Applications of confocal microscopy to the study of vascular biology

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Confocal microscopy has contributed to important findings in biology and medicine, because it enables the acquisition of fluorescence images of tissues and cells. Regarding vascular disorders, this technique has become an indispensable tool for the clarification of functions in vascular biological systems through the utilization of specific fluorescent probes. Confocal microscopy is useful for the visualization of whole cells and for the detection of the subcellular distribution of proteins, organelles, cations such as Ca^{2+}, small biomolecules like nitric oxide (NO), and enzyme activities in living or fixed cells or tissues. This technique also provides relevant information through consideration of controllable field depth, elimination of out-of-focus information, and collection of serial optical sections from thick specimens. In *in vivo* animal studies, the aortic endothelium has a thickness of 100 μm, which makes the acquisition of images under a conventional microscope difficult. The confocal fluorescence microscope is a suitable alternative for the attainment of sectioned images of not only the endothelium layer but also the smooth muscle. In this context, in this chapter we will present a mini-review of the data on vascular fluorescence confocal microscopy from our laboratory and the literature, and we will describe research on the cellular and molecular mechanisms underlying the physiology and pathogenesis related to vascular beds, including cardiac tissues.

Keywords: cardiovascular fluorescence confocal microscopy; Ca^{2+}; NO; reactive oxygen species (ROS); membrane potential

1. Introduction

Confocal laser scanning microscopy has proved to be effective at revealing details of the ultrastructure of several specimens through specific fluorescent probes (fluorophores). These probes are immunofluorescence reagents that allow for investigation of cell structure and function via their binding to specific targets. Confocal microscopy has found an important role in biology and medicine, especially with respect to the fluorescence imaging of tissues [1-5]. In the case of vascular biology studies, a relevant advantage of confocal microscopy over conventional wide-field microscopy is that it can reveal the three-dimensional structure of the layers of vascular tissues. Confocal imaging in living specimens is feasible, thereby enabling examination of dynamic processes such as vesicle trafficking [6], spine motility [7], cytoskeletal assembly and turnover, chromosome dynamics [8], and molecular binding interactions [9].

This chapter aims to provide a brief description of the laser scanning confocal microscopy (LSCM) technique with a focus on living cardio and vascular tissues. The concepts of image formation and sample preparation will be dealt with first. Next, the confocal microscopy protocols applied in our laboratory and our most important results will be presented in parallel with literature data.

2. Microscopy techniques: from optical to confocal

2.1 Microscopy techniques

Didactically speaking, it is possible to organize microscopy techniques into (1) Light Microscopy or Optical Microscopy, which include (epi)fluorescence; (2) Electronic Microscopy, which comprises both transmission and scanning microscopy; and (3) Confocal Microscopy, including laser scanning confocal microscopy (LSCM).

Light Microscopy involves the following contrast modes: bright field, dark field, phase contrast, differential interference contrast (DIC), interference reflection (IRM), polarization, and fluorescence. Comparing fluorescence microscopy with bright field microscopy, in the former case low signal levels are detected against a black background instead of a light background, with consequent improvement in the effective detection sensitivity. As for fluorescence microscopy, the intrinsic limitation is to obtain and apply the appropriate fluorescent probes. The signal-to-noise performance actually achieved by conventional fluorescence microscopy, which is often severely compromised by interference from light emitted by any fluorochrome that is situated out of the plane of focus, gives rise to the background glare [10]. Important probes have been researched and produced, so that their optical features can be modified under stimulation of specific components of their environment. Useful indicators such as probes for Ca^{2+}, pH, ATP, and membrane potential allow the cell to develop its physiological functions while it is being optically monitored. However, in conventional fluorescence microscopy the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly with the naked eye [11]. Thus, the inspection of thick specimens is another important limitation of wide-field microscopy as they are seen as the sum of sharp images of an in-focus region and

blurred images of all the out-of-focus regions [12]. These technical lacunae of conventional microscopy are brilliantly filled by confocal microscopy.

2.2 Confocal microscopy: History and principles

The inventor of the confocal microscope was Marvin Minsky, who designed it in 1955 while he was aiming to improve his work involving neural images in living cells [13]. This researcher put forward the principles of the confocal scanning microscope, and the patent came in 1957 [14]. The microscope model designed by Minsky consisted of a stage scanning system triggered by a primitive tuning fork arrangement, but construction of an image was rather slow. Nowadays, the image is either built up from the output of a photomultiplier tube or captured with the aid of a digital charge-coupled device camera (CCD), directly processed in a computer imaging system, displayed on a high-resolution video monitor, and recorded on hardcopy devices, with outstanding results [11]. Yet, it was 45 years ago that Egger and Petran presented the first experimental results by using the confocal microscope [15].

Nowadays, the most common type of microscope calibration is the confocal imaging system, which comprises a laser confocal scanning microscope (LSCM) with a pinhole aperture in the image plane objective, whereby light is emanated only from the point that is being illuminated. The unwanted fluorescence originated away from this point is blocked. In this way, most of the out-of-focus light that normally occurs in conventional fluorescence microscopy is excluded from the final image in the case of confocal microscopy. This greatly increases the contrast and hence the visibility of fine detail in the specimen. The final resolution is therefore due to some basic components such as light wavelength, objective lens, and sample properties.

When imaging experiments are conducted by conventional light microscopy, the fluorescence in the specimen away from the region of interest (ROI) interferes with the resolution of the structures in focus, mainly for specimens thicker than 2 μm (Paddock, chapter 1, vol. 122, human press). Confocal microscopy, on the other hand, selectively captures light from a thin (<1 μm) optical section at the plane of focus in the specimen. Because there is essentially no flare of light from out-of-focus areas, structures within the focal plane appear more sharply defined as compared to the image obtained with a conventional microscope [16].

The confocal approach provides a punctual control of the increase in both lateral and axial resolution [11]. A three-dimensional view of the specimen can be reconstructed from a series of optical sections at different depths [16]. The maximum depth into the specimen at which images can be captured depends on the transparency of the specimen, characteristics of the objective, and excitation wavelength. Single-photon excitation with visible wavelengths (450 to 650 nm) can typically penetrate from 50 to 200 μm. Multiphoton excitation with infrared wavelengths (>700 nm) can penetrate deeper, up to 1 mm in some circumstances.

The capability of confocal microscopy to perform optical sectioning makes it well suited for the study of cell structure and function using immunofluorescence reagents [17], organic dyes, fluorescent fusion proteins [18], quantum dots [19], and fluorescence *in situ* hybridization (FISH) [20].

There are two feasible ways of obtaining optical sections: multiple photon imaging and deconvolution [21-22].

The laser scanning confocal microscope (LSCM) captures images by scanning the specimen with a focused light beam from a laser and collecting the emitted fluorescence signals with a photodetector [23]. The photodetectors present in LSCMs consist of photomultiplier tubes (PMTs) that generate electrons at a rate proportional to the intensity of the incoming fluorescence signal. LSCMs are sometimes referred to as "spot-scanning" confocal microscopes, so that they can be distinguished from microscopes that scan the specimen with a slit of light (slit-scanning) or multiple spots of light (Spinning-disk or Nipkow disk). Spot-scanning LSCMs typically have slower image acquisition rates than the slit-scanning and the spinning-disk microscopes (<1 frame/sec vs. 30 frames/sec or higher). However, they are more versatile in a number of ways. They can accommodate lasers with a wide range of wavelengths (from the UV to the infrared) and can be configured to image multiple fluorophores either simultaneously or sequentially. Some of these microscopes include spectral detectors that can capture the entire spectrum of the fluorescence emitted at each pixel in the image. The most sophisticated LSCMs allow the user to control the illumination wavelength and intensity on a microsecond timescale. This feature enables the conduction of experiments that require selective illumination of fluorophores in a defined region of interest in order to photobleach or photoactivate them. Measurement of fluorescence recovery after photobleaching (FRAP) or fluorescence loss during photobleaching (FLIP) can provide information about molecular mobility and binding [24-27]. Photosensitive molecules include certain fluorescent proteins (e.g., see [28] for the fluorescent protein PaGFP, and [29] for the photosensitive protein Kaede), "caged" molecules such as caged Ca^{2+} chelators, neurotransmitters, and second messengers [30].

Fluorescence recovery after photobleaching (FRAP) was originally designed for measurement of the diffusion rates of fluorescently tagged proteins in organelles and cell membranes. A small spot on the specimen is continuously illuminated at a low light flux level during the regular fluorescence recovery, taking place after the photobleaching (FRAP) procedure, and the emitted fluorescence is then measured. Next, the illumination level is increased to a very high level for a brief time, to destroy the fluorescent molecules in the illuminated region by rapid bleaching.

Confocal microscopy can also be used for determination of the fluorescence resonance energy transfer (FRET) [31]. FRET is indicated in the research of kinetics; *i.e.*, when the excited-state decay kinetics of the donor or the acceptor fluorescence is measured. Fluorescence lifetime imaging microscopy (FLIM) should be used when it is necessary to

obtain a single lifetime of the fluorescence at each position in an image, since this technique makes use of the decrease in the fluorescence lifetime of the donor owing to the depopulation of its excited state by FRET [32-34]. Actually, a wide-field microscope where the spatial resolution is reduced is disadvantageous compared with intensity-based FRET measurements, but confocal fluorescence lifetime microscopy does not suffer from that drawback. An important feature of FRET imaging with FLIM is the simultaneous detection of the acceptor and the donor fluorescence decay kinetics, which allows for the utilization of spectrally similar donor–acceptor pairs [35]. FRET provides the detailed workings of the protein machines underlying the various cellular processes that are being investigated, which would otherwise be compromised by the preparative steps unavoidable in biochemical approaches. Additionally, the use of FLIM enables the simultaneous detection of multiple protein interactions [34].

The instruments equipped with 3-5 laser systems controlled by high-speed acousto-optic tunable filters (AOTFs) allow for the very precise regulation of wavelength and excitation intensity. The advantage of AOTF is that it modulates the wavelength and amplitude of the illuminating laser light in the latest generation of confocal microscopes. These filters do not suffer from the mechanical constraints, speed limitations, image shift, and vibration associated with rotating filter wheels, and they can easily accommodate several laser systems tuned to different output wavelengths. In addition, unlike fluorescence interference filters, acousto-optic filters do not deteriorate upon exposure to heat and intense light. When coupled with photomultipliers that have high quantum efficiency in the near-ultraviolet, visible, and near-infrared spectral regions, the microscopes with AOTFs are able to examine fluorescence emission ranging from 400 to 750 nanometers [36,37].

According to Klonis et al [38] and Lippincott-Schwartz et al [26], the development of AOTFs has provided substantial additional versatility to techniques such as fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching [39], and localized photoactivated fluorescence uncaging studies [37, 40].

As reviewed by Claxton, Fellers and Davidson [37], not only does AOTF allow for the near-instantaneous switching of light intensity, but it can also be utilized for the selective bleaching of randomly specified regions of irregular shape, lines, or specific cellular organelles and for the determination of the dynamics of molecular transfer into the region.

2.3 Modes of image formation and care of living specimens

The important step in practical approaches is to find a way of keeping the specimens alive on the microscope stage, or to maintain the target part of the tissue accessible to the objective lens. Our group preferentially employs the live data acquisition mode, a kind of time-lapse mode, to monitor the movement of ions and molecules such as Ca^{2+}, NO (nitric oxide), and ROS (reactive oxygen species) into the cytosol. To avoid photodamage by the laser beam, we always use the minimum laser exposure, since excess laser could accumulate over the multiple scans. According to some authors, it is safe to add antioxidants, such as ascorbic acid, in order to reduce the oxygen from excited molecules. This is because the latter could cause free radicals to form and thus impair cell activity [41]. Nevertheless, we usually perform a wide group of preliminary control experiments, mainly when our aim is to study ROS and their relation with vascular (dys)functions. Some of our procedures are described below.

Another mode of image acquisition is the use of the *Z*-series and three-dimensional (3D) imaging. A *Z*-series is a sequence of optical sections collected from specimens at differential levels. In other words, upon acquisition of the *Z*-series the researcher captures the depth of the specimen in the image. This kind of acquisition mode is ideal for further processing into a 3D representation using volume visualization techniques [42]. The series of optical sections from a time-lapse run (live data mode) can also be processed into a 3D representation of the data set, so that time is the *Z*-axis. Therefore, our preferred mode of image collection from living cells or living artery slices involves the x-y-*t* axes. For both the live data mode and the *Z*-series it is possible to process the data by means of programs available on the web, such as Image-J or Adobe Photoshop [43].

Martisek and Martisek [44] have presented an important approach that enables three-dimensional (3D) reconstructions of cells by confocal microscopes. Their work has allowed for the mathematical setup of experiments aiming at the development of direct volume measurements on cell structure by means of methods that apply 3D filters to set the output image sharpness in relation to the noises.

Besides the three spatial dimensions (*x, y*, and *z*), there is the time dimension [45]. Thus, in the laser scanning confocal microscopes (LSCM), it is also possible to collect time-lapse sequences of *Z*-series from living preparations in a four-dimensional (4D) imaging setting, namely the *x*-*y*-*z*-*t*.

2.3.1 Setup of time-lapse experiments

Our main confocal microscopy protocols concerning vascular functions are based on experiments that study the effect of drugs on living cells or tissue slices [46-49]. In this sense, we have standardized a set of procedures by using the time lapse concept, which involves: 1- the observation of specimens without any addition of drugs or control solutions for a certain time; 2- the inspection of specimens after addition of the drug, when the cells or tissue slices react and the organelles move much more rapidly and hence imaging parameters can be adapted accordingly by pre-defining short time intervals for data acquisition for a certain time; and 3- the examination of specimens when five minutes have

elapsed since drug addition in cases that the cells or tissues react slowly and data acquisition can be performed with longer time intervals.

Ensuring the quality of the protocols designed for laser scanning confocal microscopy (LSCM) is crucial, especially in the case of experiments on living cells. Zucker and Price [50] have developed quality assurance tests that guarantee the stability of LSCM in terms of maintenance of reproducible intensity measurements with excellent image quality. The authors have evaluated the performance of the dichroic reflectivity, field illumination, lens performance, laser power output, spectral registration, axial resolution, laser stability, photomultiplier tube (PMT) reliability, and system noise. The specificity of the tests allows for better recognition of instrument functionality and problems as compared to a biological/ histological sample.

3. Confocal microscopy in the cardiovascular studies

Application of the knowledge about confocal images acquisition modes has enabled the development of appropriate ways of improving cardiovascular functions. According to Niggli and Lederer [51], real-time confocal microscopy is the best approach to the study of heart muscle cells, especially in terms of Ca^{2+} measurements, due to its inherent good spatial and temporal resolution under specific conditions. Thus, live heart cells imaging is the confocal acquisition mode that offers the best prospect of this organ. The temporal resolution of the laser scan system can be configured in a very flexible way, in order to reduce spatial information and increase temporal resolution. In this way, many different image shapes and sizes can be scanned.

A selection of probes and fluorescent indicators for a spectrum of intracellular constituents is available for application in confocal microscopy using living tissue, primarily in cell cultures and isolated cells. For an overview of the main probes used in vascular studies, it is possible to consult the practical literature [52-53]. Regarding general studies, we encourage consultation of companies Web pages and/or catalogs for the search of probes that can be used during emission and excitation spectra acquisition. Anyway, the features of the probes that are frequently employed in confocal microscopy for the study of cardiovascular functions and their respective calibrations will be presented below.

The utilization of laser scanning confocal microscopy (LSCM) as a methodology for the visualization, quantification, and elucidation of the vascular structure is well established [54-59]. It has been shown how LSCM can be used for the identification of cellular aspects of morphological changes such as the subcutaneous ischemia and vessels skeletal muscle resistance occurring in patients with critical limb ischemia, a hypotensive/hypoperfusion state of the lower leg.

Arribas et al. [55-56] and Coats et al. [59] have pointed out alterations in cell number, density, size, and shape in intact resistance arteries from several rat models of hypertension and in critical limb ischemia in humans. 3D confocal microscopy allowed this group of authors to study intact blood vessels without 3D distortion and enabled them to determine the changes in cellular orientation in the *x*, *y*, and *z* axis, which might represent migratory processes. As already described, confocal microscopy produces optical sections throughout relatively thick tissues without the need for cutting thin slices. Moreover, it eliminates blur and flare from out-of-focus planes in an object, thereby greatly improving axial resolution [60]. Arribas et al [61] have put together pressure myography and confocal microscopy for evaluation of the vascular wall at the cellular level in intact arteries maintained at their physiological shape and pressurization level. The authors called this technique 'confocal myography'. According to them, five simple technical processes, namely vessel pressurization, staining, image capturing following 3D reconstruction, and quantification are necessary in a relatively short time [62].

3.1 Ca^{2+} as indicator of cardiovascular functions and its fluorescent indicators: settings for confocal microscopy

Calcium plays an important role in cell survival, proliferation, motility, apoptosis, and differentiation [63]. It also has regulatory functions since it operates as an allosteric activator or an inhibitor of several intracellular enzymes in the cytosol, organelles, and nucleus. With respect to cardiovascular functions, as in the case of other smooth muscles, the free cytosolic Ca^{2+} level is the major determinant of contractile activity activation [64]. Therefore, a relevant experiment for investigation of the properties of cardio and vascular tissues involves examination of the cellular Ca^{2+} transients.

Studies on the spatiotemporal intracellular Ca^{2+} dynamics have been improved by introduction of laser confocal scanning microscopy (LCSM). Hence, the Ca^{2+} microdomains have been revealed in excitable and non-excitable cells, and particular attention has been focused on the basis of the specificity of the calcium-mediated cellular response.

Calcium signals are produced by sudden increases in the intracellular Ca^{2+} concentration. The most effective techniques for measuring this concentration involve the use of fluorescent or luminescent probes. Two types of probes are widely employed: those that undergo a change in fluorescence upon Ca^{2+} binding and those that emit light when bound to Ca^{2+} [65].

The group of Tsien [66,67] has provided important tools for the inspection of Ca^{2+} transients by fluorescence studies. Kao et al [68] have demonstrated that, contrary to those indicators that require UV excitation and are sensitive to photolysis, fluo-3 AM can be easily loaded into cells. This allows for continuous monitoring of the intracellular Ca^{2+}

concentration without interfering with the UV-triggered release of inositol polyphosphates or Ca^{2+}. As a result, the Ca^{2+} levels can be observed by mitogen or hormone stimulations both pre- and post-photolysis.

The spatial and temporal distribution of Ca^{2+} transients has been documented in voltage clamped vertebral neurons by using the fluorescent Ca^{2+} indicator fluo-3 and a laser scanning confocal microscope [69].

Bkaily et al [53] have presented an elucidative work and protocols regarding the use of confocal microscopy in the investigation of cell structure and function in the heart, vascular endothelium, and smooth muscle cells. These authors used freshly cultured single cells from heart and vascular tissues (endothelial and smooth muscle) primary cultures for the determination of cytosolic and nuclear free Ca^{2+} using 2D and 3D confocal microscopy. Several visible wavelength Ca^{2+} dyes were tested, namely fluo-3, Fura Red, Calcium Green-1, and Calcium Orange, in order to select the ion indicator that was best suited for laser-based confocal microscopy in vascular and heart tissues. In this sense, especially in the case of Ca^{2+} studies, the following points must be carefully observed during calibration of one's own experiments: (1) cell type and origin; (2) experimental conditions (pH, temperature, etc); (3) use of 2D, 3D, or 4D measurements; (4) precise determination of nuclear and cytosolic volume and localization.

Fluo3-AM is the fluorescent indicator that is most often applied by our group in confocal experiments for evaluation of vascular tissues or cells such as aorta, tail, or mesenteric arteries [47].

Since its introduction in 1989 [70], imaging has revealed the spatial dynamics of many elementary processes involved in Ca^{2+}signaling [71-73]. Fluo-3 has also been extensively used in flow cytometry [74], in experiments involving the photoactivation of "caged" chelators, second messengers, and neurotransmitters [75,76], and in cell-based pharmacological screening [77]. The most important properties of fluo-3 in these applications are an absorption spectrum compatible with excitation at 488 nm by argon-ion laser sources and very large fluorescence intensity increase in response to Ca^{2+} binding.

Confocal microscopy is useful for both cardiomyocytes and blood vessels studies. Blood vessels, which are composed of different cells, namely endothelial, smooth muscle, fibroblasts, and other less well-characterized cell types, exhibit some elements with auto-fluorescent properties that can be used for visualization and/or quantification. Wong and Langille [78] have demonstrated that the fibrous extracellular matrix proteins collagen and elastin display auto-fluorescence in the region of 488/515 nm.

For confocal microscopy, cells are loaded with Ca^{2+} indicators by injection via patch-clamp techniques or by means of membrane crossing. The first technique is commonly applied to cultured cells, nerve cells, or brain slices, while the second is usually employed in the case of muscles cells or muscle slices (cardiac and vascular). In the latter case, the use of a permeable membrane form is necessary, so acetoxymethylester (AM) is the main fluorescent indicator complex in this case. The AM ester form allows for an indicator to cross the plasma membrane, and then the probe is metabolically trapped inside the cell by de-esterification, when it loses its AM ester radical. It is important to use cell permeable fluorescent indicators, but the main problems inherent to this technique are incomplete de-esterification, unknown compartmental distribution, and/or binding of the indicators. Actually, the presence of Ca^{2+} in the nucleus is often difficult to ascertain because the fluorescence derived from the perinuclear area interferes with that of the nucleus.

The AM esters must be reconstituted in anhydrous di-methylsulfoxide (DMSO) and used as soon as possible thereafter (within a week), to avoid decomposition with subsequent loss of the cell loading capacity. DMSO stock solutions of AM esters should be stored in the absence of light, in a freezer and desiccator. Stock solutions of the salts may be prepared in distilled water or aqueous buffers and stored at ≤ -20 °C, protected from light. These solutions should be stable for at least six months.

Using confocal microscopy Burnier et al [79] have analyzed the cytosolic and nuclear calcium distribution in vascular smooth muscle and endothelial cells using two Ca^{2+}-sensitive dyes, namely acetoxymethyl esters of fluo-3 (fluo-3 AM) and rhod 2 (rhod-2 AM), simultaneously. In the case of fluo-3 AM, the baseline fluorescence was located in the cytoplasm, but it was slightly higher in the nucleus. In the presence of all the stimuli (receptor-dependent and receptor-independent), the fluorescence intensity increased in both compartments, but it remained more pronounced within the nucleus. Yet, after calibration, the cytosolic Ca^{2+} concentration was greater than that of the nucleus at rest and was equally high after stimulation, suggesting different properties of fluo-3 in the cytosol and in the nucleus. With rhod-2 AM, the baseline fluorescence was low in the nucleus and high in the cytosol. The authors concluded that fluo-3 AM seems to be a better indicator of nuclear calcium than rhod-2 AM.

On the other hand, according to Niggli and Lederer [51], fluo-3 is primarily distributed in the cytosolic compartment, so the abovementioned problem is avoided by keeping the physiological intracellular pH constant. According to Tsien and colleagues, differences in the subcellular distribution of fluorescent indicators may be related to the properties of the dye itself, the preparation, as well as the exact loading procedure [80].

Confocal microscopy was crucial to Avedanian et al. [81] in proving the existence of two distinct structures in the nucleus of the human vascular smooth muscle cells, namely the nuclear tubular structure, which the authors called Nuclear T-Tubules (NTTs), and the nuclear reticular structure, which can be referred to as nucleoplasmic reticulum (NR). The human vascular smooth muscle cells were loaded with the Ca^{2+} probe Ca^{2+}-Orange (15 μmol/L excited with the 568 nm visible beam of the Krypton/Argon laser), and the isolated nuclei were loaded with the Ca^{2+} probe fluo-4 (13 μmol/L excited by the 488 nm Krypton/Argon laser line). The specimens were observed by the Krypton/Argon and

UV laser confocal system equipped with an inverse phase epifluorescence microscope and a 60× Nikon Oil Plan achromat objective [82-83].

Fluo-4 is an analog of fluo-3 in which the two chlorine substituents are replaced with fluorines. This fairly minor structural modification results in increased fluorescence excitation at 488 nm and consequently higher signal levels for confocal microscopy, flow cytometry, and microplate screening applications.

In the work of Avedanian et al. [81], the 363 UV or 488, 568 and 647 nm Krypton/Argon laser lines were directed to the sample via the corresponding primary dichroic filters and primary and secondary beam-splitters, followed by attenuation with a 1-3% neutral-density filter for photobleaching reduction. The pinhole size, image size, pixel size, and step size were set to 50 μm, 512x512 pixels, 0.34 μm, and 0.5 μm, respectively. The laser line intensity, photometric gain (PMT) settings, and filter attenuation were kept constant throughout the experimental procedures. The recorded serial *Z*-axis optical scans were analyzed by a Silicon Graphics O_2 analysis station equipped with the Molecular Dynamics Imagespace 3.2 analysis and Volume Workbench software modules. The captured section series were presented either as 2D images or as 3D images [84]. The real 3D top-view image allows for visualization of the fluorescence intensity of a target protein, ion, or structure in a given volume (of tissue, whole cell, organelle, etc.) altogether, hence revealing entities that otherwise would not be observed in a single section. The findings of Avedanian et al. [81] complemented other works by postulating that the nucleoplasmic reticulum and the Nuclear T-Tubules potentially corroborate the nuclear Ca^{2+} homeostasis that modulates many Ca^{2+} sensitive processes residing in the nucleus, such as nuclear enzymes regulation, nucleo-cytoplasmic transport via the pore, cell cycle, and gene transcription and expression (85-89].

Concerning the dye loading in slices (250 μm thick) from rat aortic rings and mesenteric or tail arteries, for assessment of the cytosolic Ca^{2+} concentration we usually load the preparations with fluo-3 AM (10 μmol/L) for 30 minutes, at room temperature, in Hanks buffer with the following composition in mmol/L: 145.0 NaCl, 5.0 KCl, 1.6 $CaCl_2$, 1.0 $MgCl_2$, 0.5 NaH_2PO_4, 10.0 dextrose, and 10.0 HEPES, pH 7.4. Excess dye is removed by washing out the dye with Hanks solution and allowing 30 minutes for the intracellular desterification of fluo-3 AM. Preparations are imaged in Hanks buffer (pH 7.4). With the aid of a confocal scanning laser microscope, the cytosolic Ca^{2+} concentration is determined via fluo-3 AM fluorescence. The specimen is excited with the 488-nm line of an argon ion laser, and the emitted fluorescence is measured at 510 nm. As mentioned above, a time-course software is employed for acquisition of the cells images at one-second intervals (*xyt*), using the Live Data acquisition mode at 1024 × 1024 pixel and 700 Hz. By using the laser scanning confocal microscope and appropriate computer software, the intensity of the maximum or minimum intracellular fluorescence is measured in the endothelial and in the smooth muscle sliced regions. The initial fluorescence intensity value is obtained at t= 0 and is designated *F0*, whilst the final fluorescence intensity value obtained after stimulations is designated *F*. In this way, the percentage of the difference in fluorescence intensity (*%ΔFI*) reflects the transient cytosolic Ca^{2+} concentration in the endothelial layer and in the smooth muscle layer, in relation to *F0* (100%). The intensity is calculated by the following formula: for endothelial cells: $\%\Delta FI = (F - F0)/ F0 \times 100$; for smooth muscle cells: $\%\Delta F = (F - F0/ F0) \times 100$.

An example of drug treatment and stimulations is the one applied by Rodrigues et al [47] in a study about the contribution of endothelial cell caveolae to vascular relaxation in aortas from hypertensive rats. Briefly, methyl-β-cyclodextrin (10 mmol/L) was used for elicitation of caveolae disruption. This compound was added 60 minutes before the aortic rings were loaded with fluo-3 AM in a humidified incubator at 37 °C, gassed with 5% CO_2. Control responses were obtained in experiments where methyl-β-cyclodextrin was replaced by vehicle (Ca^{2+} and Mg^{2+} free Hanks). The final fluorescence intensity value (*F*) was obtained after stimulation with acetylcholine (1 μmol/L).

When Rodrigues et al [46] studied vascular smooth muscle cells isolated from aorta by enzymatic digestion, they had utilized the procedures previously described by Lunardi et al [90] during their study of whether the cytosolic calcium concentration was reduced by photolysis of a nitrosyl ruthenium complex in vascular smooth muscle cells. To this end, aortas were dissected and longitudinally opened. The endothelium and the adventitia were removed, and the tissue was minced into small pieces and incubated in Ca^{2+} and Mg^{2+} free Hanks solution with the following composition (in mM): 145.0 NaCl, 5.0 KCl, 0.5 NaH2PO4, 10.0 dextrose, and 10.0 HEPES (pH 7.4) and containing 0.03 mg/mL collagenase Type II-S. The tissue was gently shaken in this solution for 25 minutes at 37 °C, and bubbled with carbogen mixture (5% CO_2 and 95% O_2). After that, 10 mg/mL bovine serum albumin (type I) was added to the vessel fragments in Ca^{2+} and Mg^{2+} free Hanks solution, and the cells were released by mechanical dispersion with the aid of a Pasteur pipette. The resulting cell suspension was centrifuged at 1000 rpm for three minutes, followed by suspension in Ca^{2+} and Mg^{2+} free Hanks. The cells were plated on glass coverslips pretreated with poly-L-lysine solution and kept in a humidified incubator gassed with 5% CO_2 and 95% O_2, at 37 °C. The cells were used three hours after plating and were maintained in a serum-free medium [46].

3.1.1 Sparks and Ca^{2+} fluxes in cardiac and in vascular tissues

To evaluate the cardiac and vascular functions, it is important to analyze Ca^{2+} sparks, which are thought to control the cytosolic Ca^{2+} concentration and its complementary mechanisms of Ca^{2+} activation, such as calcium-induced calcium release-CICR, and inactivation (Ca^{2+}-dependent inactivation) [91-94].

The fluorescent indicators must have a K_d (Dissociation Constant) compatible with the Ca^{2+} concentration range of interest. Indicators have a detectable response in the concentration range from approximately 0.1mol/L K_d to 10 mol/L K_d. The K_d values of Ca^{2+} indicators depend on many factors, including pH, temperature, ionic strength, viscosity, protein binding, and the presence of Mg^{2+} and other ions. Consequently, K_d values for intracellular indicators are usually significantly higher than the corresponding values measured in cell-free solutions. Cheng et al. [95] have described a pseudo-ratio equation used for the estimation of $[Ca^{2+}]i$ using a K_d value of 345 nmol/L for fluo-4 and assuming basal $[Ca^{2+}]i$ to be 100 nmol/L.

Collier et al [94] have applied fluo-4 fluorescence to demonstrate that L-type Ca^{2+} channels activate ryanodine receptors for production of calcium-induced calcium release (CICR) in the form of Ca^{2+} sparks and propagated Ca^{2+} waves in smooth muscle cells. In their experiments, the authors conducted confocal and voltage clamp experiments at the same time. Briefly, cells loaded with fluo-4 AM were excited with 488-nm light emitted from a Krypton/Argon laser and measured with a high speed laser scanning confocal head, using a plan-apo, 60X water-immersion objective lens and the indicated software for graphs. The authors acquired the *x–y* images every 8.3 milliseconds (256 X 240 pixels), and *x–t* images were obtained with line scans at 4.16-millisecond intervals for 2.13 seconds (512 X 480 pixels). Pixel size in the *x* and *y* axes were equal to 0.252 and 0.248 μm, respectively. In all the *x–y* images, a mean background fluorescence value was determined and subtracted from each pixel, and the images were smoothed by using a 3 X 3 pixel median filter. The mean baseline fluorescence intensity (*F0*) of a cell was obtained by averaging the first six to eight images that did not exhibit transient rises in intracellular Ca^{2+}. Profiles of line-scan images were obtained over a 1-μm region, and *F0* was achieved by averaging the fluorescence of 30 pixels before a depolarizing step. Ratios of images (*F*/*F0*) and profiles were constructed, in order to reflect changes in fluorescence intensity over time. The Ca^{2+} spark criteria were a localized increase in fluorescence ($F/F0 \geq 1.5$), occurring in 20–30 milliseconds, with a decay time of 50–80 milliseconds. Ca^{2+} spark latencies were calculated as the time from the start of the voltage pulse to the point at which the fluorescence exceeded 5% of *F0*, and they were calculated for Ca^{2+} sparks occurring within the first voltage-clamp step of an experiment, so as not to bias results by an increase in [Ca2+]i resulting from preceding voltage-clamp steps. The Ca^{2+} spark probability was calculated in the following way: voltage-clamp steps were measured at the same time of the confocal experiments, to determine whether or not a Ca^{2+} spark occurred during a specific clamp step. Currents associated with each step were integrated for determination of the net Ca^{2+} flux, the fluxes were binned, and the probability was calculated by dividing the number of experiments in which a Ca^{2+} spark was evoked by the total number of experiments in the bin. Thus, the likelihood of a Ca^{2+} spark occurring in voltage-clamp steps after clamp steps in which no Ca^{2+} spark occurred is probably somewhat higher due to accumulation of Ca^{2+} from previous steps.
Specific parameters, such as the amplitude, width, and kinetics of the imaged Ca^{2+} sparks are confocally affected by errors when the spark source is not in focus, especially in cardiomyocytes. Confocal microscopy was the main technique that allowed Avedanian et al [81] to consolidate the findings that the nucleoplasmic reticulum has the potential to act as a nuclear organelle, possibly as a source of generation of Ca^{2+} sparks, puffs, and waves upon agonist stimulation at nuclear envelope G-protein coupled receptors, or upon activation of nuclear envelope membranes Ca^{2+} channels. The presence of Nuclear T-Tubules in intact tissues of excitable (heart) and non-excitable (liver) cells confirmed that the presence of these structures is not due to cell isolation and culturing.

To identify the sparks that were in focus, Vyacheslav et al [96] used fast scanning (LSM 5 LIVE; Carl Zeiss) combined with fast piezoelectric focusing for acquisition of *x–y* images in three planes with 1-μm separation (*x-y-z-t* mode or 4D confocal imaging). Four-dimensional scanning eliminates the out-of-focus error because it allows for the identification of sparks that are imaged in focus. The authors demonstrated a method for the imaging and morphometric characterization of a large group of sparks in focus based on scanning fluorescence in three spatial dimensions. The Ca^{2+} release current underlying in-focus sparks was 11 pA and required between 20 and 30 open channels, a number at the high end of earlier estimates. The verified spark frequency was greater than that observed in earlier imaging studies of permeabilized ventricular cells. This suggests a greater susceptibility to excitation, which could have functional relevance for atrial cells. The Ca^{2+} release flux peaked earlier than the time of peak fluorescence and then decayed, which is consistent with significant sarcoplasmic reticulum (SR) depletion. The evolution of fluorescence and release flux were remarkably similar for in-focus sparks of different rise time (*T*). The authors concluded that spark termination involves Ca^{2+} depletion in both the SR and channel closure, which may be synchronized by depletion. With the 4D scanning of the properties of local events in focus, including spatial associations, event propagation and location-specific aspects of Ca^{2+} release can be well understood. This is because the experiments provide well-determined measures that clarify aspects of Ca^{2+} release control and produce a strict test of the theory of scanning in lower dimensions.

In an elegant video recorded by Golebiewska and Scarlata [97], it is possible to see the line-scan mode of a laser scanning confocal microscope for the measurement of fast Ca^{2+} fluxes in living cardiomyocytes. By using confocal microscopy, Guo, Golebiewska, and Scarlata [98] were the first to observe that caveolae play a direct and active role in regulating basal Ca^{2+} activity in cardiomyocytes via the caveolae-G-αq family of heterotrimeric G-proteins. These authors found that the plasma membrane protein domains known as caveolae can entrap activated Gα(q). By using LSCM, the authors demonstrated that entrapment stabilizes the activated state of Gα(q;) and results in prolonged Ca^{2+} signals in cardiomyocytes and other cell types. The authors employed Ca^{2+} green AM (5 μM) as the fluorescent Ca^{2+}

indicator, due to its ability to mark an increase in the fluorescence emission intensity upon the binding of calcium ions. The method developed by Golebiewska and Scarlata [97] permits rapid acquisition of the time course of the fluorescence intensity in pixels along a selected line, producing several hundreds of time traces on the microsecond timescale. To differentiate the Ca^{2+} responses of different flux rates, the authors performed a histogram analysis that binned pixel intensities with time. Binning was important for the grouping of over 500 traces of scans and for the spatial and temporal visualization of the compiled results in a single plot. Thus, the binned histograms allowed for the prompt detection of the slow Ca^{2+} waves. The latter are difficult to discern when the scans are overlaid, due to different peak placement and noise. The authors verified very fast fluxes in the timescale of the measurement, which revealed a narrow distribution of intensities in the very short time bins, whereas longer Ca^{2+} waves exhibited binned data with a broad distribution over longer time bins. These different time distributions enable the determination of Ca^{2+} fluxes timing in the cardiomyocytes and help establish their impact on various cellular events.

Still considering cardiac myocytes, Sassi et al [99] have used fluo-4 AM (5 μmol/L) loading in ventricular myocytes for 30 minutes at room temperature. Their aim was to demonstrate that multidrug-resistant protein 4 is the predominant isoform in the plasma membrane, and that it mediates the cAMP efflux in these cells. Cells were field-stimulated at 1 Hz with a 1- to 2-millisecond current pulse delivered via two platinum electrodes. Changes in fluo-4 fluorescence were recorded using the laser scanning confocal microscope (X63 water-immersion objective, numerical aperture: NA= 1.2). Measurements were conducted in the line-scan mode (1.5 ms/ line), and scanning was performed along the long cell axis. An excitation wavelength of 488 nm was used, and the emitted light was collected through a 505-nm long-pass filter. Regarding practical applications, it is noteworthy that the laser intensity test (3–6% of the maximum) did not evidence any deleterious effects on the fluorescence signal or on the cell function over the time course of the experiment. To enable comparisons between cells, the change in fluorescence (ΔF) was divided by the fluorescence detected immediately before the 0.5-Hz stimulation pulse (*F0*).

Confocal microscopy is also useful for the determination of Ca^{2+} concentration in the organelles. The probe fluo-5N is usually employed in this case. Gan et al. [100] have evaluated the post-conditioning protection in rat apoptotic cardiomyocytes via attenuation of the calcium-sensing receptor-induced sarcoplasmic reticulum stress by using fluo-5N AM for the measurement of Ca^{2+} concentrations in the sarcoplasmic reticulum. Briefly, after a specific treatment, the myocytes were loaded with 10^{-1} mol/L fluo-5N AM for 90 minutes at room temperature, washed with standard Tyrode's solution for removal of the extracellular fluo-5N AM, and diluted to the required concentration. Next, cells with bar-shaped and clear striae and loaded with fluo-5N AM were used in the experiment. Excitation was set at 488 nm, and emission was monitored at 530 nm, at room temperature. Fluorescence images reflecting $[Ca^{2+}]i$ were recorded with the aid of a laser confocal scanning microscope. The fluorescence (*F*) was normalized to baseline fluorescence (*F0*), and the data were quantitated using analysis software. The cardiomyocytes were loaded with fluo-5N and permeabilized with saponin. Fluo-5N is a low-affinity Ca^{2+} indicator (K_d = 400 μmol/L) that only appears bright where the intracellular Ca^{2+} concentration is very high; *e.g.*, in the sarcoplasmic reticulum, as previously described by Fischer et al [101] and Chatterjee [102]. The fluo-5N signal was stable at the beginning of ischemia, with *F0* representing the fluorescence intensity. One hour after reperfusion, the authors detected the fluo-5N signal in the sarcoplasmic reticulum of the cardiomyocytes.

3.1.2. Nitric oxide and superoxide anion measurements in vascular tissues and cells

By using fluorescence kits and antibodies together with confocal microscopy, Arribas et al (2007) [103] have identified the contribution of the different layers of the vascular wall to the remodeling process in terms of cell proliferation/death. These authors reported that the adventitia is the most active layer in terms of cell turnover. In intact rat cerebral arteries stained with a fluorescent derivative of Tunel, they found that the majority of Tunel-positive cells are located in the adventitia. These authors used 'confocal myography' and reconstruction of the serial sections as previously standardized experiments for the attainment of 3D images, where the target structures were localized and quantified. This work showed that confocal microscopy is useful not only for imaging of the vascular wall structure, but also for visualization and quantification of the fluorescence intensity. The latter provides information on the generation of vascular cell factors such as nitric oxide (NO) and the superoxide anion (O_2^-), since confocal microscopy and image analysis software give a perspective of the vascular wall structure and function as well as of the active process of vascular remodelling in physiological and pathological situations. These authors used dyes such as DAF2-Diacetate [104], which binds to NO or O_2^- like dihydroethidium [105]. This kind of dye produces fluorescent compounds, which, together with confocal microscopy, enable visualization of the bioavailability of these compounds in the vascular wall and quantification of the relationship between them. DAF2-DA enters the vascular cells and is cleaved by vascular esterases thereof, to furnish DAF2. The latter is then retained in the cell. In the presence of oxygen, the combination of DAF2 with NO generates a highly fluorescent compound that can be detected in the cytoplasm through the band at 488 nm/515 nm and can be utilized for NO real-time bioimaging with fine temporal and spatial resolution. On the other hand, DHE permeates the cell and is directly oxidized by oxygen radicals (being especially sensitive to O_2^-), to form ethidium bromide. The latter combines with DNA and is trapped in the nucleus, emitting fluorescence in the 590-nm wavelength [106]. Obviously, DAF2-DA and DHE have to be incubated in intact, non-fixed blood vessels. However,

they survive at 4% paraformaldehyde post-fixation and embedding in optimum cutting temperature (OCT), with no alteration in their fluorescent properties, even after long storage periods.

The NO bioavailability can be quantified by conventional image analysis software based on DAF2-DA fluorescent intensity levels, which increases proportionally to the amount of NO released by the vascular cells in basal or stimulated conditions. The reaction is specific, because the NO synthase inhibitor L-NAME blocks it. The superoxide availability can be quantified by the number of stained cells per unit length [105]. We have observed that under stimulated conditions – as in the case of the superoxide anion donor pirogallol – the amount of produced superoxide is so high that the fluorescence is located in all the vascular cells and is even released in the cytoplasm. For quantification purposes, it is important to establish a profile of laser power, brightness, and contrast for all the experimental groups. Whenever possible, it is also advisable to study control and experimental groups simultaneously, so as to avoid variability caused by day-to-day laser fluctuations of the confocal microscope.

Nitric oxide (NO) plays a pivotal role in the vascular tone control. It is well known that NO has multiple functions on vascular relaxation, platelet aggregation, and cell proliferation. The deficit in NO production or the failed NO bioavailability in the vascular smooth muscle cells can give rise to vascular problems such as atherosclerosis and hypertension.

Confocal microscopy can be a useful method for investigation of the intracellular NO concentration ([NO]c). NO is a highly reactive molecule that reacts with many biomolecules and has a short half-life in physiological conditions.

Endogenous NO can be produced in the vascular tissue by nitric oxide synthases (NOS) localized in the endothelial cells or in the vascular smooth muscle cells. The activation of endothelial cells can induce relaxation of the vascular smooth muscle by production of the endothelium-derived relaxing factor (EDRF), or it can induce contraction of the vascular smooth muscle by release of endothelium-derived contractile agents (EDCFs).

Several cardiovascular diseases such as hypertension have endothelial dysfunction in common. This can be due to endothelial NO-synthase (NOS3) uncoupling, which in turn impairs NO signaling in the vascular smooth muscle cells to render endothelium-dependent and endothelium-independent vasodilation. Indeed, it is a multifactorial process characterized by increased production of reactive oxygen species (ROS) in the endothelial cells and decreased NO bioavaialbility. The impaired ability of endothelial cells in terms of NO release and endothelium-dependent hyperpolarization, together with the enhanced production of EDCFs culminates in endothelium dysfunction, which appears to be the first step in the chain of events leading to to atherosclerosis and coronary disease [106]. In the case of NO failure, the synthesis of NO-donors could have an important therapeutic effect in the treatment of vascular diseases.

Confocal microscopy and the discovery of the NO-sensitive dye (DAF-2/DA) have allowed for the study of NO release inside the cells and investigation of the cellular effects of NO. As reported by Rodrigues et al. [48], DAF-2/DA fluorescence was excited with the 488-nm line of the argon ion laser, and the emitted fluorescence intensity (FI) was measured at 515 nm. Time-course software was used for acquisition of the cells image at two-second intervals (*xyt*), 1024 x 1024 pixels, and 700 Hz. This technique enabled comparison of NO release from three compounds: a new ruthenium-derived complex (DCPBY 10 μmol/L), SNP (0.1 μmol/L), and nitrite (300 μmol/L). These compounds were used for imaging at the concentrations that they produced the maximum relaxing effect. The intensity of the intracellular maximum and minimum fluorescence was measured in the smooth muscle regions. The final fluorescence intensity was achieved after addition of the given compound (F), and the initial fluorescence (F0) was obtained at time zero. Therefore, ΔFI reflects the increase in [NO]c. In comparison, DCBPY releases more NO than SNP, and the rise in [NO]c for SNP was greater compared with nitrite.

The use of DAF-2DA for [NO]c imaging by confocal microscope inside the cells is possible because it is known that this fluorescent dye is membrane permeable. It enters the cell and is subsequently hydrolyzed by cytosolic esterases, releasing DAF-2, which in turn does not leak into the medium. At physiological pH, DAF-2 is relatively non-fluorescent, but in the presence of NO and oxygen it forms DAF-2 triazole (DAF-2T), a fluorescent product.

We have previously reported that the vascular smooth muscle cells membrane is more depolarized in the 2-kidney-1-clip hypertensive rat than in normotensive control (2K) rat aorta [107]. This study was performed in isolated rat aortic rings containing the vascular smooth muscle and endothelial cells. The development of potential-sensitive dye permitted the investigation of the membrane potential of isolated vascular smooth muscle cells from 2K-1C and 2K rats using fluorimetric techniques [108]. With the aid of the confocal microscope (LEICA TCSNT) coupled to a LEICA DMIRBE inverted microscope equipped with a 63x objective, we investigated the membrane potential by using the potential-sensitive dye *bis*(1,3-diethyl-tiobarbiturate)trimethine oxonol ($DiBAC_2$) [108]. The wavelength excitation for this dye was the 488-nm line of a krypton/argon laser. The fluorescence emission was detected by means of a BP 530/30 filter, and time course studies were conducted for up to 20 minutes after the addition of 10-60 mmol/L KCl. There was an almost linear change in membrane potential following the increase in external KCl concentration. The resting membrane potential was less negative in 2K-1C than in 2K cell membranes. Fluorimetric and confocal microscopy imaging of single cells consist of a non-invasive technique for the study of cell function.

Several enzymes can produce ROS in endothelial cells, in which the major species is O_2^-. The enzymes involved in ROS production are NADPH oxidase, xanthine oxidase, cyclooxygenase, and endothelial nitric oxide synthase (NOS3). NOS3 can produce superoxide instead of NO when it is uncoupled by lack of substrate (L-arginine) or the essential cofactor tetrahydrobiopterin (BH_4) [109]. Superoxide can be dismutated by superoxide dismutase (SOD) to hydrogen

peroxide (H_2O_2), which can induce endothelium-dependent relaxation or be cleaved by catalase [110], thereby diminishing the NO bioavailability [111]. Therefore, an increase in ROS is associated with impaired endothelium-dependent vascular relaxations. On the other hand, treatment with antioxidants improves endothelium-dependent relaxation.

Vasorelaxation induced by the NO donor is impaired in aorta isolated from renal hypertensive rats as compared to aorta isolated from normotensive rats. Incubation of these vessels with the antioxidant vitamin-C enhanced the relaxation induced by TERPY in renal hypertensive rat aorta [46]. In a similar way, Vitamin-C increased the effect of NO released from TERPY in isolated smooth muscle cells imaged by confocal microscope [46].

In order to improve the lack of EDRFs impaired production by the endothelial cells, for several years we have studied the vascular effects of new NO-donors such as nitrosyl ruthenium complexes, which offer several advantages. In fact, NO donors are believed to elicit NO release and relaxation in an endothelium-independent way. However, we have reported that the classical NO donor sodium nitroprusside (SNP) is an endothelium-dependent agent. By using confocal microscopy, we demonstrated that SNP enhances Ca^{2+} concentration in the endothelial cells, which is essential for NOS activation. In fact, the relaxation induced by SNP in vascular smooth muscle cells is potentiated by the endothelial production of NO by NOS in rat aorta [112]. In our laboratory, the arterial rings (150 μm thick) were placed vertically in a coverslip treated with poly-L-lysine for imaging of the arterial cross- section.

When we investigated the vascular relaxation induced by a new ruthenium compound (TERPY) in intact endothelium rat aorta, we verified that the endothelium negatively modulates the relaxation induced by TERPY. This effect was abolished by a superoxide scavenger, an NOS3 inhibitor, and the cofactor BH_4 [112]. Therefore, the NO donor TERPY induces NOS3 uncoupling.

In order to measure the superoxide production, we have imaged the rat aortic rings cross-sections by confocal microscopy. The aortic rings (150 μm) with the intact endothelial layer were placed in a coverslip covered with poly-L-lysine. The tissue was loaded with the fluorescent superoxide sensitive dye, DHE. This dye was excited with the 488-nm line of an argon ion laser, and the emitted fluorescence was measured at 610 nm. Time course software was used for acquisition of cell images at 2.974-second intervals (*xyt*), 1024-1024 pixels, and 700 Hz. [112]. The protocol was designed for the measurement of superoxide in slices of rat aorta with endothelium, since this study aimed to investigate whether superoxide was produced in the endothelial cells and/or in the smooth muscle cells.

In fact, nitrovasodilators are pro-drugs that release NO and thus mimic the vasodilating effect of the NO endogenously produced by NOS. Interestingly, when we imaged the vascular smooth muscle cells isolated from rat basilar artery, we verified that the NO donor TERPY does not release NO in the case of those cells [49]. These results are in accordance with the functional vascular reactivity studies showing that TERPY does not induce relaxation of the rat basilar artery. For comparison purposes, we used the NO donor SNP, which presents both effects; that is, NO release into the basilar artery isolated cells and vasorelaxation. To measure the cytosolic NO concentration, basilar artery smooth muscle cells were loaded with the NO indicator DAF-2DA as previously reported for rat aorta smooth muscle cells in reference [46].

4. Conclusion

Confocal microscopy and specific probes optimize cardiomyocytes inspection as well as blood vessels studies. Many researchers are concerned about the visualization of an intact blood vessel in which the thickness is the main obstacle, especially in living tissues. In this sense, even small arteries are relatively thick specimens for conventional microscopy. Confocal microscopy also contributes to the understanding of not only the muscular and endothelial (dys)function, but also cell death and proliferation events involved in important physiological mechanisms and diseases taking place in cardiac and vascular tissues. The researchers have in their hands a remarkable tool due to the laser scanning confocal microscopy's ability to promptly reproduce with high-quality optical sections. Considering the characteristics of fluorescent probes and the ability of confocal microscopy in terms of optical sectioning, this technique is well suited to the study of both *in vivo* and *in vitro* specimens.

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