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Intravital Microscopy in Mammalian Multicellular Organisms

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Abstract

Imaging subcellular processes in live animals is no longer a dream. The development of Intravital Subcellular Microscopy (ISMic) combined with the astounding repertoire of available mouse models makes it possible to investigate processes such as membrane trafficking in mammalian living tissues under native conditions. This has provided the unique opportunity to answer questions that cannot be otherwise addressed in reductionist model systems and to link cell biology to tissue pathophysiology.

Introduction

Membrane traffic is a fundamental process in eukaryotic cells that ensures the exchange of proteins, lipids and other molecules between the cell and the extracellular space, and among intracellular organelles. At the core of these networks of trafficking routes are membranous containers of different sizes and shapes (e.g., vesicles, tubules, pleiomorphic structures), termed transport intermediates. Deciphering the molecular mechanisms regulating the dynamics of biogenesis, transport, and remodeling of transport intermediates is fundamental to understanding cell, tissue, and organ pathophysiology. For almost half a century, this has been the focus of cell biologists, who have approached this issue using a broad variety of techniques and experimental systems. The pioneering work carried out in the sixties by electron microscopy (EM), which described the ultrastructure of the intracellular compartments in tissues [1], has been followed by the use of biochemistry and time-lapse imaging in reductionist model systems (i.e., cell-free systems and cell cultures) [2–4]. These approaches lead to the discovery of an astonishing number of novel molecules and the identification of their roles inside the individual “model cell.” As our toolbox has been expanding to enable a more detailed structural analysis of molecular complexes and their dynamics, it has become evident that we have to redirect our efforts towards understanding how membrane trafficking operates at the tissue level and how it is linked to organ pathophysiology. This requires adding to the equation the contribution of factors that are unique to the native tissue environment of multicellular organisms, including three-dimensional spatial organization, the combination of signaling molecules coming from the

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vasculature, central nervous systems, extracellular matrix, and diversity of cell populations. This task has been greatly facilitated by the extensive use of smaller organisms such as Drosophila, C. Elegans, and Zebrafish, which can be easily and rapidly genetically manipulated and are amenable to high-resolution imaging [5–8]. However, in the last decade, the development of ISMic in mice and rats has permitted visualization of the dynamics of several biological processes as well as the dissection of their molecular machinery under in vivo conditions that are more relevant to human disease [9,10]. The focus of this mini-review is to illustrate the most recent advances in imaging membrane traffic in mammalian model systems in vivo. This approach has been utilized, so far, to investigate primarily two trafficking steps, namely endocytosis and regulated exocytosis. We hope to convince the reader that this technology is mature enough to be successfully extended to other trafficking processes and in general to other areas of cell biology.

Endocytosis during physiological and pathological states

Endocytosis is a crucial cellular process that enables the cell to uptake nutrients, control cell signaling, and polarity, and to remodel the plasma membrane, thus regulating several activities such as cell motility, metabolism, signaling, neurotransmission, and immune response [25–28]. De-regulation of endocytic pathways has been implicated in a variety of diseases including cancer, immunodeficiencies, neurodegeneration, and pathogen infection [29]. Although endocytosis has been investigated in multicellular organisms in several model systems, most studies were carried out by either using biochemical approaches or static imaging methods (e.g., EM or indirect immunofluorescence, IF), which do not provide information on the complex dynamics of this process. One of the major driving forces behind using in vivo imaging to investigate endocytosis has been the understanding of its contribution to physiological and pathological events at the organ level. Indeed, the first insight into the dynamics of the endosomal system *in vivo* came from the Molitoris group which systemically injected fluorescent dextrans, folate receptors, or albumin in live mice and rats and imaged their uptake in the kidneys proximal tubuli [30,31]. This work lead to a paradigm shift in renal physiology, with the discovery that proteins in the kidney are recaptured through transcytosis by the tubular epithelium and not just filtered out by the glomeruli. The importance of this discovery, which fueled several controversies [32,33], is to have sprung research efforts based on intravital microscopy, thus leading to a better understanding of the renal endocytic pathways during pathological conditions. Recently, for example, it has been shown that 1) megalin-dependent endocytic pathways are linked to autophagy-mediated tubule glomerular injury and podocyte degradation in high-fat diet mouse model of obesity and diabetes [34], 2) angiotensin II-mediated transcytosis of plasma albumin is involved in the impairment of podocyte function [35], and 3) that albumin uptake in podocytes in regulated by caveolin 1-dependent endocytosis via the scaffolding protein Shank2 [36]. These findings represent three examples of processes that cannot adequately be reconstituted in reductionist model systems and provide a direct link between membrane trafficking and disease state.

Another area that has stimulated advances in the use of ISMic to investigate endocytosis in vivo is cancer biology. Internalization and recycling of key receptors such as EGFR and β1-integrin from and to the plasma membrane have been implicated in various aspects of tumor

progression. For example, in a mouse tongue orthotopic model for head and neck squamous cell carcinoma (HNSCC), β1-integrin has been linked to invasion and metastasis through the activity of Rab25, a small GTPase which is implicated in endosomal recycling and is downregulated in head and neck cancer patients [37]. Intravital microscopy has been instrumental in unraveling that downregulation of Rab25 triggers the formation of membranous invasive protrusions at the edge of the primary tumors, which are enriched in F-actin. Another example is the EGF/EGFR signaling axis which has been clearly shown to play a fundamental role during cancer both in vivo and in patients [38,39]. Extensive work in cell culture has suggested that EGFR internalization and trafficking control cell proliferation, motility, and invasion in tumor cells. However, these aspects have been scarcely investigated in vivo, thus leaving open important questions, such as whether and how EGFR is internalized in primary tumors. This last aspect is particularly crucial since EGFR can be internalized via clathrin or non-clathrin pathways depending on the local concentration of the ligand [40], which in primary tumors is not known. One of the models that have been exploited to address these questions is a flank xenograft based on HNSCC cells, which are known to overexpress EGFR. In this system, carbon nanotubes coupled to EGF and cis-platinum were shown, for the first time, to be specifically internalized by the tumor cells in a receptor-mediated fashion [41]. More recently, the Sorkin lab developed primary tumors using genome-edited HNSCC, which express GFP-tagged EGFR. They showed that in vivo EGFR is internalized in a clathrin-dependent manner, similar to cultured cells exposed to levels of EGF in the pM range, and determined that a small pool of activated EGFR is sufficient to drive tumorigenesis by signaling through the Ras-MAPK pathway [42]. The importance of this study is two-fold. It: 1) enabled, for the first time, the rigorous in vivo investigation of quantitative aspects of internalization, trafficking and signaling of the EGFR without the confounding effect of receptor overexpression; 2) provided information on the modality of internalization that could be invaluable for therapeutic purposes.

Another important process that intravital microscopy has allowed the investigation of for the first time is the endocytosis-mediated remodeling and intracellular degradation of components of the extracellular matrix (ECM), which are fundamental for a variety of cellular events. A breakthrough has been the identification of different subpopulations of resident macrophages in the dermis, such as the M2-like type, specialized in the uptake of collagen [43], and the CCR2-expressing type, specialized in extravascular fibrin uptake [44]. These studies revealed novel receptor-mediated pathways that operate under basal conditions to maintain ECM homeostasis, and are significantly enhanced in pathological states. Indeed, it was recently shown that tumors of various origin elicit robust endocytic collagen degradation by tumor-associated macrophage-like cells that degrade collagen in a mannose receptor-dependent, as demonstrated by the increase in intra-tumoral collagen in mannose-receptor-deficient mice [45].

To date, the most successful model organ to study endocytosis in vivo are the salivary glands, which are ideal for performing intravital microscopy for a variety of reasons. First, this organ is located in the neck area, which is less affected by motion artifacts than other organs within the body cavity, thus ensuring the stability required to perform continuous imaging at a high spatial and temporal resolution for several minutes. Second, taking

advantage of the access provided by the salivary ducts that open up into the oral cavity, the salivary glands can be selectively manipulated, both pharmacologically and genetically. Fine cannulae inserted in the ducts enable the delivery of drugs, genes, and fluorescent probes, thus permitting studies under a broad repertoire of experimental conditions. The heterogeneity of these organs, that are formed by polarized epithelium and stromal cells, has also allowed the visualization of various aspects of endocytosis. This pioneering work has allowed for the first time the progression of internalized cargoes to be followed throughout the endo-lysosomal system in the stromal cells surrounding the salivary acini in vivo [46]. Differences in the trafficking of molecules between in vitro and in vivo model systems have also highlighted that the tissue environment significantly affects the modality of internalization [47]. Further, this model has also provided evidence that endocytosis in polarized epithelia under basal conditions proceeds at much slower rates than shown in cultured models. Small molecular weight dextrans or plasmid DNA are internalized with very slow kinetics from the apical plasma membrane under basal conditions [48,49], and are minimally taken up from the basolateral pole. On the other hand, apical plasma membrane uptake is enhanced during stimulation of regulated exocytosis, to recapture the excess of membranes delivered during the secretion of large secretory vesicles [50].

The development of several conditional mouse models has recently added an essential set of tools to further investigate the molecular machinery regulating endocytic processes *in vivo*. Recently, the role of the small GTPase Cdc42 in controlling the maintenance and the establishment of the apical plasma membrane in tubular organs was investigated by ablating this protein either in adult mice or at the embryonic stage using Cre-Lox technology [51]. Although these processes have been extensively studied, ISMic has uncovered a novel role for this small GTPase that was not appreciated before in reductionist model systems and animal models [52]. Specifically, Cdc42, but not RhoA and Rac1, was discovered to negatively control a still unidentified endocytic pathway which when upregulated induces an imbalance in the membrane flow from and to the apical plasma membrane. This imbalance resulted in defects in membrane remodeling that affected maintenance and establishment of plasma membrane polarity. This study represents an example of the ability to study in vivo the integration of membrane traffic signaling, actin cytoskeleton, and membrane remodeling during tissues homeostasis and development.

Regulated Exocytosis

A fundamental trafficking process whose essential molecular and mechanistic underpinnings have been unraveled through interrogation by ISMic is regulated exocytosis. During this process, which occurs in specialized secretory organs, molecules destined for secretion are packaged within secretory vesicles that constitutively bud from the Trans-Golgi Network, reach the plasma membrane, and fuse with it upon the appropriate stimulation [53,54]. Exocrine organs have been an ideal model to visualize and study the dynamics of this process in live animals due to: 1) the large size of the secretory vesicles (1–2 μm), 2) the relatively slow duration of the exocytic process (around 60 sec), and 3) the exact localization of the exocytic events that are restricted to the apical plasma membrane.

While *ex vivo* and *in vitro* approaches have provided essential information on regulated exocytosis, it is important to note that this process cannot be wholly and faithfully recapitulated in *in vitro* or *ex vivo* systems. For example, *ex vivo* studies using acinar preparations from the salivary gland proposed that either β-adrenergic or muscarinic receptors can trigger the release of secretory proteins from the granule. Intravital microscopy of the salivary gland, however, confirmed that in vivo only β-adrenergic stimulation elicits exocytosis of secretory granules, thus indicating a significant alteration of the signaling circuits due to the explant and culture procedures [55]. Similarly, secretory granules in explanted salivary gland slices and acinar preparations from other exocrine tissues were previously shown to occur with at least three distinct modalities: 1) complete fusion 2) kiss-and-run, and 3) compound exocytosis [56,57]. ISMic revealed that under true physiological conditions, neither kiss-and-run nor compound exocytosis were observed [55,56].

Most recent insights involve deciphering the biomechanics, bioenergetics, and molecular players involved in the integration of the granule membrane into the apical plasma membrane as exocytosis is completed. Within the salivary gland, the large secretory granules (~1–1.5 um) integrate into the apical plasma membrane, which envelops a network of canaliculi whose diameter is almost an order of magnitude smaller (~ 300 nm) [58,59]. The complete integration of the granule membrane into the canalicular apical plasma membrane is thus highly energetically unfavorable, raising the fundamental question of how this energy barrier is overcome [59].

The development of mice expressing fluorescent reporters for F-actin (i.e., Lifeact [60]) and the isoforms of the actin-based motors myosin II (NMII,[61]), have made it possible to decipher the machinery regulating this process. It was shown that after the fusion of the secretory granules with the plasma membrane, F-actin and later NMIIA and NMIIB are recruited on the granule membranes to form an actomyosin complex which drives their integration to completion [23,55] (Fig. 2A). Further, the use of mice floxed for NMIIA and/or NMIIB showed that these two isoforms play two distinct roles, consistent with their different biophysical characteristics. With NMIIA being more suitable to facilitate rapid contractile events, and NMIIB more fit to generate sustained contraction, it was discovered that 1) NMIIB is required to stabilize the actin filaments around the granules, thus creating a contractile scaffold to prevent membrane expansion; and 2) NMIIA is necessary to generate fast contractions to drive the integration of the granules into the plasma membrane. The same study unraveled a novel mechanism for the recruitment of the actomyosin complex in which both NMIIA and NMIIB are recruited on the granule membrane in an F-actin independent fashion, thus challenging a long-standing paradigm in the actomyosin field. Also, novel pieces of evidence have been presented about the existence of multiple populations of actin filaments on the secretory granules characterized by the presence of different members of the tropomyosin family of actin-binding proteins [62,63]. These findings support recent reports in live Drosophila salivary glands which described the recruitment of the secretory granules of two distinct sets of actin nucleators thus highlighting the exquisite complexity of this mechanism [8,64].

As for the bioenergetics of this process, a very recent report linked the increase in energy demand that is required to complete exocytosis to mitochondrial dynamics, structure, and

function [65]. It was discovered that in acinar cells in vivo, distinct from cell culture, mitochondria are organized in two populations: one, static and localized to the basolateral plasma membrane and the other dynamic and dispersed in the cytosol. Remarkably, by using a combination of pharmacological approaches and mice harboring a mitochondria-targeted photo-convertible probe, it was shown that stimulation of exocytosis induced the increased in microtubule-dependent motility and fusion of the central mitochondria. This study reveals a novel mechanism linking mitochondrial response to an increase in energy demand to support membrane trafficking in vivo.

A final example of the power of ISMic is the discovery and characterization of a different modality of lipid droplet (LD) exocytosis in mouse mammary glands during milk secretion. Several aspects of this process have remained uncharacterized for the longest time, due to the inability to reconstitute this process in vitro. For the first time, it was shown that after their biosynthesis, LDs are transported from the basolateral to the apical membrane undergoing, first several rounds of fusion, and later a final expansion at apical nucleation centers [24] (Fig. 2B). Later, LDs are extruded into the luminal space where they are attached to the cell membrane until oxytocin-mediated contraction of the myoepithelium surrounding the acinar cells triggers their release. This approach is destined to have broad application for investigating trafficking events within the mammary epithelium in real time.

Overall, these latest breakthroughs have begun to unravel integrated networks of processes including signaling, cytoskeletal dynamics, membrane remodeling, and bioenergetics that are temporally and spatially coordinated throughout the tissue. New and exciting questions arise, and ISMic provides a very powerful tool to address them.

Conclusions and future perspectives

It is clear that intravital subcellular microscopy in live rodents has finally transitioned from an exotic technique capable of providing spectacular movies of biological process in live animals to a mature approach that relies on rigorous quantitative analysis of the data and enables unraveling novel and complex molecular mechanisms in tissues in vivo. However, this approach is far from being wide-spread, and it is still somewhat lagging behind. The reason is primarily that ISMic is viewed as a technique that requires extensive resources and it is technically very challenging. We argue that this is no longer the case. Indeed, whereas initially intravital microscopy was performed on expensive multi-photon microscopes, it is now clear that data can be acquired with more conventional platforms including confocal [18,55], spinning disk [66], or even epifluorescence microscopes [67]. A major limitation of these alternative approaches is the imaging depth, that is restricted to 50–100 μm from the surface of the imaged organ. However, if the process of interest can be visualized within this range, these techniques provide a superior spatial and temporal resolution, and, in addition, make the use of fluorescent recovery after photobleaching (FRAP), Foster resonance energy transfer (FRET), photoactivation and photoconversion of various probes more accesible. Another perceived limitation is the availability of mice models suited for live imaging. The revolution of genetic engineering through CRISPR/Cas9 technology has significantly boosted the development of new models that can now be generated in a substantially shorter time and at much lower costs. As for the technical challenges posed by this approach, it is

important to realize that in the last ten years a substantial plethora of methods aimed at performing surgical procedures targeted to intravital microscopy has been developed for virtually every organ, published, and made available as videos, therefore greatly facilitating their implementation.

Finally, we want to emphasize that although we advocate for more extensive use of intravital microscopy, we firmly believe that biological processes need to be studied using a complementary approach based on all the tools and experimental models available. ISMic provides a formidable tool to verify under true physiological conditions hypotheses and mechanisms generated in in vitro models systems and at the same time to discover new biology that can be reconstituted in reductionist model systems to gain molecular insights.

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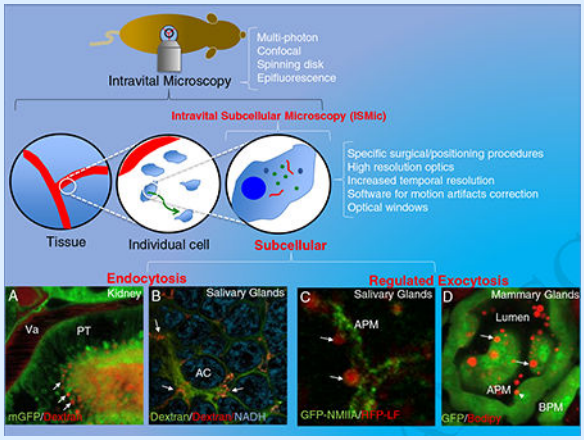
Box fig. 1



Box. 1 -

Intravital Subcellular Microscopy (ISMic).

Intravital microscopy encompasses a variety of light microscopy techniques which include multi-photon, confocal, spinning disc, and epifluorescence microscopy that are used to image biological processes in live animals. Since its introduction in the early 1800s this technique has been used primarily to image tissue behavior and with the development of better optics it has been instrumental to track individual cells, with significant applications in cancer biology, immunology, neuroscience, and stem cell research [11–14]. In the last decade intravital microscopy has been further improved to image subcellular structures in the sub-micron range (Intravital Subcellular Microscopy or ISMic). This has been accomplished by a combination of different approaches which include the development of 1) specific surgical procedures tailored to minimize the motion artifacts due to the heartbeat and respiration while maintaining the target organ under physiological conditions; 2) microscopes capable of a higher temporal resolution, 3) high-magnification optics, 4) installation of optical windows [9,15–19], and 5) software that facilitate the correction of motion artifacts. Motion artifacts have been corrected either by software that couple image acquisition with the heartbeat and respiration, or post-processing. Drifts in the XY plane have been easily corrected with commercially available programs such as Image J, whereas Z-shifts or distortion within individual frames have been corrected through specialized software [20,21]. The lower panels show few examples of ISMic application to investigate membrane trafficking. A. Endocytosis of dextran in the kidney of a mouse expressing a membrane targeted peptide (mGFP) and injected with with Texas-Red Dextran which from the vasculature (Va) is uptaken by the epithelium of the Proximal Tubuli (PT) in vesicles (arrows) [9]. B. A low (red) and a high (green) MW dextrans injected systemically in a mouse are internalized by the stromal cells (arrows) suroounding the acini (AC) of the salivary glands. Mitochondria are revealed by highlighting NADH through 2-photon microscopy [22]. C. Secretory granules (Arrows) fused with the apical plasma membrane (APM) in acinar cells of the salivary glands of a transgenic mouse expressing the F-actin sensor LifeAct and GFP-NMIIA during regulated exocytosis [23]. D. Lipid droplets labeled with Bodipy (RED) in the acini of a lactating moouse expressing GFP. Droplets localized at the APM (arrowhead) or in the lumen (arrows) after secretion. Basolateral plasma membrane (BPM) [24]