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Full Length Research Paper

Intravital microscopy: Taking a close look inside the living organisms

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The opportunity to visualize biological phenomena in living organism has fascinated and improved the research field for decades. The most common way to perform these experiments consist in anesthetize small animals (such as mice and rats) and expose the tissue of interest to a light or laser source and acquire the images using a microscope. This method is denominated "intravital microscopy" and there are several studies which have wisely used this technique and improved our knowledge in several fields of life sciences. Here, we review the basic concepts necessary to perform intravital microscopy studies, describing briefly several procedures in different organs.

Key words: Intravital microscopy, *in vivo* imaging, microscopy, microbiology.

INTRODUCTION

In the last few decades, the field of cell biology has experienced extraordinary breakthrough discoveries that significantly contributed to the understanding of several processes within the cell. In part, this may be explained by the technology-driven development of new techniques, especially those regarding genomics and proteomics. On one hand, there are several studies dissecting gene and protein expression in single cells or at particular processes and diseases; on the other, how these complex pathways, tissues and systems function in the living body is still poorly understood. In this regard, a major limitation to widen the knowledge of biological processes consists in the technical approach to perform *in vivo* studies.

To overcome this limitation, several techniques and analytical tools have been developed to image and study cellular and biological events in living animals (Weigert et al., 2010). Among these techniques, an outstanding tool is imaging biological phenomena in living organisms by using the intravital microscopy (IVM). The first report of the use of this technique date to the nineteenth century, and has led to the understanding of numerous biological processes (Wagner, 1839; Sumen et al., 2004). However, with the improvement of fluorescent cell markers, and the confocal and multi-photon microscopies (Phan and Bullen, 2010; Ishii and Ishii, 2011), the intravital technique has been taken to an extraordinary level, including non-invasive, non-labeling requiring techniques, such as orthogonal polarization spectral imaging - OPS (Buchele et al., 2007). For example, search for "*in vivo* imaging" or "intravital microscopy" in Pubmed database returns more than 3.000 papers since 60's.

Although intravital microscopy can be performed in virtually all types of tissues within an organism, most studies are focused in unraveling immunological questions. In addition, intravital microscopy can be used

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to visualize host-pathogen interaction by using fluorescent or bioluminescent labeled microorganisms, including bacteria (Lee et al., 2010; Wong et al., 2011), viruses (Doceul et al., 2010) and protozoa (Peters et al., 2008). The aim of this review is to present the methodological approaches related to intravital microscopy in different organs and to present what has been done in these several systems of the organism. In this sense, microbiologists may apply labeled organisms and the procedures described here to perform intravital microscopy studies, expanding the knowledge in specific research areas.

GENERAL EXPERIMENTAL PROCEDURES

The majority of intravital microscopy studies are done using mouse models, although almost all IVM approaches cited below can also be performed in rats. This may be explained by the relative abundance of mice in research institutions throughout the world; the availability of genetically modified mice, which include a variety of knockout and fluorescent protein-expressing animals; and their small size, allowing mice to be used in standard microscopes and laboratory facilities.

To perform *in vivo* imaging of biological events, it is necessary to gain access to the tissue of interest. In most cases this means to expose surgically the organ to allow access of the microscope objectives. Before starting the experiments, one must have in mind that the procedures have to be set to provide the conditions for the animals to tolerate surgery, avoiding excessive physiological disturbances that could interfere with the results.

In this regard, animals are usually anesthetized by injection of a mixture of ketamine (80 – 200 mg/kg) and xylazin (10 – 40 mg/kg). Although other anesthetics may be used, such as urethane (2 g/kg) or halothane (3 – 5% v/v), the ketamine/xylazin mixture generates very steady hemodynamic parameters (Cara and Kubes, 2004). In cases of long anesthesia periods (several hours), additional procedures can be used to monitor and maintain the basic physiological parameters of the animals, such as jugular cannulation for intravenous drug administration (Nacul et al., 2010), rectal temperature monitoring by using an electrothermometer (Soriano-Izquierdo et al., 2004) and artificial ventilation of the lungs by trachea intubation, aiming to maintain a patent airway throughout the experiment (Sintara et al., 2010). In addition, the arterial blood pressure and heart rate can be continuously monitored through a catheter placed in the carotid artery (Geerts et al., 2006; Henriksnas et al., 2009). Several procedures can be performed during the surgery, depending on the aim of the study. For instance, a polyethylene catheter can be also inserted in the femoral (Katayama et al., 2003) or tail (Soriano-Izquierdo et al., 2004) veins to permit injection of substances such as antibodies, anesthetics, fluorescent dyes or drugs.

The definition of the type of imaging that is going to be performed is a major point. Some IVM models using transparent tissues, such as the cremaster muscle, mesentery and liver edge, can be directly observed by conventional light microscopy (Menezes et al., 2008). However, fluorescent labeling may be necessary in solid, non-transparent organs, such as knee joint and brain, which also allows specific cell type identification and localization, and evaluation of the vascular architecture and its alterations (for example, permeability increase/plasma leakage). Some very commonly used fluorophores are rhodamine 6G and fluorescein isothiocyanate (FITC), which can be injected directly into the blood stream (Cara et al., 2000). When these fluorophores are conjugated to vasculature-impermeable, high-molecular weight molecules such as albumin, dextran (150-500 kD) or antibodies, they can be used to delimitate blood vessels and to define vascular permeability increase phenomena (McDonald et al., 2010). Also, bacteria and cells expressing the green fluorescent protein (GFP) and their relatives (RFP, YFP, CFP, etc.) are very popular and time-saving tools (Lee et al., 2010).

INTRAVITAL MICROSCOPY: A LIVING WORLD IN MOTION

As aforementioned, a major outcome of the development of intravital microscopy was the possibility to study and understand biological processes *in vivo*. We will review some of these techniques and discuss their applications in several areas of the biomedical science.

Cremaster and striated muscles

The striated muscle is a contractile tissue of animals primarily responsible for their ability of locomotion. The striated muscle tissue has a remarkable malleability and can adjust its metabolism and contraction in response to alterations in functional demands. A very important member of this class is the cremaster, a thin layer of striated muscle derived from the internal oblique and transverse abdominal muscles, formed as the result of testes descent through the inguinal canal (Grant, 1966). The cremaster is involved in the support and temperature maintenance of testicles. The thinness and high transparency of this muscle, which allows direct microcirculation observation under conventional microscopes (Menezes et al., 2008) can explain its extensive use in intravital microscopy studies (Figures 1 and 2, supplementary video 1– Intravital microscopy of cremaster muscle using an epifluoresence microscope. Note rolling and adhered leukocytes on the vessel wall).

The open cremaster muscle preparation can be executed in rat and hamsters, as it allows a more magnified and complete examination of the tissue vasculature. In addition, it has been used to dissect cellular pathways involved in regulation of microvascular function and real-time imaging of intercellular signaling. In this preparation, the animal is placed in supine position, onto the viewing plexiglas stage. An incision is made in the skin and fascia above the final portion of the scrotum. The underlying connective tissue fascia is carefully separated from the cremaster sack while it is gently pulled off the scrotum using a delicate forceps. The preparation needs to be moistened with PBS at 37ºC regularly during the whole procedure to keep it warm and moist. The final portion of the cremaster is then attached with a nylon thread to hold down and slightly extend the end of the sack. An incision is made below the center of the cremaster muscle using a thermal cautery, opening the sack. The edges of the tissue are attached with threads to hold the cremaster opened onto the stage. The

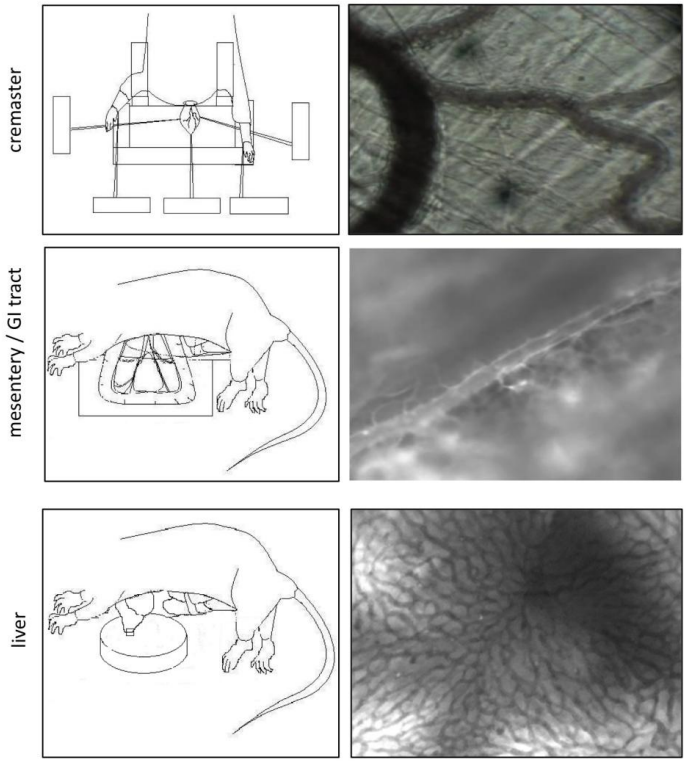

Figure 1. Representative chart showing the basic procedures to perform intravital microscopy studies in cremaster, mesentery and liver tissues. The right column shows representative snapshots from videos obtained by the procedures described in the left column. Cremaster images were captured by using a white light source coupled to a conventional microscope, while mesentery and liver images were captured by a fluorescence microscopy setup, and leukocytes were labelled by rhodamine 6G.

epididymis is clamped and lifted and the connecting tissue is cut using the thermal cautery. Finally, the epididymis is pushed into the abdominal cavity. The preparation is, at this point, ready for evaluation under light microscopy. As the muscle stays stable on stage, it is possible to perform long-term observations without disruption (Gavins and Chatterjee, 2004).

GASTROINTESTINAL TRACT

The gastrointestinal tract generally refers to the stomach, intestines and mesentery. Intravital microscopy has been used to explore the interference in the permeability of gastrointestinal tract microvessels and the different steps involved in the process of leukocyte extravasations in face of new drugs, infectious and harmful agents, physiological and pathological processes (Cserni et al., 2011). Intravital microscopy has been widely used in various studies and experimental models of cirrhosis and portal hypertension (Geerts et al., 2006) and allows the analysis of the effect of administering anti-inflammatory drugs (Tailor et al., 1999), mucosal-damaging agents (Yoshida et al., 1995; Saeki et al., 2004), new treatments to prevent gastropathies (Suzuki et al., 2001; Henriksnas et al., 2005; Mota et al., 2007), gastroduodenal disease (Polenghi et al., 2007), among other factors.

To perform *in vivo* microscopic observations of the intestinal wall and mesentery microvessels, a small surgery is necessary (Dong et al., 2009; Harris et al., 2009; Hyun et al., 2010; Katada et al., 2010; Konerding et al., 2010; Byrne et al., 2011; Castor et al., 2011). For this purpose, after a midline incision, the intestinal loop of interest is gently exposed and mounted on a plastic stage (Figure 1) for microscopic examination (Katayama et al., 2004; Harris et al., 2009; Katada et al., 2010). On the other hand, whether the interest relies on the observation of the gastric mucosa, a laparotomy is performed through a midline incision in the abdomen utilizing a microcautery and the stomach is gently extended and placed on a designed board (Suzuki et al., 2001; Saeki et al., 2004; Sintara et al., 2010). The greater curvature of the stomach is incised longitudinally leaving the greater omentum attached to the posterior wall while the anterior

wall is resected (Saeki et al., 2004). The incision in the anterior wall is opened using microclamps and covered with Saran wrap to allow visualization of the posterior mucosal surface (Kalia et al., 1997; Saeki et al., 2004).

LIVER

The liver is considered a metabolic, glandular and lymphoid organ, comprising functions such as metabolism, detoxification of exogenous and endogenous substances, bile secretion, and clearance of bacterial products from the blood (Thomson and Knolle, 2010). Weighting 1.5 kg in an adult, the liver is the second largest organ in the body and is located in the abdomen, where it receives a unique blood supply consisting of 80% venous blood from the portal vein and 20% oxygenated blood from the hepatic artery. This nutrient-rich and low oxygen tension mixture flows at low perfusion pressure through an enormous network of thin-walled hepatic microvessels called sinusoids. They are covered by fenestrated liver sinusoidal endothelial cells, which separate hepatocytes from the bloodstream and form a compartment named space of Dissé. The liver is divided microscopically in structures named lobules, which contain the hepatocyte, the major cellular and metabolic unit of the liver. The hepatocytes lie in threads inside the lobule, between the sinusoids and the non-parenchymal populations, namely the Kupffer, Ito, NK and NKT cells.

Intravital microscopy has revealed many unique features of the diseased liver in the past decades, and still offers countless applications. This *in vivo* technique has been used in a variety of animal models, ranging from bacterial infection (Lee et al., 2010), hepatocellular cancer (Takeichi et al., 2010), drug-induced liver injury (Ito et al., 2003), ischemia-reperfusion injury (Teoh et al., 2007), ageing (Ito et al., 2007), sepsis (Huynh et al., 2010), TNF-α-induced hepatic microcirculatory dysfunction (Katagiri et al., 2008), spinal cord injury-induced collateral liver damage (Hundt et al., 2011) and focal necrotic injury (McDonald et al., 2010). In this way, understanding the microscopic alterations that occur in the diseased liver through the intravital microscopy technique offers valuable mechanistic insights and novel therapeutic possibilities to many pathological conditions.

The liver has some characteristics that favor its use in intravital microscopy. Its abundant vascularization provides a very rich field for observation. Also, it can be imaged through epi-illumination and trans-illumination, that is, with or without the use of fluorescent probes, which are necessary in intravital microscopy of the brain and bone marrow, for example. In addition, several parameters besides leukocyte movement and sinusoidal perfusion can be assessed, such as presence of extrasinusoidal red blood cells (Ito et al., 2003), hepatocyte or sinusoidal endothelial cell morphology alterations (Ito et al., 2007) and phagocytosis of fluorescent microorganisms and particles (Lee et al., 2010).

The experimental setup is usually very similar throughout the literature, allowing the use of upright or inverted microscopes. The objectives used vary depending on the degree of resolution required (4x, 10x, 20x and 80x water immersion). Basically, mice or rats are anesthetized and, if necessary, cannulated for administration of drugs or additional anesthesia. The animal is then submitted to a midline laparotomy or subcostal incision to allow liver exteriorization (Figure 1, supplementary video 2 – Liver intravital microscopy using a fluorescence microscope. Leukocytes were stained by rhodamine 6G. Note rolling and adhered leukocytes on the vessel wall and the blood flow into liver sinusoids). Skin or abdominal muscle may be removed using a cautery to reduce bleeding. Following, the hepatic ligaments are dissected, the animal is placed in right lateral position and the organ exteriorized laterally, aligned to the microscope objective for imaging. The liver must be moistened with physiological solution or wrapped in plastic to avoid dehydration at all times. Both upright and inverted microscopes may need stages designed to adapt the animal's position or collect leaking fluid from the procedure. Once the liver is correctly placed, the images can be acquired and recorded for a time ranging from ten minutes to four hours, and analyzed a *posteriori* through specific software for the chosen parameters.

ADIPOSE TISSUE

The adipose tissue plays a central role in energy and endocrine homeostasis, as well as in immunity. It has been associated not only with lipid storage and metabolism, but also with the production of the hormones leptin, adiponectin, resistin and estradiol. Besides its well-established participation in diseases such obesity and diabetes, the mechanisms behind the adipose tissue role in these conditions have remained incompletely defined. This tissue produces a wide variety of pro-inflammatory cytokines and chemokines, including Interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) (Ferrante, 2007). These locally produced cytokines recruit immune cells such as lymphocytes and monocytes toward the adipose tissue, which is important to start and sustain a systemic inflammation. The recruitment of leukocytes to the white adipose tissue (WAT) is an essential event in the development of chronic metabolic diseases such as obesity and insulin resistance (Gregor and Hotamisligil, 2011). This is also seen during inflammation that happens at the WAT in an experimental model of food allergy (Dourado et al., 2011) and acute weight loss where the recruited leukocytes to WAT regulate lipolysis (Kosteli et al., 2010). Therefore, it is very important to study fundamental features of

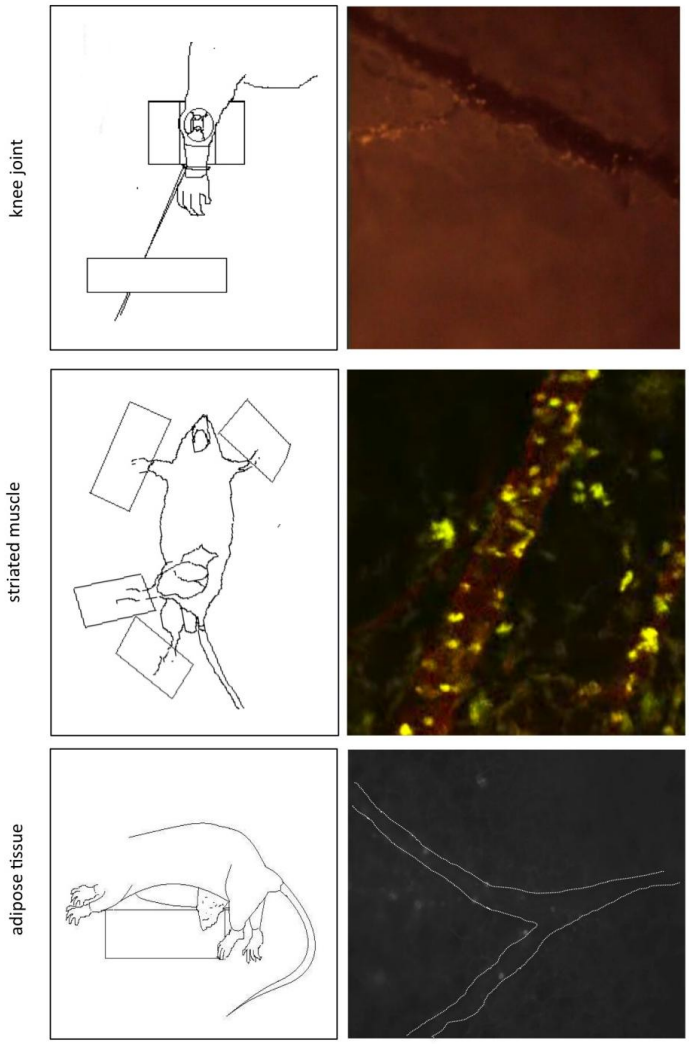

Figure 2. Representative chart showing the basic procedures to perform intravital microscopy studies in knee joint, striated muscle and epididimal adipose tissue. The right column shows representative snapshots from videos obtained by the procedures described in the left column. Knee joint and adipose tissue images were made using a fluorescence microscopy setup, and leukocytes were labelled by rhodamine 6G. Striated muscle images were made by a confocal microscopy setup (in green: eGFP expressing neutrophils and in red : PE-labelled endothelial cells (anti-CD31).

inflammation, such as leukocyte-endothelial cell interactions, in order to assist the development of pharmacological therapies for chronic metabolic diseases such as obesity (Nishimura et al., 2008).

Intravital microscopy can be performed in the epididymal or periuterine adipose tissue microcirculation (Myrhage et al., 1973) (Figure 2). For this procedure, mice are anesthetized and an unspecific cell marker as rhodamine 6G is injected intravenously to visualize the leukocyte–endothelial cell interactions (rolling and adhesion). A cautery incision is made along the abdominal region and the abdominal fat pad is exteriorized by gently teasing it out of the abdominal cavity, then placing it on a viewing pedestal. The preparation is mounted in a fluorescence microscope and the exposed tissue should be kept warm and wet. 2–4 regions of interest (unbranched venules with 20–40 µm in diameter) in each mouse are selected by using a ×20 objective. A less invasive way to make it is through small dermal windows where is possible to observe the fat pads without being exteriorized and the visualization of the microcirculation is made with an inverted microscopy

(Nishimura et al., 2008).

The respiratory system

The respiratory system is constituted by the lungs and a sequence of airways that conduct oxygen and carbon dioxide. A remarkable characteristic of this system is the presence of a great amount of lymphocytes associated to its mucosa.

Despite the extensive research on inflammatory lung diseases such as asthma and chronic obstructive pulmonary diseases, it has been difficult to directly visualize and analyze leukocyte recruitment into the airways *in vivo* (St Croix et al., 2006). Thus, the use of intravital microscopy to study the physiology of the respiratory tract has being increasing in the last years. To illustrate this scenario, the technique was recently used to analyze the pulmonary microvascular response to hypoxia or hypoxic pulmonary vasoconstriction (HPV) (Tabuchi et al., 2008), airway inflammation (Cortez-Retamozo et al., 2008, 2010), acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Chagnon et al., 2010).

The use of intravital microscopy to study patophysiological conditions of the respiratory system, especially the lungs, was restricted basically due to technical problems. When compared to other imaging techniques, a major limitation of intravital microscopy of the lungs was that the analysis could only be performed at a small observation area, making it unachievable to analyze areas located deep inside the organ. The alveolar-capillary region accessible to IVM consists of alveoli and associated juxta-alveolar arteriolar, septal, and venular microvessels. However, new methodological approaches overcame these restrictions. On this sense, two models have been proposed for chronic studies of inflammatory processes in the lung (Kuebler et al., 2007). First, it was developed a miniaturized laser scanning microscope that operates in the visible and near-infrared ranges at up to four wavelengths (three of which can be acquired simultaneously at any time). The optics of the system relies 1.3 mm-diameter “stick optics” that can be inserted into the animal through a tiny keyhole incision. Furthermore, the biopsy needle–sized objectives have a distal flush mechanism that allows their insertion deep (1–2 cm) into tumors or organs as the lung. Another different approach would be through a microscopic access to the surface of the right upper lung lobe in anesthetized and ventilated mice by a surgical excision of a 7 to 10 mm diameter window from the right thoracic wall. The window is covered by a transparent polyvinylidene membrane and sealed with α-cyanoacrylate. Then, the intrathoracic air removal through a transdiaphragmal intrapleural catheter allows the coupling of the lung surface to the window membrane (Tabuchi et al., 2008).

The aforementioned approaches can be powerful tools for the understanding of biological processes in the respiratory system under normal and pathological conditions.

Central nervous system

The central nervous system (CNS), which is constituted by the encephalon and medulla, is a site of selective and modified immune reactivity, in which leukocyte entry is restricted, in part, due to the blood-brain barrier (Ransohoff et al., 2003). Under physiological conditions, leukocyte traffic into the CNS is low, however leukocyte recruitment represents a critical step in the inflammatory pathologies, like cerebrovascular diseases, infections (Vilela et al., 2008), autoimmune diseases, traumas, and degenerative processes (Steffen et al., 1994; Soares et al., 1995; Buckwalter et al., 2006). Intravital microscopy studies have had remarkable importance in the study of cell trafficking into the CNS (Ransohoff et al., 2003). This technique allows the access to the mouse pial microcirculation during acute and chronic physiological and pathophysiological conditions (Cabrales and Carvalho, 2010). Although the visualization is restricted to the pia mater microvessels, histological analysis revealed that leukocyte recruitment of pial microvasculature was similar to that found in all parts of the brain (Liu and Kubes, 2003).

In addition to the extensive use of intravital microscopy for analysis of leukocyte-endothelial interactions in brain, this technique has been used to analyze cerebrovascular parameters like: angiogenesis, microvessels density, microvessel diameter, red cell velocity, blood flow, assessment of cerebral perfusion and vascular leakage by fluorescence-labeled markers such as Albumin-FITC (Niimi et al., 2000; Nageswari et al., 2002; Cabrales and Carvalho; 2010, Herz et al., 2011; Zanini et al., 2011).

To perform intravital studies in the CNS, a craniotomy is performed in anesthetized mice with a high-speed drill in the right parietal bone, and the tissues are continuously perfused with artificial cerebrospinal fluid. Then, animals are placed in prone position on a stereotaxic frame and the head is carefully secured using ear bars. A similar surgical procedure is used to study the spinal cord. If this is the case, after a midline skin incision of 3–4 cm, the paravertebral musculature is detached from the cervical spinous processes and retracted laterally, exposing the vertebral laminae. A laminectomy is performed, and the dura matter is opened over the dorsal spinal cord avoiding trauma to the parenchyma and the spinal microvasculature.

A major advantage of using intravital microscopy to study the spinal cord is that vessels in the central white matter are visualized directly, owing to the topography of the spinal cord (Ransohoff et al., 2003).

Reproductive system

In general, basic research in the reproductive field is

limited by the lack of available techniques to conciliate the observation of the behavior and the crosstalk between different cell types at a cellular level with the maintenance of their native microenvironment (that is, hormonal availability, proper blood perfusion, growth factors, cell-cell and cell-extracellular matrix interactions, among others). The recent development of the intravital microscopy technique with the consequent emergence of time-lapse imaging of individualized cells in *in vivo* conditions allowed the investigation of dynamic processes involving different cell types in the biology of reproduction field.

The gametogenesis, which is the process that culminates in the formation of spermatozoa within the testes or in the oocytes within the ovaries, occurs in several sequential steps that are tightly regulated. The spermatogenesis within the testis is based on a stem cell system and, therefore, depends on the balance between stem cell self-renewal and differentiation to support sperm production throughout the male reproductive life. The behavior of the spermatogonial undifferentiated cells and their fate to self-renewal or commitment to differentiation can be addressed by intravital microscopy, which allows the observation of these cells *in vivo* for a prolonged period of time. In this sense, with this methodological approach, it is possible to demonstrate the existence of a functional niche for the undifferentiated spermatogonia within the seminiferous tubules epithelium, as well as its spatial relationship with other testicular components, such as the vascular network and the different stages of the seminiferous epithelium cycle (Yoshida et al., 2007). Although the niche hypothesis was already proposed and studied in the literature (Chiarini-Garcia et al., 2001; Chiarini-Garcia et al., 2003), the detailed information of its structural basis was only possible by using time-lapse imaging. Later on, a similar methodology was used to show that the population of the putative stem cells within the seminiferous tubules is not homogeneous and that their differentiation is not linear and strictly irreversible, as the cellular bridges between aligned Type A spermatogonia (A_{al}) are disrupted to form smaller groups of cells with distinct gene expression profile (Nakagawa et al., 2010). The observation of such dynamic pathway would be extremely limited by using standard immunohistochemical and histological methodology. On the other hand, intravital microscopy can also be used to address, *in vivo*, the entire ovulatory process, making possible the comparison of the available biochemical and physiological data with the sequential morphological events that reflect follicle maturation and oocyte release (Dahm-Kahler et al., 2006; Petersen et al., 2008).

Moreover, several other reproductive processes can be addressed by using intravital microscopy. The study of the capacitation of spermatozoa that takes place within the female reproductive system by using this technique allowed the quantitative analysis of the trajectory of individualized spermatozoa as well as alterations in the motility pattern of these cells during their transit in the oviduct (Druart et al., 2009). This assay was also important for the comparison between different protocols of sperm storage and evidenced that most of the techniques routinely used to estimate the quality of stored spermatozoa destined to assisted reproduction are not completely satisfactory, as they are based in *in vitro* assays and do not consider the interactions of the male gametes with the female reproductive tract (Druart et al., 2009). In addition, the possibility to visualize the intact blood-perfused vasculature of reproductive organs raised the perspective of understanding the placental exchange of substances between maternal circulation and the fetus at a much higher level of topographical and functional precision and detail (Solder et al., 2009; Burke et al., 2010).

Intravital microscopy is a useful tool not only to study physiological mechanisms. In fact, it is a powerful approach to investigate alterations of the reproductive organs under pathological conditions as well as the development of new therapies. In line with this interpretation is, for example, the assessment of testicular alterations (Nagler et al., 1987; Bajory et al., 2011; Turner, 2011) regulation of blood pressure in pregnancy (Burke et al., 2010) and the investigation of prostate cancer development/progression and new therapies (Frost et al., 2005; Abedinpour et al., 2011).

Usually, the intravital microscopy methodology requires a small surgery to expose the organ to allow the assessment of the microscope objectives to the tissue. However, this procedure may be limited by the size of the animals, being used in small species such as mice and rats. In order to perform intravital microscopy in large species or in organs that cannot be easily exposed, an interesting alternative is the use of fiber confocal fluorescence microscopy, where the microscope objective is replaced by a flexible fiber-optic miniprobe. This miniprope is introduced into the organ's lumen and is capable to excite fluorochromes bound to specific targets (Laemmel et al., 2004; Thiberville et al., 2007; Druart et al., 2009). Another alternative for organs with difficult access is the usage of dorsal skinfold chamber, in which two symmetrical titanium frames are implanted in a dorsal skinfold and permits the growth of tissues within the chamber (Frost et al., 2005; Abedinpour et al., 2011) and to follow biological processes. Because these methodologies for time-lapsing imaging allows analysis in 4 dimensions (X-Y-Z axis during a time period), another important requirement is the development of new analytical methods that reliably describe the data obtained.

The current knowledge of physiological and pathological processes both in male and female reproductive systems was significantly impacted by the development of intravital microscopy. In the future, this technique can be used to provide functional information

about several other processes involved in fertility, such as sperm maturation within the male excurrent ducts and the participation of secretions from the accessory glands in reproduction, as well as the interactions between sperm and oocyte during fertilization. In addition, intravital microscopy can be an interesting tool to investigate the morphological and functional effects of hormones, drugs and environmental toxicants in reproductive organs.

Hemostatic system

The hemostatic system comprises platelet aggregation, coagulation and fibrinolysis also termed primary, secondary and tertiary hemostasis (Stassen et al., 2004). Following activation, these components lead to the formation of a clot or thrombus, a very well described physiological process that is responsible for limiting the blood loss from sites of injury. However, this process is also responsible for several diseases, such as deep vein thrombosis, myocardial infarction, hemophilia and stroke (Borissoff et al., 2011). Given the complexity of the hemostatic process and its consequences, the study of hemostasis biology in living systems by intravital microscopy is interesting, as it allows the visualization of the thrombotic obstruction as it happens (Kurz et al., 1990). This has assisted not only the elucidation of hemostatic pathological mechanisms, but also reveals possible sites for therapeutic interventions in the prevention and treatment of hemostatic disorders.

Numerous studies have described the formation of microvascular thrombi. To study hemostatic dysfunctions in the brain, for example, some of the methods most commonly used involve the observation of a $FeCl_3$-induced clot (Kurz et al., 1990) by intravital microscopy through a cranial window prepared over the parietal cortex of mice (Mostany and Portera-Cailliau, 2008). Using this technique, it was recently observed that beta-amyloid plaques, well-known to be associated with Alzheinmer's disease, are directly related to thrombosis and fibrinolysis in the brain vessels, and this relationship has been found to affect cognition in animal models (Cortes-Canteli et al., 2010). Although invasive models are commonly used to obtain intravital images of the brain's blood flow, a noninvasive stroke model using a $FeCl_3$-induced clot was recently described (Karatas et al., 2011). Another noninvasive model of thrombus formation observable by intravital microscopy is the photochemical thrombus induction in the ear microvessels of hairless mice, caused by local continuous mercury light exposure followed by injection of fluorescein isothiocyanate–dextran (FITC-dextran) (Roesken et al., 1997). This model allowed for distinct *in vivo* analysis of arteriolar and venular thrombus formation and elimination, representing an interesting tool for the study of novel antithrombotic and thrombolytic strategies.

Moreover, a frostbite injury model was recently developed to study reperfusion and angiogenesis after photochemical injury in the ear skin of hairless mice (Goertz et al., 2011). The evaluation of the thrombus formation process was recently reported by Koike et al. (2011). The authors used two-photon laser-scanning microscopy in the microvessels of the arterial smooth muscle and the intimal layer of GFP-expressing mice to assess the thrombolytic effect of anticoagulant drugs.

Knee joint

The synovial tissue is a thin vascular connective tissue, surrounding the articular capsule and the joint cavity (Veihelmann et al., 1998). The synovial tissue presents a high-density honeycomb-like capillary network, containing some post capillary venules and a few arterioles. This vascular network is similar to the one present in the subcutaneous fat tissue (Veihelmann et al., 1998). Diseases affecting joints and its structures, such as rheumatoid arthritis and gout, can severely affect articular functionality leading to pain and incapacitation (Amaral et al., 2011). The intravital microscopy technique can be performed in the joint cavity to assess the microcirculation of the synovial tissue in the knee joint. Considering the worldwide importance of rheumatoid arthritis and gout (Coelho et al., 2008), the elucidation of the inflammatory mechanisms behind articular injury, by using intravital microscopy, are of major importance in the development of new and more effective treatments.

To start the intravital assay in mouse synovial tissue, the hind limb is immobilized with the knee slightly flexed (Figure 2). After an incision distal to the patellar tendon, a partial skin resection is carried out and the patellar tendon is mobilized and partly resected. Then the intraarticular synovial tissue of the knee joint is visualized. After superfusion with sterile saline, a cover glass is placed on the knee capsule and the intravital microscope is directed onto the synovium (Gregory et al., 2004; Zysk et al., 2004; Pinho et al., 2011).

Interestingly, the first evidence that platelets accumulate in arthritic vessels, indicating platelet activation due to antigen induced arthritis, was provided by intravital microscopy (Schmitt-Sody et al., 2005). Furthermore, this technique has already been useful to elucidate several inflammatory responses in arthritis models, such as the involvement of the CXC chemokine receptors in neutrophil recruitment and platelet P-selectin mediating leukocyte-endothelial interactions (Veihelmann et al., 2001; Schmitt-Sody et al., 2007; Coelho et al., 2008).

Tumors

Tumor development at the primary site is cause of the onset and progression of neoplastic disease, whereas the metastatic colonization of secondary tissues is clearly the most lethal aspect of clinical disease as it is responsible

for more than 90% of cancer-associated deaths (Weiss, 1990; Hanahan and Weinberg, 2000; Mehlen and Puisieux, 2006). Metastasis is a multi-step process that includes the detachment of malignant cells from the primary tumor, their entry into the blood circulation, interaction with platelets and leukocytes, adhesion to the endothelium and finally the growth of disseminated tumor cells within the blood vessel (Al-Mehdi et al., 2000) or in the surrounding tissue after their extravasation (Monzavi-Karbassi et al., 2007).

The use of multi-photon intravital microscopy enables the observation of angiogenesis in the primary tumor, the migration of tumor cells across the endothelial barrier into blood vessels and its interaction with the immune cells and extracellular matrix (Wyckoff et al., 2000, 2007). All the performed studies so far were done in rodents where minimal surgery, namely the generation of a skin flap, is used to expose the tumor. However, following tumor development over a longer period of time with repeated imaging sessions raises additional challenges (Lohela and Werb, 2010). In addition, it is observed a great effort on performing long term imaging of tumors in the same animal in order to provide valuable information about the angiogenesis and the invasive process of slowly migrating tumors (Alexander et al., 2008; Fukumura and Jain, 2008).

In order to perform intravital microscopy to investigate tumors, an elegant approach is the usage of implanted imaging windows. This methodology allows access to the tumor without the need for repeated surgery. To illustrate this scenario, two successful possibilities are the dorsal skin chamber and the optical window installed in the mammary fat pad (Alexander et al., 2008; Kedrin et al., 2008; Gligorijevic et al., 2009). These techniques have been combined with the use of novel fluorescent proteins expressed by the tumor cells that can be either photoactivated or photo-switched (Patterson and Lippincott-Schwartz, 2002; Lippincott-Schwartz and Patterson, 2008; Gligorijevic et al., 2009) and have provided novel information about the modality of migration of invasive cells *in vivo* and their relationship with the local microenvironment, particularly the vasculature and the lymphatic system (Weigert et al., 2010).

Conclusion

Imaging has gained a crescent significance in the last years, and the importance of combining and confirming data with imaging techniques is patent. In this review, we aimed to describe briefly some ways of performing intravital microscopy of several organs and tissues, illustrating advantages and limitations. The increasing availability of fluorescent-protein expressing organisms (mice, insects, and parasites) and new labeling alternatives (such as quantum dots) will certainly facilitate *in vivo* imaging and tracking. Finally, despite of laborious efforts to perform intravital microscopy studies, imaging always bring the opportunity to visualize more than previously expected, opening windows for very relevant discoveries.

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