Imaging the Dynamics of Endocytosis in Live Mammalian Tissues

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In mammalian cells, endocytosis plays a pivotal role in regulating several basic cellular functions. Up to now, the dynamics and the organization of the endocytic pathways have been primarily investigated in reductionist model systems such as cell and organ cultures. Although these experimental models have been fully successful in unraveling the endocytic machinery at a molecular level, our understanding of the regulation and the role of endocytosis in vivo has been limited. Recently, advancements in intravital microscopy have made it possible to extend imaging in live animals to subcellular structures, thus revealing new aspects of the molecular machineries regulating membrane trafficking that were not previously appreciated in vitro. Here, we focus on the use of intravital microscopy to study endocytosis in vivo, and discuss how this approach will allow addressing two fundamental questions: (1) how endocytic processes are organized in mammalian tissues, and (2) how they contribute to organ physiopathology.

Endocytosis is a fundamental process used by the cell to internalize molecules from the plasma membrane (Mellman 1996; Doherty and McMahon 2009), and its dysregulation is the cause of several pathological conditions, such as cancer and neurodegenerative, metabolic, and storage diseases (Lanzetti and Di Fiore 2008; Mosesson et al. 2008; Ballabio and Gieselmann 2009).

In mammals, endocytosis has been primarily studied in cell culture, which has been instrumental in identifying various endocytic pathways and elucidating the trafficking of internalized molecules throughout the endolysosomal system (Conner and Schmid 2003; Maxfield and McGraw 2004; Donaldson et al. 2009;

Hurley and Stenmark 2011). The degree of complexity in the organization and the regulation of the endocytic processes have been shown to substantially increase in polarized cells (Mostov et al. 2003; Folsch et al. 2009) and in organ cultures (Dunn et al. 1980; Kandimalla et al. 2009; Khandelwal et al. 2010), which recapitulate some of the architectural features of the intact tissue. The scenario is further complicated in live animals, where tissues are continuously exposed to a specific combination of cues coming from the vasculature, the central nervous system, and the extracellular environment, which are difficult to reconstitute accurately in vitro. Therefore, although our knowledge of the molecular machineries controlling mammalian

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endocytosis has substantially increased in the last decades, there are still fundamental issues that have not been explored yet, such as how endocytic pathways are organized and regulated in mammalian tissues. Specifically, it is fundamental to establish whether *in vivo* cells show the same regulation of endocytic pathways that has been reported *in vitro*, or how molecules are internalized and trafficked in the presence of physiological levels of ligands and regulatory molecules. Another question is what is the contribution of the endocytic pathways to the pathophysiology of a specific tissue or organ. For example, it is of paramount importance to determine whether and how endocytic pathways are altered in epithelial and stromal cells during tumor development and progression, and which specific cell function is affected by their dysregulation.

Investigations of endocytosis in live mammals (i.e., rodents) were extensively performed during the 1980s and 1990s by using conventional techniques (e.g., biochemical assays, EM, and indirect immunofluorescence). However, the advent of the green fluorescent protein (GFP) technology, which has enabled imaging subcellular organelles in real time, has significantly shifted the focus toward cell cultures.

The recent advancements in intravital microscopy (IVM), which encompasses a series of light microscopy-based techniques, have now made possible imaging biological processes in live animals at a subcellular resolution (Weigert et al. 2013). In this perspective, we focus on reviewing most of the recent data on IVM and endocytosis and try to convey to the reader a sense of the potential, challenges, and limitations of this approach. However, before discussing the “heart of the matter,” we start by briefly pointing out the advantages of using animal models versus the more popular and well-established *in vitro* model systems.

ENDOCYTOSIS IN LIVE ANIMAL MODELS: WHY TAKE THIS DIFFICULT ROUTE?

So far, endocytosis has been primarily investigated in cell and organ cultures. Cell cultures, in

particular, are amenable to several manipulations and have provided fundamental information on the regulation of the endocytic pathways at a molecular level. Moreover, they can be imaged in real time by using state-of-the-art techniques, such as total internal reflection (TIRF) microscopy and laser spinning disk microscopy, which have revealed the dynamics of the early internalization steps at the level of individual molecules (Cocucci et al. 2012). However, cells in culture are typically grown either on a solid surface (e.g., glass or plastic) or in a few cases in suspension, and both conditions do not fully recapitulate the complex architecture of the native tissue *in situ* (Fig. 1A). Notably, some of the mechanisms regulating the internalization steps have been shown to be dependent on the culture conditions. For example, the role of the actin cytoskeleton in receptor-mediated endocytosis has been found to be dependent on whether cells are grown adherent to a substrate or in suspension (Fujimoto et al. 2000), whereas the lifetime of the clathrin-coated pits has been shown to be strongly affected by membrane tension that ultimately depends on whether the plasma membrane is engaged in interactions with adjacent cells, as in the case of polarized epithelium (Gottlieb et al. 1993; Boulant et al. 2011). These limitations have been partially overcome by the use of *ex vivo* model systems such as perfused liver (Dunn et al. 1980; Wall and Hubbard 1985) and kidney (Birn et al. 1997), explanted bladder (Khandelwal et al. 2010), brain slices (Kandimalla et al. 2009), or intestinal mucosa (Fig. 1B) (Hansen et al. 2009). However, although these systems maintain the proper tissue architecture, they lack several signals provided by the vasculature (i.e., oxygen, nutrients, and hormones), the nervous system (i.e., neurotransmitters), and the immune system (i.e., cytokines, chemokines) (Fig. 1C). Do these cues affect the physiology of the tissue of interest and the endocytic pathways? Various studies conducted in live rodents suggest that in some instances this is the case, as shown for the kidney and the liver. In the kidney epithelium, apical endocytosis is critical for the reabsorption and degradation of proteins that traverse the glomerular filtration

Figure 1. Increasing morphological and functional complexity from in vitro to in vivo models. (A) Diagram of cells in vitro, which grow adherent on glass or plastic surfaces and are exposed to the culture medium. (B) Diagram of the liver illustrating the complex 3D architecture of an organ. In the hepatocytes (light brown), the apical plasma membrane forms the bile canaliculi (green), and the basolateral membrane is in contact with the endothelium of the blood vessels (orange). Kupffer cells (burgundy) line the wall of the sinusoids. (C) Diagram illustrating the salivary glands with the acini (dark brown), the ducts (light brown), myoepithelial cells (light orange), and the stromal cells (blue). In situ, cells are constantly exposed to molecules coming from the circulation (e.g., oxygen, glucose, hormones), the central nervous system (e.g., neurotransmitters), and the cells in the stroma, which may produce various structural or signaling molecules (extracellular matrix, cytokines).

barrier and is also involved in the recycling of functionally important apical plasma membrane transporters (Birn et al. 1993; Maranda et al. 2001). Caveolins have been implicated in this process based on data from isolated proximal tubule or proximal tubule cells in culture (Trivedi et al. 2004). However, it was recently shown that both caveolin 1 and 2 are not expressed by proximal tubuli in situ and that their expression is activated upon explant and in culture conditions (Zhuang et al. 2011). In the liver, the endocytic activity of Kupffer cells was analyzed in situ, in freshly explanted tissue, and in isolated cultured cells. Interestingly, the reduction in microvilli projections and cellular roundness that occurs upon the isolation procedures significantly reduced the internalization

of glycoproteins with a progressive decrement of both binding and uptake capacity from in situ, freshly isolated, and cultured Kupffer cells (Dini et al. 1998).

In some instances, the use of live animals as experimental models has been the only viable approach to study endocytosis in those tissues that cannot be reconstituted in vitro or ex vivo owing to their tight dependence on the activity of the vasculature and the lymphatic system. Two examples are the blood–brain barrier and the choroid plexus epithelium, which perform a neuroprotective function in the brain by allowing the transport of selected molecules. As for the blood–brain barrier, the transcytosis of receptors involved in iron metabolism (e.g., transferrin, p97 melanotransferrin) through the en-

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endothelial cells and their uptake by perivascular cells and neurons have been characterized in situ by using electron microscopy and radiolabeled ligands (Broadwell et al. 1996; Demeule et al. 2002). These studies have provided the ground for designing therapies targeting neurodegenerative diseases (Yu et al. 2011). Similarly, in the choroid plexus epithelium, molecules have been shown to be continuously removed from the cerebrospinal fluid and either transported to the lysosomes or to the basolateral surface (Van Deurs et al. 1981). A similar transcytotic pathway is used to clear the amyloid- β from the cerebral tissue, and a recent study has shown that this process is regulated by the megalin pathway and the insulin-like growth factor I, thus providing novel insights into Alzheimer's disease (Carro et al. 2005).

Other examples, summarized in Table 1, highlight the advantages of studying endocytosis in vivo and underscore that some of the

conclusions derived from in vitro model systems may have to be reevaluated. However, although the use of conventional approaches generated a plethora of groundbreaking information, their main limitation, so far, has been their inability to provide data on the dynamics of the endocytic processes in vivo.

INTRAVITAL MICROSCOPY: A POWERFUL APPROACH TO STUDY THE DYNAMICS OF ENDOCYTOSIS IN LIVE RODENTS

IVM encompasses a series of light microscopy-based techniques that have been successfully applied to image several biological processes in live animals including tissue dynamics, cell migration, immune response, and synaptic plasticity (Svoboda and Yasuda 2006; Amornphimoltham et al. 2011; Beerling et al. 2011; Ritsma et al. 2012). Recently, IVM has been successfully used to image the dynamic of subcellular struc-

Table 1. Endocytosis in live rodents by conventional approaches

Organ	Molecule	Technique	Reference
Brain			
Blood–brain barrier	Ferritin, transferrin	EM, BA	Broadwell et al. 1996; Demeule et al. 2002
Choroid plexus	β -Amyloid, ferritin	EM	Van Deurs et al. 1981; Carro et al. 2005; Yu et al. 2011
Endothelium			
Aorta	LDL	EM, BA	Vasile et al. 1983
Lung	Albumin		Schnitzer 1994
Salivary glands			
Ducts	Ferritin	EM	Webster et al. 1994; Matsuoka et al. 2000
Acini	HRP, CFTR, transferrin receptor	EM, IF	Oliver and Hand 1978
Liver			
Kupffer cells	Glycoprotein	EM, IF	Dini et al. 1998; Okaya et al. 2012
Hepatocytes	Poly-IgA, Rab5	EM, IF	Rahner et al. 2000; Zeigerer et al. 2012
Kidney			
Proximal tubuli	Caveolin, folate receptor	IF, EM	Birn et al. 1993; Maranda et al. 2001; Zhuang et al. 2011
Stomach			
Mesothelial cells	Plasmid DNA, Rac	IF	Fumoto et al. 2009
Pancreas			
Acini β -cells	Vasoactive intestinal peptide (VIP)	EM, BA	Anteunis et al. 1989
Teeth			
Alveolar bone	Epidermal growth factor (EGF)	EM	Martineau-Doize et al. 1988

EM, Electron microscopy; IF, indirect immunofluorescence; BA, biochemical assays.

tures in live rodents (Weigert et al. 2010, 2013; Pittet and Weissleder 2011; Masedunskas et al. 2012c), also providing quantitative data on the process of interest (Sandoval and Molitoris 2008; Masedunskas et al. 2011). This has been accomplished by the development of specific surgical techniques and organ holders that have enabled considerably reducing the motion artifacts due to the heartbeat and respiration, which are the major challenge in performing subcellular imaging in vivo (Masedunskas et al. 2012a). Subcellular IVM has been performed by using either confocal or two-photon microscopy. Confocal microscopy provides an excellent spatial resolution that enables resolving subcellular structures, as shown by its extensive use in cell cultures. However, because it is based on UV-visible light as the excitation source, which has a limited tissue penetration and may induce tissue photodamage, its application for IVM has been limited to imaging a few tens of micrometers below the surface of exposed organs (three or four cell layers) and for short-term observations (30 min to 1 h). On the other hand, two-photon microscopy, which is based on the use of IR light, ensures deeper tissue penetration and reduced phototoxicity, and therefore it is best suited for deep-tissue imaging and to perform long-term studies. Because the technical details regarding this technique are not the focus of this article, we refer interested readers to a more specialized literature (Zipfel et al. 2003; Masedunskas et al. 2012a).

Imaging endocytosis in live animals has been successfully attempted for the first time in the proximal tubuli in the rat kidney (Dunn et al. 2002). The researchers followed the internalization of systemically injected fluorescently labeled dextrans, as a marker for bulk endocytosis, and the antibiotic gentamycin, which is internalized via a megalin- and clathrin-dependent pathway (Dunn et al. 2002, 2003). This study opened the door to a series of investigations aimed at addressing the crucial role of endocytosis in the context of the physiopathology of the kidney. Subsequently, the same group discovered that the filtration of albumin from the urine is due to its extensive internalization from the apical plasma membrane of the prox-

imal tubuli and not to a barrier in the glomerular capillary wall (Russo et al. 2007b, 2009). Although this finding has been extensively debated in the field (Russo et al. 2007a; Peti-Peterdi 2009), it has spearheaded an extensive characterization of the endocytic activity in the kidney of live animals. This is shown by several studies investigating the apical uptake and transcytosis of folate (Sandoval et al. 2004), the internalization of albumin and transferrin (Ohno et al. 2005), and the uptake of siRNAs (Molitoris et al. 2009), which have provided invaluable information on the role of endocytosis in renal diseases.

A very powerful model to study endocytosis has been developed by our group using the salivary glands as a model organ. Salivary glands are ideal to perform IVM and to study endocytosis for various reasons. First, the glands are located in the neck area, which is less affected by motion artifacts than other organs located in the body cavity. This has enabled extending the time of observation of the endocytic processes from a few seconds up to several minutes (Masedunskas and Weigert 2008). Second, the salivary glands can be selectively manipulated both genetically and pharmacologically. Indeed, fine polyethylene cannulae can be inserted in the major excretory ducts and used to inject pharmacological agents (Masedunskas and Weigert 2008) or to perform transient transfections (Sramkova et al. 2009). Finally, because the salivary glands are a heterogeneous tissue, endocytic processes can be studied in different groups of cells at the same time. Indeed, we have characterized the uptake of systemically injected dextrans and their trafficking both in rats and in mice (Masedunskas and Weigert 2008). In vivo, these molecules are primarily internalized by stromal cells, which show a much higher endocytic activity than the adjacent epithelium (Fig. 2A) (Masedunskas and Weigert 2008). Dextrans, which are internalized very rapidly via small endocytic vesicles and in an actin-dependent manner, are immediately transported to the early endosomes, which undergo extensive homotypic fusion, as previously described both in vivo and in vitro (Bright et al. 2005; Zeigerer et al. 2012). Interestingly, transferrin

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Figure 2. Intravital microscopy to study endocytosis in live rodents. (A) Model to study endocytosis in the salivary glands of live rats. (Left panel) The submandibular salivary glands of anesthetized rats are exposed and imaged by intravital microscopy as previously described (Masedunskas et al. 2013). (Center panels, *) 500-kDa FITC and 70-kDa Texas-Red dextrans are injected systemically and immediately detected in the circulation. (Inset) Only the 70-kDa dextrans diffuse out from the vasculature and are internalized in a stromal cell. (Center panels, arrows) Within a few minutes, the dextran is observed in endosomal structures. (Right panel, arrows) After 30 min, dextran is primarily observed in stromal cells and not in the acini (Ac). Scale bars, 10 μ m. (B) Model to study apical endocytosis in the rat salivary glands. (Left panel) 70-kDa Texas Red dextran was administered through the salivary duct as previously described (Sramkova et al. 2009) in order to access the apical plasma membrane of the salivary epithelium. The side view (xz) of a Z-stack realized by intravital two-photon microscopy reveals that the dextran (red) is distributed in large ducts (center panel, arrowhead) and in the acinar canaliculi (center panel, arrows) that are visible below the surface of the glands (highlighted by the collagen, cyan). In the right panels, an xy view of an individual acinus shows that dextran is localized at the apical plasma membrane (*). Isoproterenol is injected subcutaneously to stimulate compensatory endocytosis, and small vesicles are formed from the canaliculi (right panels, arrows). Scale bars, 10 μ m. (C) Endocytosis in tumor cells. HN12 cells, a human oral squamous cell carcinoma, were implanted in the back of immunocompromised mice. (Left panel). After 3 wk, the tumor was exposed and imaged by IVM, as previously described (Bhirde et al. 2009). Hoechst (blue) and EGF conjugated to Q-dots and carbon nanotubes (red) were injected systemically. After a few minutes, the EGF complex was internalized in tumor cells and localized in small endocytic structures (right panels, arrows). Scale bar, 10 μ m.

endocytosis is less apparent than dextran uptake in vivo (Masedunskas et al. 2012b), whereas in cultured cells this scenario is reversed. This is not a peculiarity of the salivary glands because in stromal cells explanted and plated on glass coverslips, transferrin is robustly internalized within a few seconds, whereas dextran internalization, which occurs via small endocytic vesicles, is significantly slowed down. These data support the notion that the environment in a multicellular organism strongly affects the regulation of the endocytic pathways, and this should be the subject of further investigations. Along this line, another study highlighted the advantages of using IVM to investigate caveo-

lae-mediated endocytosis under physiological conditions. In vitro, the regulation of caveolae has been controversial, with some studies describing them as static structures that do not constitutively traffic cargo (Thomsen et al. 2002) and others showing different degrees of endocytosis depending on whether stimulated by viruses or by triggering specific signaling pathways (Pelkmans et al. 2004; Parton and del Pozo 2013). Recent data in the lung endothelium of live mice suggested that they are, indeed, very dynamic and regulate the transcytosis of molecules such as aminopeptidase under basal conditions (Oh et al. 2007). The salivary glands also offer the unique opportunity to study en-

docytosis in a polarized epithelium. Basolateral endocytosis can be followed either by delivering fluorescent probes from the vasculature or by expressing specific reporter molecules (Sramkova et al. 2009, 2012b), whereas apical endocytosis can be studied by retrodiffusing a fluorescent probe through the salivary duct (Fig. 2B) (Sramkova et al. 2012a). Interestingly, we found that the basal levels of apical endocytosis are quite low and differ considerably in the various components of the epithelium. This may reflect the fact that epithelial cells in tissues are engaged in cell–cell interactions and that the extensive cortical cytoskeletal network may limit the endocytic activities. Apical endocytosis can be selectively stimulated in large ducts or in the acini, by stimulating protein secretion (compensatory endocytosis) (Fig. 2B). Apical uptake has been shown to be clathrin independent and to be sensitive to amiloride but not to actin-disrupting agents. Interestingly, molecules such as dextran or plasmid DNA transiently localize in early endosomes after apical internalization, but subsequently they do not reach the degradative compartments or traffic through the retrograde pathway to the Golgi, thus suggesting a novel trafficking route that needs to be further characterized (Sramkova et al. 2012a).

IVM has been extensively used to study several aspects of the immune response, pathogen infection, and tumor progression. However, the role played by endocytosis in these processes has only recently begun to be investigated in vivo. Indeed, a study aimed at following the clearance of the immune complexes from the vasculature by neutrophils has unraveled the precise role of FcγRIIa and FcγRIII, which has been elusive because of the inability to fully reconstitute this process in in vitro model systems. Specifically, FcγRIIa mediates binding with the immune complexes, and FcγRIIIb mediates its clathrin-independent internalization (Chen et al. 2012). A similar study was performed in macrophages in the subcapsular sinus (Phan et al. 2009). IVM has also enabled imaging of the dynamics of pathogen infections at a single-cell level, as documented in a few systems such as (1) dermal dendritic cells, which internalize the protozoan parasite *Leishmania major* via long,

highly dynamic pseudopods and rapidly incorporate it into cytosolic vacuoles (Ng et al. 2008); (2) macrophages resident in the popliteal lymph nodes, which rapidly internalize the influenza virus (Gonzalez et al. 2010); (3) neutrophils in the kidney, which phagocytose uropathogenic *Escherichia coli* (Mansson et al. 2007); and (4) macrophages and trophoblastic fetal-derived cells in the mouse placenta that engulf plasmodium-infected erythrocytes (de Moraes et al. 2013). As for cancer cells, as of now, only a few studies have documented the internalization molecules in tumor models in live animals. For example, we reported that the uptake of carbon nanotubes conjugated with EGF and Q-dots is very selective for human squamous cell carcinomas overexpressing EGFR and implanted in the back of immunocompromised mice (Fig. 2C) (Bhirde et al. 2009). The uptake of antibodies directed against either EGFR and EpCAM in colon cancer tumors has revealed differences in endocytic activity between micrometastasis and vascularized primary tumors (Thurber and Weissleder 2011; Thurber et al. 2013). Moreover, phagocytosis of fluorescently labeled human fibrosarcomas by macrophages in live animals was imaged by using a dorsal skin chamber (Yamauchi et al. 2012). Finally, the role of endocytic recycling in tumor invasion and metastasis was recently highlighted in the context of head and neck cancer. Indeed, it was shown that the small GTPase Rab25, which regulates endosomal recycling, controls the ability of tumor cells to invade and metastasize to cervical lymph nodes in vivo. IVM has been instrumental in determining that the role of Rab25 in this process is linked to its ability to regulate the assembly of the actin cytoskeleton at the plasma membrane (Amornphimoltham et al. 2013).

These examples should convey the sense that IVM can be used to image endocytosis in a variety of tissues and that it can potentially address several questions on the endocytic processes in live animals. The application of IVM to this field is still at the very beginning and soon should become one of the main techniques to look at the dynamics of the endocytic pathways in vivo.

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CONCLUDING REMARKS AND FUTURE DIRECTIONS

IVM is clearly an invaluable tool to study subcellular structures in vivo and in real time. Its application to study endocytosis will enable answering several fundamental questions related to the molecular machinery regulating this process and its link to organ pathophysiology. Indeed, IVM overcomes the shortcomings of in vitro and ex vivo systems and allows us to investigate processes that cannot be otherwise reconstituted outside a living animal. In addition, IVM has evolved from an exotic approach used to qualitatively visualize a process in live animal to a technique that provides quantitative and molecular details on the process of interest (Sandoval and Molitoris 2008; Masedunskas et al. 2011). However, it is important to emphasize some of the limitations and possible areas of improvement for this approach. First, IVM is currently limited in terms of spatial and temporal resolution. For example, although trafficking throughout the endolysosomal system, macropinocytosis, and phagocytosis has been clearly visualized in vivo, very fast processes involving small structures, such as the biogenesis of clathrin-coated vesicles, cannot be captured by conventional IVM. Unfortunately, TIRF microscopy, which has been used for this purpose, cannot be implemented in live animals because even the most superficial cells are well beyond the evanescent field. Superresolution microscopy (e.g., PALM) is not suited for animal work, as well, because it requires the complete stability of the specimen and long acquisition times. However, this issue will probably be overcome by the development of confocal and two-photon microscopes equipped with fast resonant scanners (Kirkpatrick et al. 2012) or by using spinning disk microscopy, which has been recently introduced to in vivo imaging (Jenne et al. 2011).

So far, subcellular IVM has been applied to a few organs, such as the kidney and the salivary glands, and to a limited extent to skeletal muscle, vascular endothelium, the immune system, and a few tumor models. A main area for improvement will be the development of novel sur-

gical procedures and tools to reach every organ and to ensure their stabilization. In this respect, a few major breakthroughs have been recently made. For example, organs such as the heart and the lung have been recently imaged in situ with the use of devices coupling both heartbeat and respiration to the scanning device (Presson et al. 2011; Li et al. 2012). Microlenses with diameter of 350 μm have been developed and used for IVM microendoscopy, thus minimizing the extent of the surgical procedures (Llewellyn et al. 2008; Barretto et al. 2009). Microstages for the stabilization of organs in the body cavity have been developed (Cao et al. 2012), and chronic ports of observation such as mammary, cranial, and abdominal windows, or the dorsal skin chamber have been installed in mice, making it possible to perform long-term imaging experiments (Alexander et al. 2008; Kedrin et al. 2008; Ritsma et al. 2013). These advancements may also help to address one of the other current limitations of IVM, which is the inability to study the role of endocytosis during the developmental process in embryos, an area that has been primarily explored in smaller organisms, such as *Drosophila*, *Caenorhabditis elegans*, and zebrafish (Fares and Grant 2002; Fischer et al. 2006; Fleming and Rubinsztein 2011).

In terms of probes and molecular tools, a significant effort has to be devoted by the scientific community to expand the existing repertoire of transgenic models, such as knock-in mice for fluorescently tagged endocytic molecules and conditional knockout targeting the endocytic machinery. In this respect, the new technologies developed for gene editing, including zinc-finger endonucleases and Talen, will help in generating new mouse strains at much lower costs and at a faster pace (Le Provost et al. 2010; Sung et al. 2013). In addition, a valid approach that needs to be further expanded is the use of viral- and nonviral-based methods to target siRNA and shRNA selectively in vivo (Akinc and Battaglia 2013). Finally, a coordinated effort among chemists and cell biologists should be undertaken in order to develop novel fluorescent fluorophores to be conjugated with various biomolecules with the aim of improving deep tissue imaging and photostability while

ensuring minimal interference with the organism physiology.

Overall, IVM is an exceptional tool that is destined to provide major contributions to the field of endocytosis. We believe that IVM will provide new opportunities to further explore subjects such as the link between endocytosis and signaling, endocytosis and cell metabolism, and mechanisms of drug delivery at a cellular level. This will have a significant impact on our understanding of organ physiology and will help to develop better strategies to target diseases.

In conclusion, we foresee that IVM will soon become a fundamental tool to study not only endocytosis but also cell biology, and although in vivo animal imaging is expensive in terms of resources, the benefits will more than compensate for the investment.

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