1814–1870) observations in frog's capillaries. Their discoveries were seminal for the initial understanding of endothelial physiology. Also visualizing the microcirculation, Julius Cohnheim (1839–1884) demonstrated that leukocytes immigrate across the vessel wall to the interstitial space following stimuli [2]. Researchers have explored the fact that different species may be used for intravital microscopy, leading to live imaging of worms, fish, insects, amphibians, reptiles and small mammalians.

The application of IVM in hepatology is more recent probably due to the solid structure of the liver and its location inside the peritoneal cavity, which make liver imaging more difficult. Nevertheless, the vast hepatic microvasculature and unique diversity of cell types provide a rich field for intravital studies in both health and disease [3]. In the past two decades, the development of new imaging techniques, dyes and fluorescent proteins that favor IVM resulted in an explosion of possibilities to mechanistically investigate liver biology down to the molecular level (Fig. 1). Liver IVM has been employed in studies of transplantation, ischemia/reperfusion injury, cancer, acute/chronic toxic injuries, fibrosis, infections, hemorrhagic shock and sepsis. In this review, we will focus on how *in vivo* imaging using different microscopy modalities has enhanced our understanding of liver immunology.

Keywords: Liver intravital microscopy; Liver immunology; *In vivo* imaging; Sterile injury; Liver infection.
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Abbreviations: CCD, Charge coupled devices; CMOS, Complementary metal-oxide semiconductors; DILI, Drug-induced liver injury; KC, Kupffer cell; LS, Laser scanning; LSEC, Liver sinusoidal endothelial cells; NET, Neutrophil extracellular traps; OPS, Orthogonal polarization spectral; SD, Spinning-disk; SDF, Stream dark field; SHG, Second harmonic generation; SIM, Structured illumination microscopy; SPECT, Sporozoite microneme protein essential for cell traversal; STED, Stimulated emission depletion; STORM, Stochastic optical reconstruction microscopy.

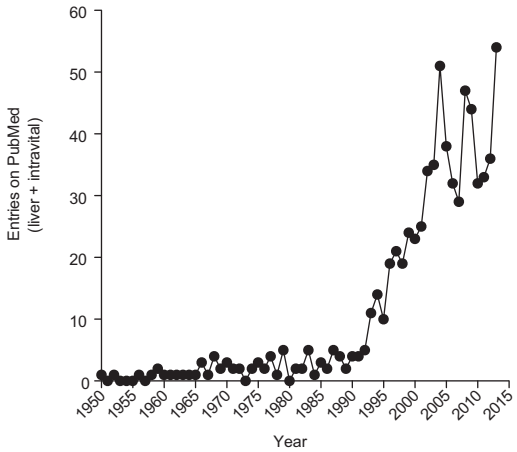

Fig. 1. Number of entries on Pubmed containing the words "liver" and "intravital" from 1950 to 2014. Note that the abrupt increase in published papers in 1990's is coincident with the increased availability of fluorescence and laser scanning confocal microscopes. Source: Alexandru Dan Corlan. Medline trend: automated yearly statistics of PubMed results for any query, 2004. Web resource at URL:http://dan.corlan.net/medline-trend.html. Accessed: 2012-02-14. (Archived by WebCite at http://www.webcitation.org/65RkD48SV).

Imaging modalities for liver intravital microscopy

The liver can be imaged by intravital microscopy with most standard microscopes or even by confocal probes that fit in a surgical needle [4,5]. As the pioneers in IVM evaluated translucent organs, the liver edge can be imaged in a similar way, since it is considerably thin [6]. In this way, a regular bright field microscope would suffice to evaluate general leukocyte trafficking and behavior (Fig. 2 and Supplemental Video 1). However, deeper mechanistic approaches often require evaluation of specific cell types, prompting the use of antibodies to particular surface antigens (i.e., F4/80 in mouse monocytes and macrophages, CD3 in lymphocytes, etc.) or fluorescent protein expressing cells (Supplemental Video 2). In this case, fluorescence microscopes enable the identification of different markers (or cells) in the same animal. More recently, laser scanning confocal microscopes and newer alternatives such as spinning-disk and multi-photon microscopes emerged as higher quality intravital imaging options [7–11] (Fig. 3 and Supplemental Videos 3 and 4). Confocal microscopes provide high-resolution fluorescence imaging since they use a pinhole to exclude out of focus light [12,13] offering live tissue imaging even of thick and complex samples. However, as laser scanning confocal raster scan the sample point-by-point, faster events are challenging to capture. Field scanning confocals such as the spinning-disk microscope utilize multiple pinholes [14], enabling faster phenomena such as platelets flowing in the bloodstream or calcium waves in the liver parenchyma to be imaged with accuracy. Multi-photon microscopes are also beneficial for IVM because of their ability of imaging at great tissue depths (hundreds of micrometers) [15]. In multi-photon microscopy, only fluorophores in the in-focus-plane are excited, therefore excluding the need for pinholes while reducing phototoxicity and bleaching. Multi-photon excitation has been used to visualize tumorigenesis [16], angiogenesis [17] and related deep tissue processes that were previously difficult to assess due to inefficient fluorescence excitation and detection using standard confocal techniques. There are alternative imaging techniques such as light sheet microscopy, which provide optical sectioning capability. However, traditional light sheet microscopes involve a unique optical geometry, which poses challenges to sample mounting and therefore sample choices. Nevertheless, as these techniques evolve, intravital imaging using light sheet microscopy may become feasible in the future. With the large variety of imaging techniques currently available, choosing the best imaging approach relies on what the main research question is and the available budget (Table 1). Multi-photon and spinning-disk microscopes generally have higher costs, although they are more effective and versatile for intravital imaging. Nonetheless, reliable data can be acquired by adapting regular fluorescence or laser scanning confocal microscopes for intravital imaging [18].

Recent advances in the field of super-resolution microscopy pose interesting possibilities for IVM (Fig. 2). Techniques such as SIM (structured illumination microscopy), which utilizes structured illumination, provide two fold improvement over the conventional diffraction limit without compromising cell health or speed. SIM is currently limited to relatively thin samples, but as technology is constantly improved, it will be very interesting to adapt SIM for IVM. Indeed, multi-photon excitation has been applied to SIM to provide deeper penetration depths [19]. SIM in its standard form can be utilized to "complement" conventional IVM techniques to examine structures inside thin samples (e.g. cremaster muscle or mesentery) at higher resolution. Additional super-resolution techniques such as STORM (stochastic optical reconstruction microscopy) which utilize Gaussian fitting of emission points, provide even greater resolution improvements of ~ten fold over conventional microscopy techniques, enabling resolution down to the single molecule level. However, achieving ~ten fold improvement in resolution requires longer imaging times and more prolonged exposure of cells to light, limiting their utility in live imaging experiments. This limitation also applies to other super-resolution techniques like STED (stimulated emission depletion microscopy) that provide similar improvements in resolution.

Fluorescent labeling of hepatic cells and structures is not usually an issue when performing IVM. The same antibodies and probes used for immunofluorescence or flow cytometry are normally effective for IVM as well. Therefore, the imaging possibilities are virtually limitless as it is feasible to label multiple cells simultaneously (Fig. 3). In this sense, one can visualize collagen and extracellular matrix proteins [20], blood flow [21], bile canaliculi [22], sinusoidal endothelial cells (LSECs) [23], hepatocytes [24], stellate cells [25], Kupffer cells [26], neutrophils [27], platelets [28], lymphocytes [29], NKT cells [30], and even cellular components such as DNA [26]. Also, liver function can be assessed using fluorescent reagents, such as hepatocyte viability using Rhodamine 6G [21,31], hepatocyte bile secretion using carboxyfluorescein diacetate (CFDA) [31], endothelial viability using formaldehyde-treated albumin [32], and Kupffer cell (KC) phagocytosis using fluorescent latex beads [30].

Imaging acquisition requires detection and recording devices. The most common acquisition devices for laser scanning confocals are photomultiplier tubes (PMTs), but charge coupled devices (CCD) and complementary metal-oxide semiconductors (CMOS) are also widely used for field scanning confocals and epi-fluorescence microscopes for their high signal-to-noise and high-speed acquisition properties [33] (Fig. 2). Acquisition is usually followed by image analysis in a computer equipped with

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