22

Salivary Glands: A Powerful Experimental System to Study Cell Biology in Live Animals by Intravital Microscopy

Monika Sramkova, Natalie Porat-Shliom, Andrius Masedunkas, Timothy Wigand, Panomwat Amornphimoltham and Roberto Weigert
Intracellular Membrane Trafficking Unit, Oral and Pharyngeal Cancer Branch, National Institutes of Dental and Craniofacial Research, National Institutes of Health, Bethesda, USA

1. Introduction

Mammalian cell biology has been studied primarily by using *in vitro* models. Among them, cell cultures are the most extensively used since they make possible to study in great detail the molecular machineries regulating the biological process of interest. Indeed, cell cultures offer several advantages such as, being amenable to both pharmacological and genetic manipulations, reproducibility, and relatively low costs. However, their major limitation is that the architecture and physiology of cells *in vitro* may differ considerably from the *in vivo* environment. This reflects the fact that cells in a living organism i) have a three-dimensional architecture, ii) interact with other cell populations, iii) are surrounded by an extracellular matrix with a specific and unique composition, and iv) receive a number of cues from the vasculature and from the nervous system that are essential for maintaining their functions and differentiation state (Cukierman et al., 2001; Ghajar and Bissell, 2008; Xu et al., 2009). In the last two decades, cell biology has greatly benefited from major technological advances in light microscopy that have enabled imaging virtually any cellular process at different levels of resolution. The development of genetically-encoded fluorescently tagged proteins (Chalfie et al., 1994) has triggered the development of novel technologies such as FRAP, FLIM, FRET, BRET, photo-activation, photo-switching and photo-conversion (Diaspro, 2002; Lippincott-Schwartz, 2011a, b), and the realization of more sophisticated microscopes, which have significantly improved the limits of light microscopy in terms of both temporal (spinning disk, resonant scanners) and spatial (PALM, STORM, STED) resolution (Lippincott-Schwartz, 2011a, b). However, the application of these very powerful technologies has been primarily restricted to *in vitro* systems. One of the major breakthroughs in light microscopy is the realization of instruments based on non-linear emissions (Denk et al., 1990; Mertz, 2004; Zipfel et al., 2003b), which has opened the door to the development of intravital microscopy (IVM). IVM encompasses a series of light microscopy-based techniques aimed at studying several physiological processes in live animals (Amornphimoltham et al., 2011; Fukumura et al., 2010; Weigert et al., 2010). In particular, two-photon microscopy (TPM) has been instrumental in developing fields such as neuroscience, immunology and tumor biology For example,, TPM has made possible imaging the behavior of single neuronal populations in the brain of live animals leading to fundamental discoveries in neuronal plasticity and neurotransmission, thus

increasing our understanding of pathological conditions such as the Alzheimer's disease or ischemia-induced damages (Serrano-Pozo et al., 2011; Svoboda and Yasuda, 2006; Zhang and Murphy, 2007). In immunology, TPM has been instrumental in analyzing the interactions among the cells of the immune system during the immune response and has provided valuable information on host-pathogen interactions (Cahalan and Parker, 2008; Germain et al., 2005; Miller et al., 2002; Textor et al., 2011). Finally, the ability to image tumors *in situ* during cell growth and invasion, and to monitor the tumor microenvironment has provided with formidable tools to unravel several key mechanisms regulating tumor progression, thus leading to the design and test of novel therapeutic approaches (Andresen et al., 2009; Fukumura et al., 2010; Fukumura and Jain, 2008; Orth et al., 2011). The first attempt to image submicron structures in a live animal has been in the brain, where long term imaging of dendritic spines has been accomplished (Pan and Gan, 2008), whereas the first attempts to image the internalization of fluorescently labeled molecules into highly dynamics sub-cellular structures, such as the endosomes, has been performed in the kidney of live rats and mice (Dunn et al., 2002; Dunn et al., 2003; Sandoval et al., 2004; Sandoval and Molitoris, 2008). However, the motion artifacts due the heartbeat and the respiration of the animal have precluded a detailed analysis of the dynamics of these events. Recently, we have developed an experimental system that has enabled us to follow the dynamics of endosomes and secretory granules in the salivary glands (SGs) of live rodents by using IVM (Masedunskas et al., 2011; Masedunskas and Weigert, 2008; Sramkova et al., 2009). In this chapter, we will review some of the most recent applications of IVM, aimed at studying various aspects of cell biology in live rodents, and will highlight the fact that the salivary glands (SGs) represent a perfect model organ for these studies since they offer unique advantages: first, they can be easily externalized and positioned to completely eliminate the motion artifacts due to the respiration and the heartbeat (Masedunskas and Weigert, 2008), and second they can be easily manipulated both pharmacologically and genetically providing thus with the opportunity to dissect and unravel molecular machineries (Masedunskas and Weigert, 2008, Sramkova et al., 2009). Our goal is to persuade the readers that this approach has a wide range of applicability in different areas of the biomedical field and has the potential to address several fundamental biological questions.

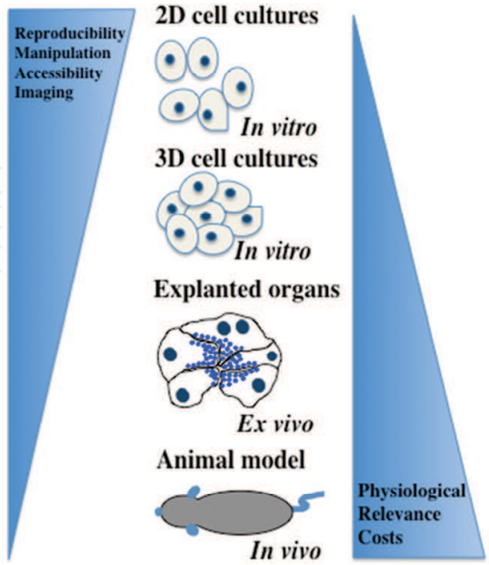


Fig. 1. Comparison among the various experimental systems utilized to study cell biology in mammalian system

Increased complexity in the architecture of the experimental model systems utilized to study cell biology: cell cultures grown on bi-dimensional surfaces (i.e. plastic or glass), cell cultures grown in three dimensions (i.e. purified components of the extracellular matrix), explanted organs, and live animals.

2. Basic principles of intravital microscopy

Biologists have been always fascinated by the possibility to observe biological process in live organisms. One of the major challenges in IVM is to expose the tissue of interest taking care of minimizing damages and maintaining its functionality during the observation period. To this aim, appropriate surgical techniques have been developed since the early days of IVM. The first intravital studies were performed in the early 30's, although they were limited to the examination of the vasculature and its cellular components by using bright field illumination (Beck and Berg, 1931). Advances in optical methods and particularly the development of fluorescence light microscopy, have increased the level of resolution, thus extending the number of biological processes that can be observed *in vivo* (Amornphimoltham et al., 2011; Weigert et al., 2010). Fluorescence light microscopy is based on the generation of contrast by the excitation of the energy levels of molecules (referred as fluorophores) that are either naturally present in the tissue of interest or are administered exogenously. The excitation is achieved by illuminating the specimen with a light source such as a mercury lamp or a laser. The emission can be either directly proportional to the excitation (linear) or exhibit a more complex dependence (non-linear). In the last two decades, microscopes based on e various non-linear processes have been developed, making possible to perform deep tissue imaging (Denk et al., 1990; Mertz, 2004; Zipfel et al., 2003b). Below, we will briefly describe and compare some of the linear (confocal microscopy, CM) and non-linear (multi-photon and harmonic generation) techniques that are commonly used to perform IVM.

2.1 Confocal microscopy

In CM, the excitation of the fluorophore is achieved by using single photons with wavelengths ranging from ultraviolet (UV) to visible light (Fig.2). In order to gather the signal coming from the focal plane and to avoid off-focus emissions that reduce the spatial resolution, the emitted light is forced to pass through a pinhole. This allows to modify the thickness of the sampled area providing an easy way to balance resolution and signal intensity. Confocal microscopes are widespread tools and have been extensively used for IVM (Guan et al., 2009; Masedunskas et al., 2011). However, CM has some limitations. First, UV and visible light are scattered by biological specimen, thus limiting the imaging to the first 50-60 μm below the surface of the specimen and making CM the optimal choice for cell cultures and optically transparent tissues. Second, long term illumination with UV and visible light may lead to photobleaching and phototoxicity, limiting the use of CM to short term imaging as documented by several reports of radiation-induced cellular damage or impairment in tissue development (Dela Cruz et al., 2010). However, when tissues are homogeneous and biological processes are not dependent on the depth, CM can be successfully used providing a better spatial resolution than other techniques (Masedunskas et al., 2011).

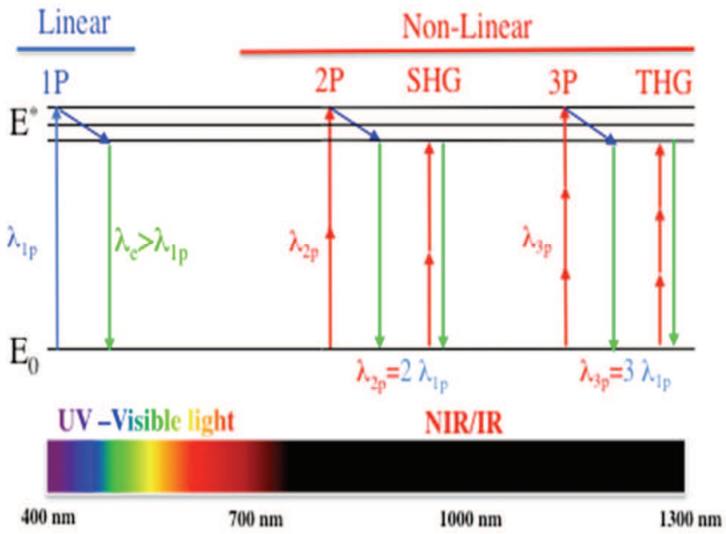


Fig. 2. Comparison between linear and non-linear modalities of fluorescence light microscopy.

Single photon excitation: the energy gap between the ground (E_0) and the excited (E*) state in the fluorophore is filled by a single photon in the UV or visible range. Following some energy dissipation, a single photon is emitted at a higher wavelength (red-shift). Two- and three-photon excitation: the same energy gap is filled by two or three photons respectively, which have half or a third of the energy required for single photon excitation (NIR or IR light). Second and third harmonic generation (SHG and THG): two or three photons interact with the fluorophore and recombine generating a photon with half or a third of the wavelength of the incident ones.

2.2 Multiphoton microscopy (MPM)

Multiphoton emission is based on the fact that a fluorophore can be excited by the almost simultaneous absorption (within femto or atto seconds) of two or three photons that have a half or a third of the energy required to fill the energy gap in the fluorophore (Fig. 2). This requires the use of infrared (IR) light which has a lower intrinsic scattering in biological specimen when compared to UV or visible light. The non-linear nature of multi-photon excitation and the low probability for a multiphoton transition to occur, require that a high number of photons are focused in a restricted volume (1 fl–1 μm^3). This is achieved with pulsed lasers, such as the tunable titanium:sapphire laser, which generates high power beams (in the order of 2-4 W), that are focused in the focal point with high numerical aperture lenses (McMullen and Zipfel, 2010). This implies that all the emitted light generated from the focal point can be utilized to generate the image without the need for a pinhole. This simplifies the geometry of multiphoton microscopes, which require detectors with high sensitivity placed as close as possible to the specimen. Another implication of the fact that photons are absorbed in a confined volume is that photobleaching and phototoxicity are reduced, extending the duration of the experimental observations without any tissue damage, and enabling the realization of long term longitudinal studies that are fundamental in fields such as tumor biology. In terms of depth, MPM enables to extend the range of observation when compared with CM. For example, by using high numerical

aperture objectives, subcellular structures can be resolved up to a depth of 100-150 μm. Lowering the level of resolution and using lenses with longer working distances cellular structures can be routinely resolved at a depth of 300-500 μm. Furthermore, some tissues, such as the brain, exhibit lower light scattering enabling imaging up to a 1 mm depth. Recently, alternative approaches based on the use of either longer excitation wavelengths through the use of optical parametric oscillators (OPO) or regenerative amplifiers, have extended the limits of imaging depth (Andresen et al., 2009; Theer et al., 2003). Two final advantages of multiphoton excitation are: first, the fact that several endogenous molecules can be easily excited providing a contrast that provides numerous information on tissue and cell architecture (Campagnola and Loew, 2003; Dela Cruz et al., 2010; Weigert et al., 2010; Zipfel et al., 2003a), and second, that due to their broad multiphoton absorption spectra, multiple fluorophores can be excited simultaneously using a single excitation wavelength. This avoids the use of multiple lasers, thus reducing further the risk of photodamage.

2.3 Second and third harmonic generation (SHG and THG)

SHG and THG do not involve energy absorption since the incident photons are scattered and recombined into a single photon in a process without energy loss (Campagnola and Loew, 2003; Schenke-Layland et al., 2008; Zoumi et al., 2002). Molecules that generate second harmonic signals such as, collagen, microtubules, and muscle myosin are usually assembled in highly ordered and repeated structures with non-centrosymmetric symmetries, whereas third harmonic signals are typically generated at the interface between optically heterogeneous biological materials (Campagnola and Loew, 2003; Debarre et al., 2006; Gualda et al., 2008). SHG has been extensively utilized to study the properties of the extracellular matrix under both physiological and pathological conditions, and shows also an incredible potential for diagnostic purposes. THG has been used to image lipid bodies in small organisms, to study early embryogenesis dynamics in zebrafish, and to study the process of demyelination in models for neurodegenerative disorders. SHG and THG have the advantage of being nontoxic since no energy is absorbed by the specimen during imaging (Fig. 2), and they can be combined with MPM providing with the opportunity to perform multimodal imaging (Campagnola and Loew, 2003; Chen et al., 2009; Debarre et al., 2006; Farrar et al., 2011; Radosevich et al., 2008).

3. The salivary glands as a versatile model to perform intravital microscopy and to manipulate cellular pathways

3.1 Architecture and physiology of the salivary glands

Salivary glands (SGs) are major exocrine glands responsible for the production and secretion of saliva into the oral cavity (Gorr et al., 2005; Melvin et al., 2005). In mammals there are two kinds of SGs: the major and the minor glands. The major SGs include: parotid glands, which secrete primarily enzymes involved in digestion (e.g. amylase), submandibular glands, which secrete enzymes required to defend the oral cavity from pathogens (e.g. peroxidases, kallikrein), and sublingual glands, which secrete molecules required to protect the oral cavity (e.g. mucins). Saliva is a mixture of water, proteins, and electrolytes that is primarily released from the acini, the main secretory units of the SGs, into the acinar canaliculi and from there discharged into the ductal system (Fig. 3). Acini are formed by polarized acinar cells, with the apical plasma membrane (APM) facing the lumen of the acinar canaliculi, and

the basolateral membrane facing the basement membrane and the stroma. The secretion of water and proteins is under the control of G protein-coupled receptors: muscarinic stimulation is the primary signal regulating water secretion, whereas protein secretion is regulated by either the beta-adrenergic (submandibular and parotid) or the muscarinic receptors (sublingual) (Gorr et al., 2005; Melvin et al., 2005). The ductal system is also formed by polarized cells and its main function is to modify the electrolyte composition of the primary saliva and convey it into the oral cavity. In rodents, a subpopulation of the ductal cells, the granular convoluted tubules, secrete large amount of growth factors (such as EGF and NGF) that are stored in large secretory granules (Peter et al., 1995).

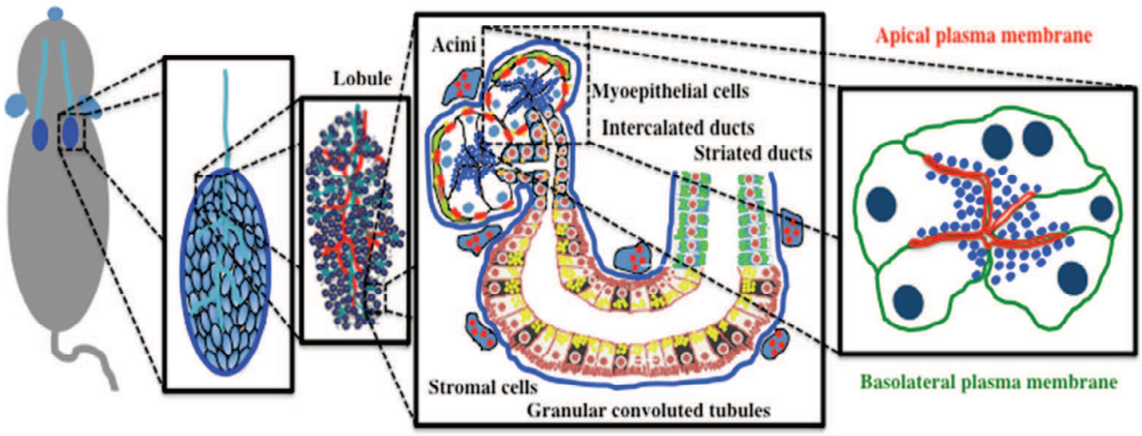


Fig. 3. Diagram of rodent submandibular salivary glands.

The SGs are formed by interconnected lobules, which contain both acini and ductal structures. Acini are formed by polarized epithelial cells, which contain secretory granules that fuse with the apical plasma membrane and release their content in the acinar canaliculi. The canaliculi merge in larger ducts, the intercalated ducts, than enlarge forming the granular convoluted tubules and later the striated ducts. The striated ducts merge with the major excretory duct

3.2 Delivery of molecules, drugs and gene transduction in the salivary glands

SGs are ideal organs to perform IVM for various reasons. First, in rodents the glands are located in the neck area, where the motion artifacts due to the heartbeat and the respiration are significantly reduced (Masedunskas and Weigert, 2008). Second, the SGs can be exposed with relatively minor surgical procedures, which do not involve the exposure of the body cavity, which may effect the overall health status of the animal. Finally, the epithelium of the SGs can be easily accessed from the oral cavity by introducing fine polyethylene tubings into the major excretory ducts (called Wharton's duct in the submandibular glands and Stensen's duct in the parotid glands) that can be utilized to selectively deliver various molecules into the ductal system (Masedunskas et al., 2011; Masedunskas and Weigert, 2008; Sramkova et al., 2009) (Fig. 4A). We have shown that fluorescent dyes can be delivered into the ductal system through injection or by gravity diffusion, and utilized to study endocytosis, exocytosis or various aspects of water secretion (Fig. 4B) (Masedunskas and Weigert, 2008; Sramkova et al., 2009, Masedunskas et al., 2011). The same route has been utilized to selectively deliver drugs to the SGs. This approach offers two advantages: 1) to specifically

target the SGs avoiding the side effects due to systemic injections, and second, to precisely control the doses of the drugs administered (Masedunskas and Weigert, 2008).

SGs have been widely used as a target organ for the viral-mediated expression and gene delivery of various transgenes both in live animals and in humans (Baum et al., 2010; Cotrim and Baum, 2008). Indeed, these organs have the potential to be utilized for gene therapy to correct various diseases including Sjogren's syndrome and protein deficiencies (Baum et al., 2004; Voutetakis et al., 2004). Notably, for viral-mediated gene therapy in humans, the SGs offer several advantages with respect to other organs: i) the encapsulation of the SG tissue prevents the dissemination of the virus in the rest of the body (Baum et al., 2004; Voutetakis et al., 2004), ii) in case of potential health issues the SGs can be removed since they are not essential for life, iii) the differentiation of the cells provides a relatively stable cell populations for non-integrating vectors, and iv) duacrine (both exocrine and endocrine) protein secretion allows to direct the expressed molecules into either the saliva or the blood stream (Baum et al., 2004). Numerous studies have shown successful gene transfer into both rat and mouse submandibular glands using viral-based approaches, which offer the advantage of a more robust expression of the transgenes (Andresen et al., 2009; Baum and Tran, 2006; Delporte et al., 1996; Honigman et al., 2001; Mastrangeli et al., 1994; Morita et al., 2011; Palaniyandi et al., 2011; Perez et al., 2011; Samuni et al., 2008; Wang et al., 2000; Zheng et al., 2009). However, non viral-mediated approaches have also been utilized, although limited to a small percentage of the cells in the parenchyma (Goldfine et al., 1997; Honigman et al., 2001; Niedzinski et al., 2003a; Niedzinski et al., 2003b; Passineau et al., 2010; Sramkova et al., 2009). Furthermore, the majority of the studies on rodent SGs were focused on submandibular glands and only few studies were performed in parotid glands. Recent studies demonstrated efficient gene transfer into rat parotid glands, as shown by the effective delivery of human erythropoietin and human parathyroid hormone (Adriaansen et al., 2010; Kagami et al., 1998; Mastrangeli et al., 1994; Zheng et al., 2009). The rationale behind developing strategies to deliver transgene into parotid glands is their use in humans as main target for clinical applications (Zheng et al., 2011).

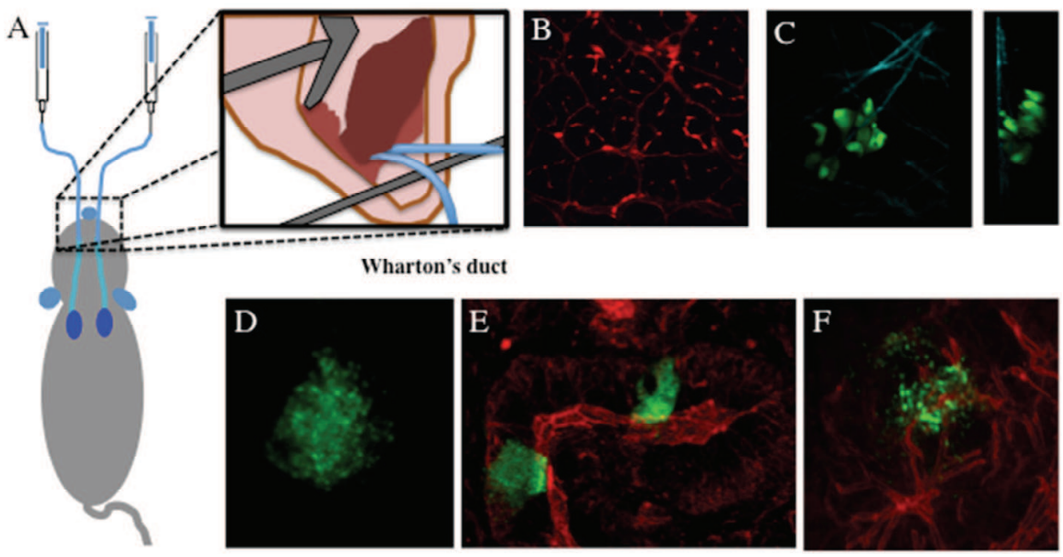


Fig. 4. Non viral-mediated gene transfer in the submandibular SGs of live rats.

A. Fine polyethylene cannulae are introduced in the oral cavity in the Wharton's duct of live rats. B– 10 kDa Texas Red-dextran is injected in the SGs and the ductal system is highlighted. C-F. Plasmid DNAs encoding for the fluorescent protein pVenus (B), GFP-ribonuclases (D), Aquaporin 5-GFP (E), and GFP-Clathrin (F) were injected in the submandibular glands. After 16 hrs, the glands were exposed and imaged by two-photon IVM (C and D) or excised, fixed, labeled with rhodamine-phalloidin, and imaged by CM (E and F). C. Cluster of pVenus expressing-cells (green) localized below the surface of the glands as shown by SHG, which reveals the collagen fibers (cyan). Excitation 930 nm. D. GFP-ribonuclease is localized in intracellular vesicles. Excitation 930 nm. E,F. GFP-Acquaporin is expressed in large ducts (E) and GFP-clathrin in acini (F) as revealed by labeling for the actin cytoskeleton (red).

We have utilized plasmid DNA in live rats and shown that the transgenes under the appropriate conditions can be targeted to specific subpopulations of the SGs (Sramkova et al., 2009). The main advantage in using naked DNA vs. viral-based vectors is the possibility to screen very rapidly for multiple genes without dealing with the time-consuming steps of designing, cloning and preparing the viral particles. We have injected plasmid DNA encoding for various fluorescent proteins into the Wharton's duct of rat submandibular glands, and after 16 hours we have observed their expression in the SGs epithelium (Fig 4C-F). Specifically, we have found that when plasmid DNA is injected alone, the reporter molecule is expressed in approximately 0.05% of the cells of the parenchyma, which we have identified as intercalated ducts. The addition of empty replication-defective adeno-viral (rAd5) particles increases the level of transduction up to 0.5-2% of the cells and notably, the fluorescent reporter is expressed primarily in the large striated and granular ducts and to a lesser extent in the acinar cells (Fig. 4C, 4E and 5) . In both instances, the expression of the reporter molecule is transient and lasts for 72 hrs, a window of time sufficient to be utilized for IVM. Furthermore, since our goal is to transduce genes primarily into acinar structures, we sought to find a more specific way to target these cells. We reasoned that plasmid DNA might be internalized by the acinar cells via the endocytic pathways and for this reason we stimulated compensatory endocytosis by activating the beta-adrenergic receptors during plasmid DNA injection (see below). Notably, under these conditions, the reporter molecule is expressed in 1% of the cells of the parenchyma and primarily in the acinar cells (Fig. 4D, 4F and 5) (Sramkova et al., 2009). It is important to emphasize, that although the efficiency of gene expression is low, the absolute number of cells that can be imaged by IVM is still very high. This implies that viral-based approaches may still have to be utilized whenever a different readout, such as a biochemical assay, needs to be used.

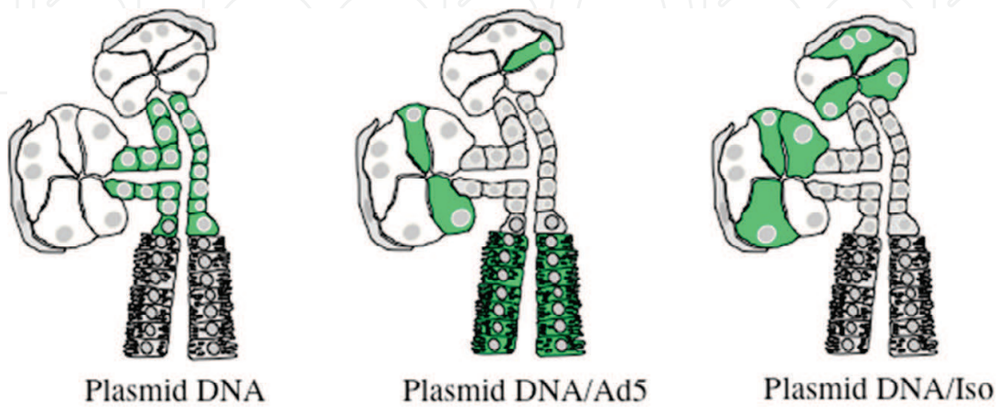


Fig. 5. Naked DNA is targeted to different cell populations of the salivary glands.

When naked DNA is injected in the absence of any other manipulation the transgene is expressed in the intercalated ducts. When naked DNA is pre-mixed with empty adenoviral particles, the transgene is expressed primarily in the large ducts but also in some acinar cells. When naked DNA is injected and compensatory endocytosis is elicited by stimulation of the beta-adrenergic receptor (sub-cutaneous injections of isoproterenol) the transgene is expressed in acinar cells.

This approach can be utilized to express any fluorescently tagged protein, enabling the expansion of the repertoire of compartments that can be visualized in a short period of time (Fig. 4C-F). Moreover, this strategy has provided us with a powerful tool to interfere with specific molecular machineries by introducing molecules acting as positive or negative regulators of the specific processes of interest. Finally, this approach can be used to genetically modify the target tissue by silencing certain genes. Small interfering RNA (siRNA) has been successfully delivered in live rats. siRNA targeting the cystic fibrosis transmembrane conductance regulator (CFTR) were injected intraductallly into rat submandibular glands resulting in the effective silencing of CFTR (Ishibashi et al., 2008; Ishibashi et al., 2006). Notably, in order to complement and confirm the results with the siRNA, a specific inhibitor of CFTR ($CFTR_{inh-}$ 172) and suramin, a non-specific P2 receptor antagonist, were also injected, further highlighting the power of this approach. Although the efficiency in siRNA delivery is low, novel approached has been introduced to overcome this issue. For example, silencing of GAPDH in rat parotid glands was performed in combination with microbubble-enhanced sonoporation, improving the efficiency of siRNA transfer by 10-50% (Sakai et al., 2009).

4. Imaging membrane trafficking and the actin cytoskeleton in salivary glands by intravital microscopy

Membrane traffic is an important field in cell biology that studies the processes and the machineries involved in the transport of various molecules among different compartments within the cell. Transport steps are mediated by membranous containers, termed "transport intermediates", which are very heterogeneous in size, shape, contents and modality of transport. Their biogenesis, trafficking, delivery to the target compartments, and dynamic behavior are dictated by the architecture of the cells and by the organization of the cytoskeletal elements (e.g. microtubules and microfilaments). Most of the data on the dynamics of the transports steps have been derived from cell culture models. As, previously discussed, the architecture of the cells in a living organism differs considerably from the architecture of cells in culture. Although IVM offers a very powerful opportunity to study membrane trafficking in physiological conditions, the challenges in controlling the motion artifacts have discouraged several investigators from pursuing this approach and only few labs have invested in high resolution imaging of live animals. For example, submicron structures were imaged dynamically in the brain of live mice, where structural changes in the architecture of dendritic spines were observed under conditions such as epileptic seizures (Mizrahi et al., 2004; Pan and Gan, 2008; Svoboda and Yasuda, 2006). In kidney, various subcellular processes were analyzed, such as endocytosis of selected molecules (Dunn et al., 2002; Dunn et al., 2003; Molitoris and Sandoval, 2006; Sandoval et al., 2004; Sandoval and Molitoris, 2008), exocytosis of renin (Toma et al., 2006), and mitochondrial function (Hall et al., 2009). Recently, mitochondrial dynamics and lipid bodies have been analyzed in live animals (Debarre et al., 2006; Roberts et al., 2008; Zhong et al., 2008).

Another area where imaging membrane trafficking in vivo has provided novel information is tumor biology. Very recently, nuclear dynamics and mitotis were observed using in murine xenograft model of human cancer and compared to cells in culture (Orth et al., 2011) with profound implications for drug development and cancer therapy (Amornphimoltham et al., 2011). Furthermore, using QD conjugated either to an anti-HER2 antibody or to EGF-conjugated nanotubes the delivery and the uptake of these molecules by tumor cells was analyzed (Bhirde et al., 2009; Tada et al., 2007).

Here, we review our work using the SGs, which represent a robust model to study several aspects of membrane trafficking, particularly because the motion artifacts can be easily reduced using various strategies described in detail elsewhere (Masedunskas et al. 2011b). Although the SGs are exocrine glands, which represent a perfect model system to study exocytosis, they are also a powerful model to study endocytic processes, such as receptor-mediated endocytosis, which occur at the basolateral plasma membrane of the epithelium, compensatory endocytosis that is triggered upon exocytosis at APM, fluid phase endocytosis in stromal cells, polarized trafficking of plasma membrane proteins in the epithelium, and mitochondrial dynamics.

4.1 Endocytosis

The endosomal system is utilized as a transport route, to shuffle proteins, lipids and membranes to and from the cell surface, and towards other sub-cellular organelles. Endocytosis occurs in every cell and is involved in several processes such as nutrient uptake, cell adhesion, migration, cytokinesis, polarity and signaling (Maxfield and McGraw, 2004; Mellman, 1996). Endocytosis and recycling mediate the removal and retrieval of membrane components from the cell surface and these tightly coupled processes are highly regulated. Notably, endocytic pathways are very diversified in terms of molecular machinery, as shown by the fact that multiple endocytic routes have been described (Conner and Schmid, 2003; Doherty and McMahon, 2009; Grant and Donaldson, 2009; Mayor and Pagano, 2007). Much of our understanding of the endosomal system is derived from studies performed in cell cultures and few studies have been performed in live organisms such as rodents. The first attempt to image endocytosis in vivo was realized in the kidney of live rats and mice, where fluorescently labeled dextrans of different molecular weight were injected systemically (Dunn et al., 2002). Similar studies were performed imaging the receptor-mediated endocytosis of the antibiotic gentamicin and the internalization of the folate-receptor (Dunn et al., 2002; Sandoval et al., 2004). Although the diffusion of the injected probes along the tubular system in the kidney was imaged, their internalization could be followed only for short period of times due to the high levels of the motion artifacts. In this respect, the SGs provide with a more controlled experimental system. Indeed, we injected fluorescently-labeled molecules in the tail artery and tracked the dynamics of the endosomal compartments for over 60 minutes (Masedunskas and Weigert, 2008) (Fig. 6A). To further distinguish among the different endocytic sub-compartments, Texas red-dextran (TXR-D) was injected systemically and allowed to accumulate into the lysosomes (Fig. 6B). After 24 hours, Alexa-488 dextran (488-D) was injected and imaged in time-lapse mode. 488-D was first internalized into small vesicles, and then delivered to early endosomes that grew over time due to homotypic fusion events. Later, 488-D was delivered to late endosomes and lysosomes and the process was imaged providing novel insight on the dynamics of the endo-lysosomal system (Fig. 6B) (Masedunskas and Weigert, 2008). In a separate study

lysosomal fusion events were captured at a higher resolution, almost comparable to that achieved in cell culture (Weigert et al., 2010).

Interestingly, Cytochalasin D and Latrunculin A, two actin-disrupting agents significantly reduced the uptake of fluorescent dextran in SGs, suggesting for a role of actin during internalization (Fig. 6C and 6D) (Masedunskas and Weigert, 2008). The requirement for actin during endocytosis has been demonstrated in yeast but has been controversial in mammalian cells (Galletta et al., 2010). The conflicting results could be due to differences in the organization of the actin cytoskeleton or to a different organization of the endocytic pathways. Additional work is required to address these fundamental questions and particularly in defining the endocytic routes *in vivo* and their reciprocal relationship.

Another important issue is the fact that different cell populations within the same organ exhibit different rate of internalization. For example we found that in the SGs, fibroblasts and dendritic cells internalized molecules at a much faster rates than the acinar or the ductal cells in the parenchyma (Fig. 6E). This may reflect the presence of barriers such as the basement membrane and the tight junctions that controls the delivery of molecules from the blood stream.

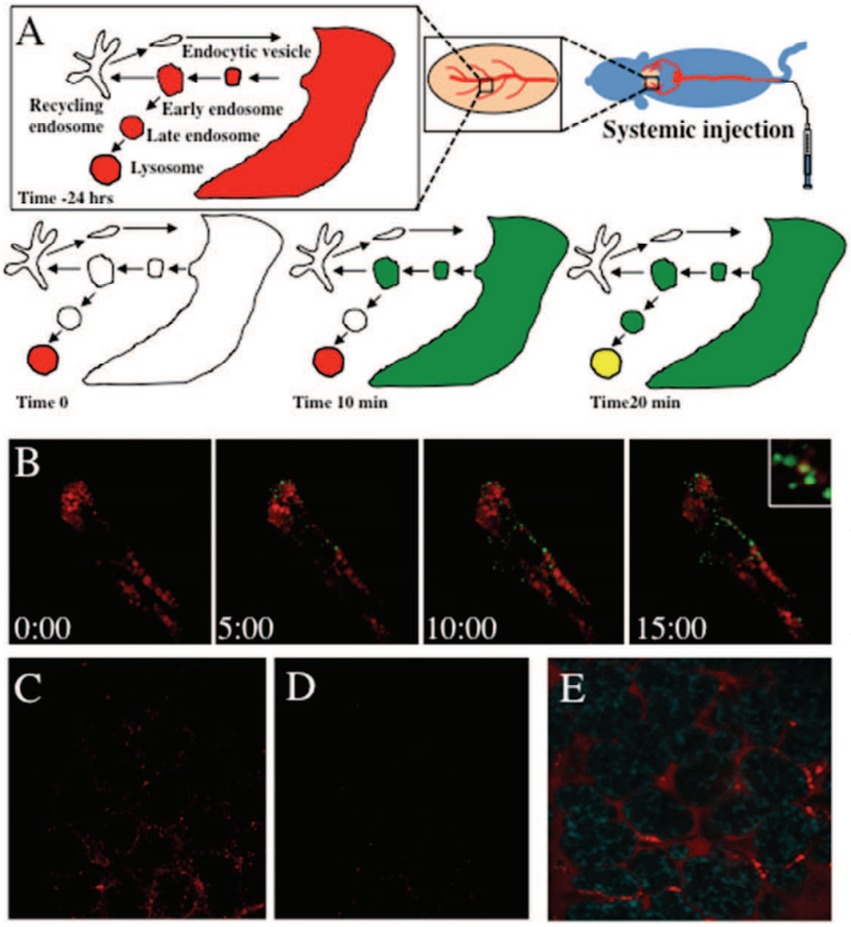


Fig. 6. Endocytosis of systemically-injected fluorescently-labeled probes in the salivary glands of live rats

A. Diagram of the experimental design to study endocytosis in SGs. The fluorescent probes are injected into the tail artery, reach the SGs through the circulation, and diffuse out of the vasculature from the fenestrated capillaries. B. TXR-D (red) is accumulated into the lysosomes after 24 hrs from the injection (time 0:00). Alexa 488-dextran is first internalized into early endosomes (time 5:00 and 10:00) and later reach the lysosome (time 15:00 and inset). C,D. TXR-D was injected and the SGs were imaged after 20 min. C. Control glands D. SGs treated with latrunculin A. E. Lower magnification of the SGs after the injection of TXR-D. The probe is accumulated in the stroma, internalized in stromal cells, and was excluded from the acini that are revealed by two-photon-stimulated intrinsic emission (Masedunskas and Weigert, 2008; Weigert et al., 2010).

Endocytosis from the APM of the SGs was also analyzed by either injection or slow gravity-mediated infusion of small molecular weight dextrans though the Wharton's duct. Under resting conditions most of the probes underwent a low but detectable level of endocytosis in both the ducts and the acinar cells. However, upon stimulation of protein but not water secretion, the probes were primarily internalized into the acini, most likely by the activation of a process known as compensatory endocytosis (Masedunskas et al., 2011; Sramkova et al., 2009). Interestingly, this process did not involve any of the currently characterized endocytic processes and studies are undergoing to further elucidate this it s machinery. Finally, in order to study receptor-mediated endocytosis at the basolateral plasma membrane we have transfected in the acinar cells of live rats, transferrin receptor, as a model for constitutive endocytosis and beta2-adrenergic receptor, as a model for agonist-induced endocytosis. The ectopically expressed receptors are properly targeted to the acinar cells and at the plasma membrane as predicted (Fig. 7C and 7D).

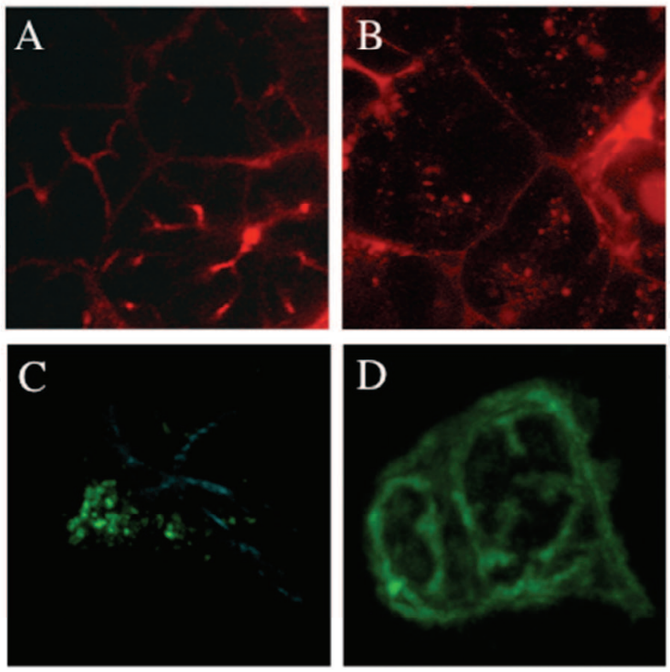


Fig. 7. Endocytosis from the apical and the basolateral plasma membrane of the salivary glands

A,B. 10 kDa TXR-D was injected into the Wharton's duct to fill the acinar canaliculi. After 20 minutes, minimal internalization of the probe was observed (A). Five minutes after the SC injection of isoproterenol smaller endocytic vesicles formed from the APM (B). C,D. GFP-Transferrin receptor (C) and YFP-beta2-adrenergic receptor were transfected in the acinar cells of live rats and imaged by using IVM. Maximal projections of Z-stacks are shown. Transferrin receptor is primarily localized in intracellular vesicles, whereas beta2-adrenergic receptor is primarily localized at the basolateral plasma membrane.

4.2 Exocytosis

SGs are a well-established model for exocrine secretion. Proteins destined to secretion are synthesized in the endoplasmic reticulum and transported through the Golgi apparatus to the trans-Golgi network (TGN) where they are packed in large vesicles, secretory granules (SCGs), which are released into the cytoplasm, and transported to the cell periphery. Here, upon stimulation of the appropriate G protein-coupled receptor (GPCR), the SCGs fuse with the APM, releasing their content into the lumen of the canaliculi. Although, exocytosis in SGs has been extensively studied in ex-vivo models (Castle et al., 2002; Castle, 1998; Gorr et al., 2005), very little is known about the molecular mechanisms regulating this process. Several studies have reported contradicting findings about the stimuli triggering exocytosis, the modalities of fusion, and the requirement for the actin cytoskeleton in this process (Eitzen, 2003; Nashida et al., 2004; Segawa et al., 1998; Segawa and Riva, 1996; Segawa et al., 1991; Sokac and Bement, 2006; Warner et al., 2008). This variability probably reflects the different experimental conditions utilized to isolate and culture *ex-vivo* the acinar cells. To overcome this issue, we have utilized IVM and studied the dynamics of the SCGs in live animals. To this aim, we used a series of transgenic mouse models expressing selected fluorescently labeled molecules, combined with the ability to transduce genes, and selectively deliver molecules and pharmacological agents, as described above and elsewhere (Masedunskas et al., 2011b). This approach has provided novel and valuable information on the structure and the physiology of the acinar cells (Masedunskas et al., 2011). We estimated that in resting conditions, the major SGs contain approximately 2500-3000 granules per acinus, most of them accumulated in the sub apical area of the PM. Our analysis on the effect of various agonists of GPCRs has revealed three major differences between in vivo and ex-vivo models: 1) the stimulation of the beta-adrenergic but not the muscarinic receptors, enhances the mobility of the secretory granules promoting their docking and subsequent fusion at the APM; 2) muscarinic receptors do not play any synergistic role with the adrenergic receptor during exocytosis; and 3) the maximal rate of fusion of the secretory granules in live animal (10-15 granules/cell/min) is 3-4 times faster than previously reported for *ex-vivo* systems. Furthermore, by using another mouse model, which expresses the Tomato fluorescent protein fused with a di-palmitoylated peptide (m-Tomato), a well-established marker for the plasma membrane, we discovered that the secretory granules after fusing with the plasma membrane completely collapse within 30-40 seconds (Masedunskas et al., 2011). This result underscores another major difference between *in vivo* and *ex-vivo* models, in which compound exocytosis (i.e. the sequential fusion of strings of SCGs), has been described as the primary modality of fusion (Warner et al., 2008). Notably, we also observed that the granules in close proximity of the APM recruit a series of cytosolic proteins including actin, suggesting a role for the cytoskeleton during granule exocytosis. To address this issue we have transduced the salivary glands of live rats with the small peptide Lifeact fused with GFP, a novel tool to label dynamically F-actin (Riedl et al., 2008). We determined that F-actin filaments are polymerized onto the surface of the granules only after

fusion has occurred, and persisted until their complete collapse. The impairment of the dynamics of the actin cytoskeleton, using pharmacological agents such as cytochalasin D (cyto D) or latrunculin A (lat A), did not affect the fusion of the secretory granules with the APM, but it blocked substantially their collapse leading to the accumulation of fused granules which often expanded in size. Finally, we found that myosin IIa and IIb, two actin-based motor proteins are recruited on the fused secretory granules and that their motor activity is required to drive the gradual collapse of the granules. These results suggest that the acto-myosin complex provides a contractile scaffold around the secretory granules that facilitates the completion of the fusion at the APM and preventing an aberrant influx of membrane inside the cell (Masedunskas et al., 2011). This novel approach provided new insights into the molecular mechanisms of exocytosis in SGs, captured the exocytosis process dynamically and established important tools to study this process. This approach can be extended to study exocytosis in other exocrine glands such as pancreas, lacrimal glands, and mammary glands

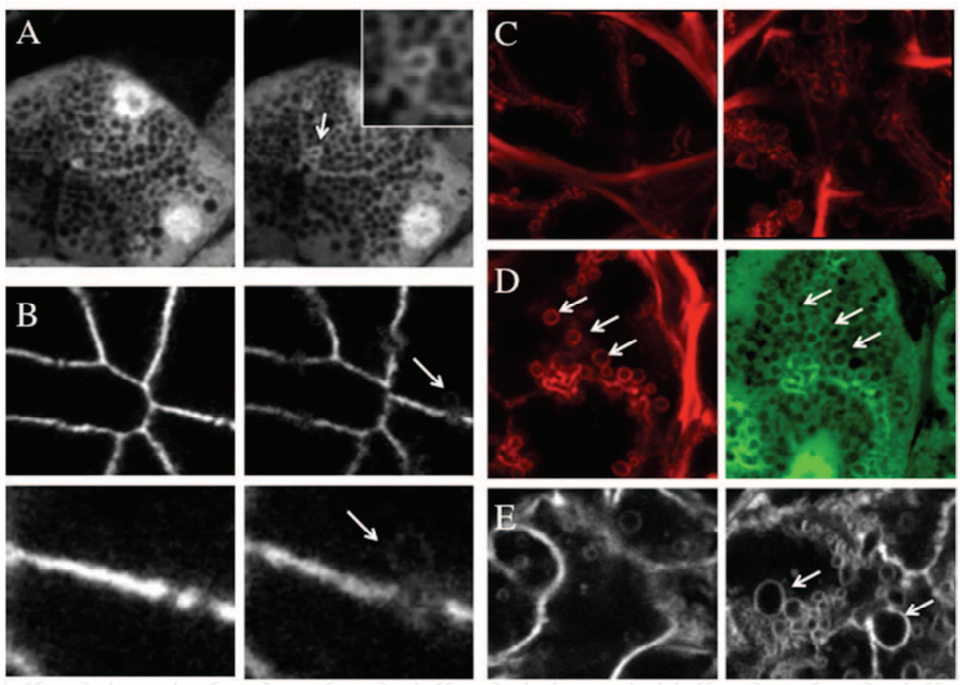


Fig. 8. Regulated exocytosis in the salivary glands of live rodents.

A. SGs of mice expressing cytoplasmic GFP. GFP is excluded from the large SCGs that appear as dark vesicles in the cytoplasm. Upon stimulation with isoproterenol, SCGs fuse with the APM. Fusing SCGs are characterized by an accumulation of GFP on the limiting membranes of the SCGs (arrow), as previously described (Masedunskas et al., 2011). B. SGs in the m-Tomato mice. The m-Tomato probe labels all the cellular membranes. Upon stimulation with isoproterenol, the m-Tomato diffuses into the membrane of the SCGs (arrows). C. The SGs of a live rat were labeled with rhodamine-phalloidin to reveal the actin cytoskeleton. The left panel shows the enrichment of actin at the APM in control conditions. The right panel shows the recruitment of actin around the SCGs upon stimulation with isoproterenol. D. A GFP mouse was stimulated with isoproterenol and labeled with rhodamine-phalloidin. Arrows point to the SCGs at the plasma membrane that are enriched in GFP and actin. E. The effect on exocytosis of actin-disrupting agents. The SGs of a live m-Tomato mouse were treated with

latrunculin A and stimulated with isoproterenol (right panel). SCGs fail to collapse at the plasma membrane and increase in size forming large vacuolar structures.

4.3 Actin cytoskeleton

The actin cytoskeleton plays a fundamental role in many cellular events. We have recently shown a role for actin in endocytosis and a novel role for actin and the actin motor protein myosin II in exocytosis in the SGs of live animals (Masedunskas et al., 2011; Masedunskas and Weigert, 2008). Notably, by using IVM we revealed a novel function for the actomyosin complex that was not completely appreciate in cell cultures. Specifically, we found that actin serves: 1) as a barrier preventing the unwanted homotypic fusion between the SCGs, 2) as a scaffold to prevent the hydrostatic pressure generated by fluid secretion to disrupt the exocytic events, and 3) as a platform to generate a contractile scaffold that facilitate the collapse of the SCGs with the apical plasma membrane (Masedunskas et al., 2011). We took an advantage of the methodology reviewed here to pharmacologically disrupt the actin cytoskeleton and fluorescently tag proteins for labeling the cytoskeleton *in vivo*. Specifically, we have used GFP-lifeact as a tool to follow the dynamics of F-actin (Riedl et al., 2008). This tool is more effective and less toxic than GFP-actin (Fig. 9A and 9C). However, one of the drawbacks of our transfection system is that the expression of the protein of interest is limited to one or two cells per acinus, limiting the possibility to study the behavior of the actin cytoskeleton in groups of cell. Recently, a transgenic mouse expressing GFP-life act has been generated (Riedl et al., 2010). These mice are superior to the GFP-actin transgenic mice in terms of viability, level of protein expression and cellular toxicity and represent a formidable tool to study several aspect of the involvement of actin in various cellular processes. These mice are nicely complemented by other transgenic mice expressing the GFP-tagged versions of the actin motor proteins myosin IIa and IIb (Bao et al., 2007).

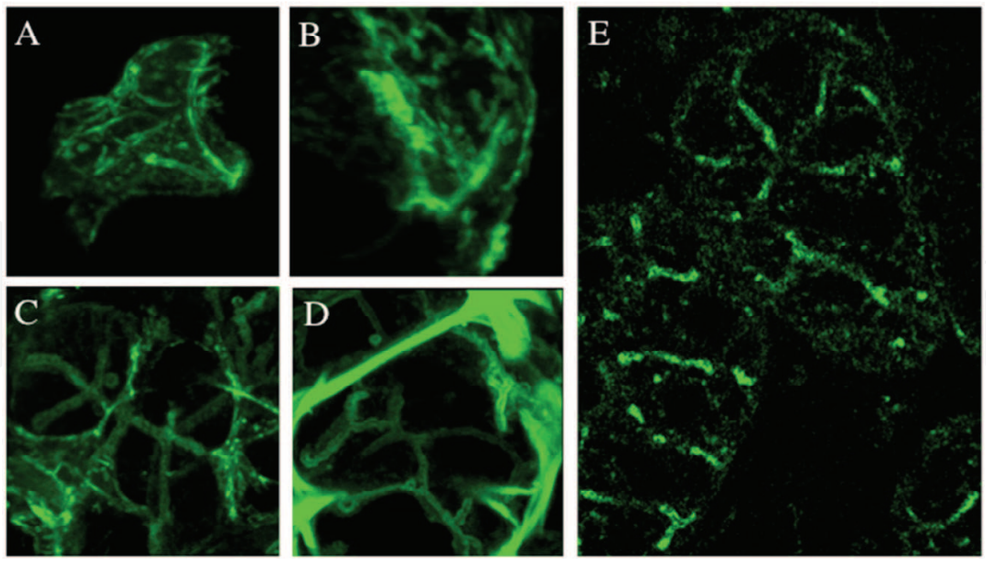


Fig. 9. Imaging the actin cytoskeleton in the salivary glands of live animals.

A, B. GFP-actin or GFP-lifeact were transfected in the SGs of live rats. Both molecules were expressed in acinar cells and exhibited filamentous cortical localization. However, the precise localization of the two probes with respect to the apical or the basolateral pole

cannot be assessed in a single cell. C, D. Transgenic mice expressing GFP-actin (C) or GFP-lifeact (D). The acinar structure in the salivary glands show the typical enrichment at the apical plasma membrane. E. Transgenic mouse expressing GFP-myosin IIb. In the acini of the SGs, GFP-myosin IIb is localized at the apical plasma membrane as previously described for the endogenous myosins (Masedunskas et al., 2011).

5. Conclusions

We have provided several examples from our recent work that the combination of IVM as imaging technique, and the SGs as a model organ is a very versatile tool that can be successfully used to address several biological questions under physiological conditions. The possibility to either express or down regulate proteins in an acute fashion, combined with the plethora of available transgenic and knockdown mouse models, offer a unique set of opportunities to study processes in live animals at a molecular level. Furthermore, the ability to image at a subcellular level in a live animal has opened the possibility to study several aspects of cell biology in a dynamic fashion. We have utilized this approach to study membrane trafficking and the dynamics of the actin cytoskeleton, which represent only a fraction of the fields that can be studied *in vivo*. We envision that soon IVM will be extended to study other processes, such as cell cycle, signal transduction, mitochondrial dynamics and metabolisms to name a few.

6. Acknowledgments

This research was supported by the Intramural Research Program of the NIH, National Institute of Dental and Craniofacial Research.

7. References

Adriaansen, J., Zheng, C., Perez, P., and Baum, B.J. (2010). Production and sorting of transgenic, modified human parathyroid hormone in vivo in rat salivary glands. Biochem Biophys Res Commun *391*, 768-772.

Amornphimoltham, P., Masedunskas, A., and Weigert, R. (2011). Intravital microscopy as a tool to study drug delivery in preclinical studies. Adv Drug Deliv Rev *63*, 119-128.

Andresen, V., Alexander, S., Heupel, W.M., Hirschberg, M., Hoffman, R.M., and Friedl, P. (2009). Infrared multiphoton microscopy: subcellular-resolved deep tissue imaging. Curr Opin Biotechnol *20*, 54-62.

Bao, J., Ma, X., Liu, C., and Adelstein, R.S. (2007). Replacement of nonmuscle myosin II-B with II-A rescues brain but not cardiac defects in mice. J Biol Chem *282*, 22102-22111.

Baum, B.J., Adriaansen, J., Cotrim, A.P., Goldsmith, C.M., Perez, P., Qi, S., Rowzee, A.M., and Zheng, C. (2010). Gene therapy of salivary diseases. Methods Mol Biol *666*, 3-20.

Baum, B.J., and Tran, S.D. (2006). Synergy between genetic and tissue engineering: creating an artificial salivary gland. Periodontol 2000 *41*, 218-223.

Baum, B.J., Voutetakis, A., and Wang, J. (2004). Salivary glands: novel target sites for gene therapeutics. Trends Mol Med *10*, 585-590.

Beck, J.S., and Berg, B.N. (1931). The Circulatory Pattern in the Islands of Langerhans. Am J Pathol *7*, 31-36 31.

Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P.W., and van Rheenen, J. (2011). Intravital microscopy: new insights into metastasis of tumors. J Cell Sci *124*, 299-310.

Bhirde, A.A., Patel, V., Gavard, J., Zhang, G., Sousa, A.A., Masedunskas, A., Leapman, R.D., Weigert, R., Gutkind, J.S., and Rusling, J.F. (2009). Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. ACS Nano *3*, 307-316.

Cahalan, M.D., and Parker, I. (2008). Choreography of cell motility and interaction dynamics imaged by two-photon microscopy in lymphoid organs. Annu Rev Immunol *26*, 585-626.

Campagnola, P.J., and Loew, L.M. (2003). Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. Nat Biotechnol *21*, 1356-1360.

Castle, A.M., Huang, A.Y., and Castle, J.D. (2002). The minor regulated pathway, a rapid component of salivary secretion, may provide docking/fusion sites for granule exocytosis at the apical surface of acinar cells. J Cell Sci *115*, 2963-2973.

Castle, J.D. (1998). Protein secretion by rat parotid acinar cells. Pathways and regulation. Ann N Y Acad Sci *842*, 115-124.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802-805.

Chen, H., Wang, H., Slipchenko, M.N., Jung, Y., Shi, Y., Zhu, J., Buhman, K.K., and Cheng, J.X. (2009). A multimodal platform for nonlinear optical microscopy and microspectroscopy. Opt Express *17*, 1282-1290.

Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. Nature *422*, 37-44.

Cotrim, A.P., and Baum, B.J. (2008). Gene therapy: some history, applications, problems, and prospects. Toxicol Pathol *36*, 97-103.

Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. (2001). Taking cell-matrix adhesions to the third dimension. Science *294*, 1708-1712.

Debarre, D., Supatto, W., Pena, A.M., Fabre, A., Tordjmann, T., Combettes, L., Schanne-Klein, M.C., and Beaurepaire, E. (2006). Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy. Nat Methods *3*, 47-53.

Dela Cruz, J.M., McMullen, J.D., Williams, R.M., and Zipfel, W.R. (2010). Feasibility of using multiphoton excited tissue autofluorescence for in vivo human histopathology. Biomed Opt Express *1*, 1320-1330.

Delporte, C., O'Connell, B.C., He, X., Ambudkar, I.S., Agre, P., and Baum, B.J. (1996). Adenovirus-mediated expression of aquaporin-5 in epithelial cells. J Biol Chem *271*, 22070-22075.

Denk, W., Strickler, J.H., and Webb, W.W. (1990). Two-photon laser scanning fluorescence microscopy. Science *248*, 73-76.

Diaspro (2002). Confocal and Two-Photon Microscopy. Foundations, Applications, and Advances.

Doherty, G.J., and McMahon, H.T. (2009). Mechanisms of endocytosis. Annu Rev Biochem *78*, 857-902.

Dunn, K.W., Sandoval, R.M., Kelly, K.J., Dagher, P.C., Tanner, G.A., Atkinson, S.J., Bacallao, R.L., and Molitoris, B.A. (2002). Functional studies of the kidney of living animals using multicolor two-photon microscopy. Am J Physiol Cell Physiol *283*, C905-916.

Dunn, K.W., Sandoval, R.M., and Molitoris, B.A. (2003). Intravital imaging of the kidney using multiparameter multiphoton microscopy. Nephron Exp Nephrol *94*, e7-11.

Eitzen, G. (2003). Actin remodeling to facilitate membrane fusion. Biochim Biophys Acta *1641*, 175-181.

Farrar, M.J., Wise, F.W., Fetcho, J.R., and Schaffer, C.B. (2011). In vivo imaging of myelin in the vertebrate central nervous system using third harmonic generation microscopy. Biophys J *100*, 1362-1371.

Fukumura, D., Duda, D.G., Munn, L.L., and Jain, R.K. (2010). Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. Microcirculation *17*, 206-225.

Fukumura, D., and Jain, R.K. (2008). Imaging angiogenesis and the microenvironment. APMIS *116*, 695-715.

Galletta, B.J., Mooren, O.L., and Cooper, J.A. (2010). Actin dynamics and endocytosis in yeast and mammals. Curr Opin Biotechnol *21*, 604-610.

Germain, R.N., Castellino, F., Chieppa, M., Egen, J.G., Huang, A.Y., Koo, L.Y., and Qi, H. (2005). An extended vision for dynamic high-resolution intravital immune imaging. Semin Immunol *17*, 431-441.

Ghajar, C.M., and Bissell, M.J. (2008). Extracellular matrix control of mammary gland morphogenesis and tumorigenesis: insights from imaging. Histochem Cell Biol *130*, 1105-1118.

Goldfine, I.D., German, M.S., Tseng, H.C., Wang, J., Bolaffi, J.L., Chen, J.W., Olson, D.C., and Rothman, S.S. (1997). The endocrine secretion of human insulin and growth hormone by exocrine glands of the gastrointestinal tract. Nat Biotechnol *15*, 1378-1382.

Gorr, S.U., Venkatesh, S.G., and Darling, D.S. (2005). Parotid secretory granules: crossroads of secretory pathways and protein storage. J Dent Res *84*, 500-509.

Grant, B.D., and Donaldson, J.G. (2009). Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol *10*, 597-608.

Gualda, E.J., Filippidis, G., Voglis, G., Mari, M., Fotakis, C., and Tavernarakis, N. (2008). In vivo imaging of cellular structures in Caenorhabditis elegans by combined TPEF, SHG and THG microscopy. J Microsc *229*, 141-150.

Guan, Y., Worrell, R.T., Pritts, T.A., and Montrose, M.H. (2009). Intestinal ischemia-reperfusion injury: reversible and irreversible damage imaged in vivo. Am J Physiol Gastrointest Liver Physiol *297*, G187-196.

Hall, A.M., Unwin, R.J., Parker, N., and Duchen, M.R. (2009). Multiphoton imaging reveals differences in mitochondrial function between nephron segments. J Am Soc Nephrol *20*, 1293-1302.

Honigman, A., Zeira, E., Ohana, P., Abramovitz, R., Tavor, E., Bar, I., Zilberman, Y., Rabinovsky, R., Gazit, D., Joseph, A., *et al.* (2001). Imaging transgene expression in live animals. Mol Ther *4*, 239-249.

Ishibashi, K., Okamura, K., and Yamazaki, J. (2008). Involvement of apical P2Y2 receptor-regulated CFTR activity in muscarinic stimulation of Cl(-) reabsorption in rat submandibular gland. Am J Physiol Regul Integr Comp Physiol *294*, R1729-1736.

Ishibashi, K., Yamazaki, J., Okamura, K., Teng, Y., Kitamura, K., and Abe, K. (2006). Roles of CLCA and CFTR in electrolyte re-absorption from rat saliva. J Dent Res *85*, 1101-1105.

Kagami, H., Atkinson, J.C., Michalek, S.M., Handelman, B., Yu, S., Baum, B.J., and O'Connell, B. (1998). Repetitive adenovirus administration to the parotid gland: role of immunological barriers and induction of oral tolerance. Hum Gene Ther *9*, 305-313.

Lippincott-Schwartz, J. (2011a). Bridging structure and process in developmental biology through new imaging technologies. Dev Cell *21*, 5-10.

Lippincott-Schwartz, J. (2011b). Emerging in vivo analyses of cell function using fluorescence imaging (*). Annu Rev Biochem *80*, 327-332.

Masedunskas, A., Sramkova, M., Parente, L. and Weigert, R. (2011b). Intravital microscopy to image membrane trafficking in live rats. Method in Mol Biol. (in press)

Masedunskas, A., Sramkova, M., Parente, L., Sales, K.U., Amornphimoltham, P., Bugge, T.H., and Weigert, R. (2011). Role for the actomyosin complex in regulated exocytosis revealed by intravital microscopy. Proc Natl Acad Sci U S A *108*, 13552-13557.

Masedunskas, A., and Weigert, R. (2008). Intravital two-photon microscopy for studying the uptake and trafficking of fluorescently conjugated molecules in live rodents. Traffic *9*, 1801-1810.

Mastrangeli, A., O'Connell, B., Aladib, W., Fox, P.C., Baum, B.J., and Crystal, R.G. (1994). Direct in vivo adenovirus-mediated gene transfer to salivary glands. Am J Physiol *266*, G1146-1155.

Maxfield, F.R., and McGraw, T.E. (2004). Endocytic recycling. Nat Rev Mol Cell Biol *5*, 121-132.

Mayor, S., and Pagano, R.E. (2007). Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol *8*, 603-612.

McMullen, J.D., and Zipfel, W.R. (2010). A multiphoton objective design with incorporated beam splitter for enhanced fluorescence collection. Opt Express *18*, 5390-5398.

Mellman, I. (1996). Endocytosis and molecular sorting. Annu Rev Cell Dev Biol *12*, 575-625.

Melvin, J.E., Yule, D., Shuttleworth, T., and Begenisich, T. (2005). Regulation of fluid and electrolyte secretion in salivary gland acinar cells. Annu Rev Physiol *67*, 445-469.

Mertz, J. (2004). Nonlinear microscopy: new techniques and applications. Curr Opin Neurobiol *14*, 610-616.

Miller, M.J., Wei, S.H., Parker, I., and Cahalan, M.D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science *296*, 1869-1873.

Mizrahi, A., Crowley, J.C., Shtoyerman, E., and Katz, L.C. (2004). High-resolution in vivo imaging of hippocampal dendrites and spines. J Neurosci *24*, 3147-3151.

Molitoris, B.A., and Sandoval, R.M. (2006). Pharmacophotonics: utilizing multi-photon microscopy to quantify drug delivery and intracellular trafficking in the kidney. Adv Drug Deliv Rev *58*, 809-823.

Morita, T., Tanimura, A., Shitara, A., Suzuki, Y., Nezu, A., Takuma, T., and Tojyo, Y. (2011). Expression of functional Stim1-mKO1 in rat submandibular acinar cells by retrograde ductal injection of an adenoviral vector. Arch Oral Biol.

Nashida, T., Yoshie, S., Imai, A., and Shimomura, H. (2004). Presence of cytoskeleton proteins in parotid glands and their roles during secretion. Arch Oral Biol *49*, 975-982.

Niedzinski, E.J., Chen, Y.J., Olson, D.C., Parker, E.A., Park, H., Udove, J.A., Scollay, R., McMahon, B.M., and Bennett, M.J. (2003a). Enhanced systemic transgene expression after nonviral salivary gland transfection using a novel endonuclease inhibitor/DNA formulation. Gene Ther *10*, 2133-2138.

Niedzinski, E.J., Olson, D.C., Chen, Y.J., Udove, J.A., Nantz, M.H., Tseng, H.C., Bolaffi, J.L., and Bennett, M.J. (2003b). Zinc enhancement of nonviral salivary gland transfection. Mol Ther *7*, 396-400.

Orth, J.D., Kohler, R.H., Foijer, F., Sorger, P.K., Weissleder, R., and Mitchison, T.J. (2011). Analysis of mitosis and antimitotic drug responses in tumors by in vivo microscopy and single-cell pharmacodynamics. Cancer Res *71*, 4608-4616.

Palaniyandi, S., Odaka, Y., Green, W., Abreo, F., Caldito, G., De Benedetti, A., and Sunavala-Dossabhoy, G. (2011). Adenoviral delivery of Tousled kinase for the protection of salivary glands against ionizing radiation damage. Gene Ther *18*, 275-282.

Pan, F., and Gan, W.B. (2008). Two-photon imaging of dendritic spine development in the mouse cortex. Dev Neurobiol *68*, 771-778.

Passineau, M.J., Zourelias, L., Machen, L., Edwards, P.C., and Benza, R.L. (2010). Ultrasound-assisted non-viral gene transfer to the salivary glands. Gene Ther *17*, 1318-1324.

Perez, P., Adriaansen, J., Goldsmith, C.M., Zheng, C., and Baum, B.J. (2011). Transgenic alpha-1-antitrypsin secreted into the bloodstream from salivary glands is biologically active. Oral Dis *17*, 476-483.

Peter, B., Van Waarde, M.A., Vissink, A., s-Gravenmade, E.J., and Konings, A.W. (1995). Degranulation of rat salivary glands following treatment with receptor-selective agonists. Clin Exp Pharmacol Physiol *22*, 330-336.

Radosevich, A.J., Bouchard, M.B., Burgess, S.A., Chen, B.R., and Hillman, E.M. (2008). Hyperspectral in vivo two-photon microscopy of intrinsic contrast. Opt Lett *33*, 2164-2166.

Riedl, J., Crevenna, A.H., Kessenbrock, K., Yu, J.H., Neukirchen, D., Bista, M., Bradke, F., Jenne, D., Holak, T.A., Werb, Z., *et al.* (2008). Lifeact: a versatile marker to visualize F-actin. Nat Methods *5*, 605-607.

Riedl, J., Flynn, K.C., Raducanu, A., Gartner, F., Beck, G., Bosl, M., Bradke, F., Massberg, S., Aszodi, A., Sixt, M., *et al.* (2010). Lifeact mice for studying F-actin dynamics. Nat Methods *7*, 168-169.

Roberts, M.S., Roberts, M.J., Robertson, T.A., Sanchez, W., Thorling, C., Zou, Y., Zhao, X., Becker, W., and Zvyagin, A.V. (2008). In vitro and in vivo imaging of xenobiotic transport in human skin and in the rat liver. J Biophotonics *1*, 478-493.

Sakai, T., Kawaguchi, M., and Kosuge, Y. (2009). siRNA-mediated gene silencing in the salivary gland using in vivo microbubble-enhanced sonoporation. Oral Dis *15*, 505-511.

Samuni, Y., Zheng, C., Cawley, N.X., Cotrim, A.P., Loh, Y.P., and Baum, B.J. (2008). Sorting of growth hormone-erythropoietin fusion proteins in rat salivary glands. Biochem Biophys Res Commun *373*, 136-139.

Sandoval, R.M., Kennedy, M.D., Low, P.S., and Molitoris, B.A. (2004). Uptake and trafficking of fluorescent conjugates of folic acid in intact kidney determined using intravital two-photon microscopy. Am J Physiol Cell Physiol *287*, C517-526.

Sandoval, R.M., and Molitoris, B.A. (2008). Quantifying endocytosis in vivo using intravital two-photon microscopy. Methods Mol Biol *440*, 389-402.

Schenke-Layland, K., Xie, J., Angelis, E., Starcher, B., Wu, K., Riemann, I., MacLellan, W.R., and Hamm-Alvarez, S.F. (2008). Increased degradation of extracellular matrix structures of lacrimal glands implicated in the pathogenesis of Sjogren's syndrome. Matrix Biol *27*, 53-66.

Segawa, A., Loffredo, F., Puxeddu, R., Yamashina, S., Testa Riva, F., and Riva, A. (1998). Exocytosis in human salivary glands visualized by high-resolution scanning electron microscopy. Cell Tissue Res *291*, 325-336.

Segawa, A., and Riva, A. (1996). Dynamics of salivary secretion studied by confocal laser and scanning electron microscopy. Eur J Morphol *34*, 215-219.

Segawa, A., Terakawa, S., Yamashina, S., and Hopkins, C.R. (1991). Exocytosis in living salivary glands: direct visualization by video-enhanced microscopy and confocal laser microscopy. Eur J Cell Biol *54*, 322-330.

Serrano-Pozo, A., Mielke, M.L., Gomez-Isla, T., Betensky, R.A., Growdon, J.H., Frosch, M.P., and Hyman, B.T. (2011). Reactive Glia not only Associates with Plaques but also Parallels Tangles in Alzheimer's Disease. Am J Pathol *179*, 1373-1384.

Sokac, A.M., and Bement, W.M. (2006). Kiss-and-coat and compartment mixing: coupling exocytosis to signal generation and local actin assembly. Mol Biol Cell *17*, 1495-1502.

Sramkova, M., Masedunskas, A., Parente, L., Molinolo, A., and Weigert, R. (2009). Expression of plasmid DNA in the salivary gland epithelium: novel approaches to study dynamic cellular processes in live animals. Am J Physiol Cell Physiol *297*, C1347-1357.

Svoboda, K., and Yasuda, R. (2006). Principles of two-photon excitation microscopy and its applications to neuroscience. Neuron *50*, 823-839.

Tada, H., Higuchi, H., Wanatabe, T.M., and Ohuchi, N. (2007). In vivo real-time tracking of single quantum dots conjugated with monoclonal anti-HER2 antibody in tumors of mice. Cancer Res *67*, 1138-1144.

Textor, J., Peixoto, A., Henrickson, S.E., Sinn, M., von Andrian, U.H., and Westermann, J. (2011). Defining the quantitative limits of intravital two-photon lymphocyte tracking. Proc Natl Acad Sci U S A *108*, 12401-12406.

Theer, P., Hasan, M.T., and Denk, W. (2003). Two-photon imaging to a depth of 1000 microm in living brains by use of a Ti:Al2O3 regenerative amplifier. Opt Lett *28*, 1022-1024.

Toma, I., Kang, J.J., and Peti-Peterdi, J. (2006). Imaging renin content and release in the living kidney. Nephron Physiol *103*, p71-74.

Voutetakis, A., Kok, M.R., Zheng, C., Bossis, I., Wang, J., Cotrim, A.P., Marracino, N., Goldsmith, C.M., Chiorini, J.A., Loh, Y.P., *et al.* (2004). Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. Proc Natl Acad Sci U S A *101*, 3053-3058.

Wang, S., Baum, B.J., Yamano, S., Mankani, M.H., Sun, D., Jonsson, M., Davis, C., Graham, F.L., Gauldie, J., and Atkinson, J.C. (2000). Adenoviral-mediated gene transfer to mouse salivary glands. J Dent Res *79*, 701-708.

Warner, J.D., Peters, C.G., Saunders, R., Won, J.H., Betzenhauser, M.J., Gunning, W.T., 3rd, Yule, D.I., and Giovannucci, D.R. (2008). Visualizing form and function in organotypic slices of the adult mouse parotid gland. Am J Physiol Gastrointest Liver Physiol *295*, G629-640.

Weigert, R., Sramkova, M., Parente, L., Amornphimoltham, P., and Masedunskas, A. (2010). Intravital microscopy: a novel tool to study cell biology in living animals. Histochem Cell Biol *133*, 481-491.

Xu, R., Boudreau, A., and Bissell, M.J. (2009). Tissue architecture and function: dynamic reciprocity via extra- and intra-cellular matrices. Cancer Metastasis Rev *28*, 167-176.

Zhang, S., and Murphy, T.H. (2007). Imaging the impact of cortical microcirculation on synaptic structure and sensory-evoked hemodynamic responses in vivo. PLoS Biol *5*, e119.

Zheng, C., Cotrim, A.P., Sunshine, A.N., Sugito, T., Liu, L., Sowers, A., Mitchell, J.B., and Baum, B.J. (2009). Prevention of radiation-induced oral mucositis after adenoviral vector-mediated transfer of the keratinocyte growth factor cDNA to mouse submandibular glands. Clin Cancer Res *15*, 4641-4648.

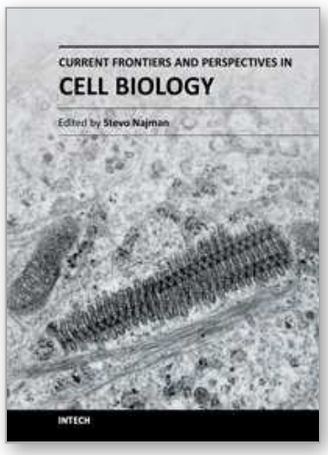
Zheng, C., Shinomiya, T., Goldsmith, C.M., Di Pasquale, G., and Baum, B.J. (2011). Convenient and reproducible in vivo gene transfer to mouse parotid glands. Oral Dis *17*, 77-82.

Zhong, Z., Ramshesh, V.K., Rehman, H., Currin, R.T., Sridharan, V., Theruvath, T.P., Kim, I., Wright, G.L., and Lemasters, J.J. (2008). Activation of the oxygen-sensing signal cascade prevents mitochondrial injury after mouse liver ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol *295*, G823-832.

Zipfel, W.R., Williams, R.M., Christie, R., Nikitin, A.Y., Hyman, B.T., and Webb, W.W. (2003a). Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. Proc Natl Acad Sci U S A *100*, 7075-7080.

Zipfel, W.R., Williams, R.M., and Webb, W.W. (2003b). Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol *21*, 1369-1377.

Zoumi, A., Yeh, A., and Tromberg, B.J. (2002). Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. Proc Natl Acad Sci U S A *99*, 11014-11019.



Current Frontiers and Perspectives in Cell Biology
Edited by Prof. Stevo Najman

ISBN 978-953-51-0544-2
Hard cover, 556 pages
Publisher InTech
Published online 25, April, 2012
Published in print edition April, 2012

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Monika Sramkova, Natalie Porat-Shliom, Andrius Masedunkas, Timothy Wigand, Panomwat Amornphimoltham and Roberto Weigert (2012). Salivary Glands: A Powerful Experimental System to Study Cell Biology in Live Animals by Intravital Microscopy, Current Frontiers and Perspectives in Cell Biology, Prof. Stevo Najman (Ed.), ISBN: 978-953-51-0544-2, InTech, Available from: http://www.intechopen.com/books/current-frontiers-and-perspectives-in-cell-biology/novel-imaging-approaches-to-study-cell-biology-in-live-animals

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen