Imaging receptors with Laser Scanning Confocal Microscopy: qualitative and quantitative analysis

J.B. Sousa[1,2], **P. Fresco**[1,2] and **C. Diniz**[1,2]

[1]REQUIMTE/FARMA, Department of Drug Sciences, Laboratory of Pharmacology, University of Porto, Porto, Portugal
[2]MedinUP- Center for Drug Discovery and Innovative Medicines, University of Porto, Porto, Portugal

Receptors are the molecular targets through which drugs produce their beneficial effects in a disease state. Receptors location, in the cell membrane or within the cell, contributes for the understanding of the subsequent signalling events triggered by their activation. The subcellular localization and physiological functions of receptors are closely related and thus it is crucial to precisely determine the distribution of different molecules inside the intracellular structures. This is usually studied by fluorescence microscopy and posterior evaluation of the colocalization of the molecule of interest in a definite region in the cell. Laser Scanning Confocal Microscopy (LSCM) imaging is a powerful tool allowing to distinguish a variety of unexpected locations for receptors, manly in subcellular locations such as mitochondria, lysosomes, nuclei [1] turns crucial rethinking the current knowledge of the pharmacological role ascribed to receptors. Moreover, the use of fluorescent ligands in tissues will also be addressed since it allows a multidimensional analysis and provides new insights to receptor dynamics and intracellular traffic[2].This chapter describes some of the the strengths, limitations and potentialities associated with receptors Laser Scanning Confocal Microscopy (LSCM) imaging, giving some practical examples concerning our current research line. Emphasis is placed on the importance of appropriate image acquisition guiding the reader through the basic and advanced knowledge of major quantitative image analysis approaches. Quantitative evaluation of receptors in tissues provides information about receptor over, subexpression or redistribution contributing for the understanding of the impairment/deregulation associated with receptors putative physiological and/or pathophysiological role.

Keywords: receptors distribution, Laser Scanning Confocal Microscopy, molecular targets, quantitative evaluation

1. Introduction

In cell biology a structure on the surface of a cell (or inside a cell) that selectively receives and binds a specific substance triggering a cellular response is called a receptor. Receptors can be divided into four main classes: ligand-gated ion channels, tyrosine kinase-coupled, intracellular steroid and G protein coupled receptors. The latter represent the largest of the several families of plasma membrane receptors, comprising more than a thousand genes and regulating virtually all known physiological processes in mammals. Because G protein coupled receptors play specific roles in human disease, they have provided useful targets for drug development, being, at present, the commonest molecular target for currently used drugs. Accordingly, receptor physiology in a specific tissue/organ can be better understood if information on the expression of receptors or other type of proteins/ligands combined with experiments that allow to establish their respective function contributes for an integrative view of ligands/receptors role both in physiology and pharmacology. The knowledege of receptors location, distribution and function has been advanced through continous improvement of methodologies and equipments. In this sense, the first approach developed to establish receptors location was based on the use of radioligands and their binding visualized by *in vitro* autoradiography in tissue sections[3-5]. However, limitations in the accuracy of this method, at the cellular level, lead to the refinement of techniques such as RT-PCR or Western-blot, classic and fluorescent immunohistochemistry microscope visualization.

1.1 Fluorescence microscopy

The application of fluorescence microscopy technologies to physiology and pharmacology is growing bringing together insights from three separate existing fields (chemistry, immunology and histology). Indeed, immunohistochemistry has grown as genomics and bioinformatics have provided unique sequences against which to raise antibodies for identifying proteins. Vital chromophores indicate localised chemical composition such as pH or Ca^{2+} concentration by changing the characteristics of their excitation or emission. Finally, highly selective ligands have been labeled covalently with fluorescent moieties to provide the potential for ligand binding studies at the subcellular level, thereby providing pharmacologically interesting insights beyond those provided by immunohistochemistry. These rejuvenated old technologies are enhanced further by the new opportunities offered by luminescent or fluorescent organic ligands found in plants or marine organisms. These can be used as conventional chromophores but, more powerfully, can be spliced to

known proteins, by genetic manipulation, to provide a covalently bonded fluorescent moiety whose chromatic properties can be exploited.

Fluorescence microscopy, therefore, allows the detailed mapping of ligands/receptors in cells/tissues by multiple fluorescent staining. Fluorescent techniques can be divided in two categories: the conventional fluorescent staining and the molecular tagging. In the context of receptor biology, these technologies offer the chance to visualise receptors, their antagonist or agonist ligands, including natural hormones and neurotransmitters, the transducing proteins with which they interact, and the indicators of the cellular response that they mediate or modify.

The majority of studies on G protein coupled receptors rely on qualitative determinations where apparent fluorescence increases or decreases in cells are taken as changes in receptor fate or activity. Other inportant information can be extracted when colocalisation occurs, i.e. two or more antigens staining patterns labeled by corresponding antibodies with different excitation spectra, visualized in different colors, overlaps. Colocalization correspond to a coexistence of molecules in a very close physical location providing valuable information to clarify their common features. Furthermore, it is of upmost importance to find a way of getting quantitative data that will help to define magnitude, concentration dependency or time-course of the processes under study. Currently, several methodologies, including laser scanning confocal microscopy (LSCM) have been used to characterize receptor dynamics: immunohistochemistry and analysis of signal transduction pathways, including visualization of protein binding to other molecules or their folding by using fluorescence ressonance energy transfer (FRET) or bioluminescence ressoance energy transfer (BRET)[6], live-cell imaging, including molecular dynamics by fluorescent protein-fusion probes[7, 8], traffic analysis of individual proteins, such as receptors, by fluorescence recovery after photobleaching (FRAP) techniques[9] and protein turnover.

1.1.2. Laser Scanning Confocal Microscopy (LSCM)

The major application of LSCM in biomedical sciences is imaging of fixed or living tissues previously labeled with fluorescent ligands. The reader is referred to Paddock's review[10], a chapter that will provide an excellent introduction to the principles and practices of LSCM.

The quality of an image dataset acquired by LSCM, depends mainly on two aspects, the correct processing of the sample and the microscope performance. Sample processing, in turn, depends on several factors such as, fixation, loading of fluorescent dyes or immunostaining, and the correct mounting of the specimen. It is essential to consider the preparation of the samples (specimens) as a subject of particular relevance particularly if quantitative analysis is on our purpose: procedures, light conditions and magnification must be similar. Ideally, from the technical point of view, the specimen calibration step (performed by the computer), requires homogeneous specimens in chemical, dimension and structural features. Microscope performance is crucial for the quality of image acquisition, mainly through its ability to eliminate the "out-of-focus" from thick fluorescently labeled specimens. Since the illumination in a confocal microscopy is achieved by scanning one or more focus beams of light across the specimen using a laser, the stability of this laser, as well as efficient reflecting mirrors and sensitive low noise photodetectors, are key factors that need to be considered. Moreover, there have been considerable improvements in the digital image systems, now allowing that multiple fluorochromes to be imaged simultaneously by frame or, alternatively, sequentially on a line-by-line basis.

In modern systems such as LSCM, 'Z' axis is now usually associated to the collection of a series of images of various focal planes that can be, subsequently, used to construct 3D representations of the specimen. Sometimes, lower image stacks acquired by LSCM have lower voxel intensities when compared with the upper ones, i.e., there is intensity attenuation with depth. To reduce this, it is recommended to start the Z series at the deepest part of the specimen or, alternatively, it is also possible to correct this problem during image processing[11]. Therefore, it is possible to process images stacks to obtain both 2D or 3D reconstruction (by rotating the specimen and imaging it from several different angles) of a specimen, from a single view, using algorithms that have been in continous improvement. 4D reconstructions are also possible if time is considered. The latter is crucial for receptor signalling events or traffic studies such as visualization of receptor internalization processes (using tagged fluorescent proteins). LSCM 2D, 3D and 4D accurate reconstructions of multiple points offer better depth understanding and measurement of features located on the specimen.

2.Imaging receptors with LSCM: qualitative analysis

Fluorescent ligands bound to receptors can evidence their location and pharmacological information such as affinity or proximity between interacting molecules. Such information can be obtained, if binding is reversible, overcaming some limitations, namely low molecular concentration and diverse location of receptors. As such, relationship between receptor location and function are becoming better understood, contributing to new insights relative to drug action mechanisms and new therapeutic targets. Nevertheless, spatial precise distribution of receptors and their function

relationships are still largely unknown, making this an expanded area of study. In the last decade, studies using LSCM have been used to better understand the physiological/pathophysiological function of receptors, most of them using antibodies to identify, characterize and define the location of proteins/receptors, by immunohistochemistry, in different cells[12] and tissues[13], including in vascular tissues[14]. In the vascular wall three layers can be identified (intima, media and adventitia), since their cells present different morphologies and different functions. In this respect, the use of fluorescent nuclear stain will enable identification of cell orientation, number, organization and cell type allowing layer vessel recognition[15, 16]. Receptors can be located in any of these cells and may interplay with other receptors, enzymes or transporters located in the same cell or in neighbouring cells. In this respect, LSCM resolution is crucial to distinguish between two points on a specimen as separate entities, which is mainly dependent on the numeric aperture of the objectives, of the substage condenser and the wavelength spectrum of light used[10].

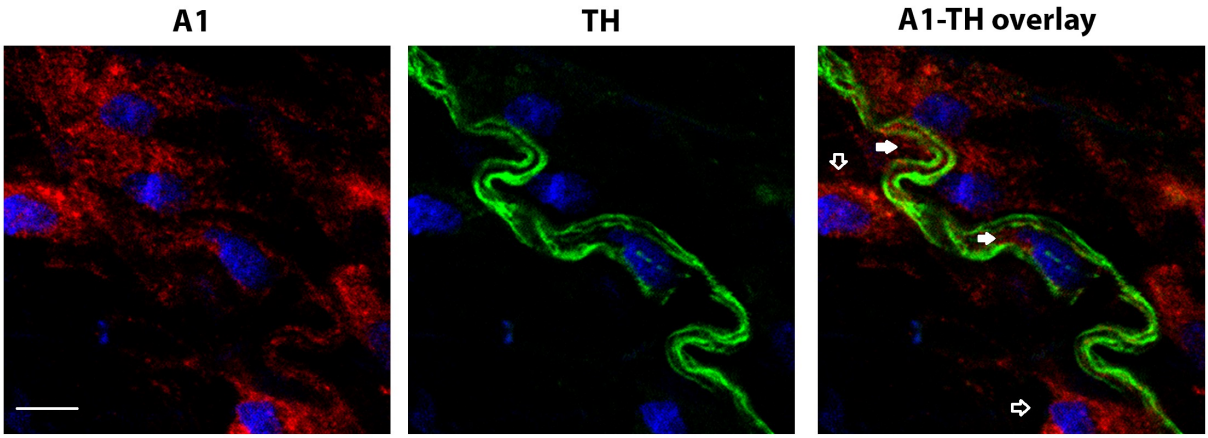

Figure 1. Laser scanning confocal immunofluorescence images of rat mesenteric artery adventitia immunostained for adenosine A_1 receptors (rabbit primary polyclonal adenosine A_1 receptor antibody and a species specific secondary Alexa 647 antibody: red), tyrosine hydroxylase (mouse primary monoclonal tyrosine-hydroxilase (TH) antibody and a species specific secondary Alexa 488 antibody: green) and DAPI (cell permeable fluorescent minor groove-binding probe for DNA, blue). Scale Bar = 20 μm. White open arrows indicates adenosine A_1 receptor population in non-neuronal cells while white filled arrows indicate adenosine A_1 receptor population on the sympathetic nerves.

Taken adenosine A_1 receptors as an example, it is possible to identify two populations of adenosine A_1 receptors in images acquired by LSCM (Figure 1A). One population is shown to be in the proximity of an enzyme involved in the generation of the neurotransmitter, noradrenaline, inside the sympathetic nerves, thyrosine hydroxilase, whereas other receptors are located in non-neuronal cells. This type of identification is possible since qualitative image analysis is a task for which the human eye is well suited, once qualitative data is based on recognition patterns (overall arrangements of elements within the specimen).

Such information complements the knowledge that adenosine A_1 receptors are involved in sympathetic neurotransmission, as previously determined by transmitter release (ref) and vascular reactivity studies (refs). On the other hand, the presence of A_1 receptors in non-neuronal cells, evidenced in these images, clearly suggest additional effects mediated by this population of A_1 receptors. This information further indicates the need to identify the type of cells where these adenosine A_1 receptors are present. Indeed, since it is well known that several type of cells can be found in adventitia, such as fibroblasts, macrophages and other immune cells or peripheral glial cells (Schwann cells) [17], it would be valuable to known the type of cell where adenosine A_1receptors are present in order to clarify their physiological/pathological role.

Other type of insights on the interplay between receptors and signalling molecules can be drawn using LSCM imaging. Indeed, this type of studies can, for instance, reveal substances that may influence receptor triggered signalling pathways/events such as reactive oxygen species (ROS), nitric oxide (NO), etc. Figure 2 shows two examples of images acquired using specific fluorescent probes for NO and ROS (DAF-FM DA and DHE, respectively), in the vicinity of adenosine A_1 receptors, suggesting the occurrence of an interplay between these receptors and those signalling molecules. This type of data can, therefore, be correlated with information in the literature to enhance knowledge. The example above mentioned correlates well with reported data indicating that adenosine receptor activation promote the increase of ROS generation[18], and can activate eNOS leading to an increase of NO production[19].

Nevertheless, qualitative analysis may present limitations, for instance, when it is required the precise description of differences in staining patterns among sets of tissues and/or cells, comparison of staining among experimental groups, etc. In this sense, applicability of LSCM imaging observed, *per se,* is, however, limited because it is perceived differently by the eye and thus can frequently be misleading. Under ideal conditions, a person with excellent eyesight can distinguish between 30 to 35 levels in comparison to the 256 or more detected by LSCM, detecting intensity variations invisible to the human eye while for a computer, an image is just an array of values of individual pixels (for 8-bit monochrome image, this would be a sequence of numbers, with values ranging from 0 to 255). To take advantage of this ability, i.e., to achieve infomation related with this intensity variations, computer assisted methods of image analysis, particularly, quantification is required.

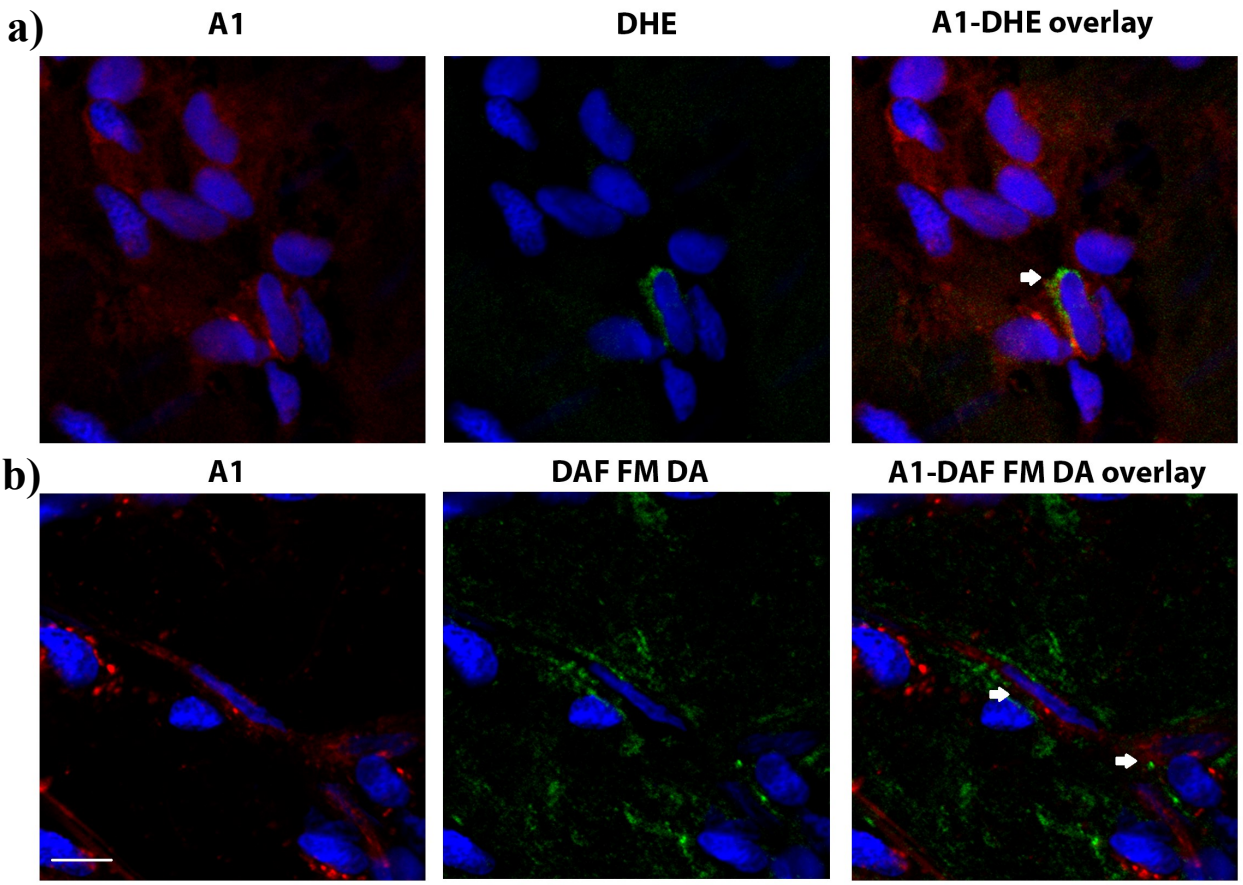

Figure 2. Laser scanning confocal immunofluorescence images of rat mesenteric artery adventitia: a) immunostained for adenosine A_1 receptors (rabbit primary polyclonal adenosine A_1 receptor antibody and a species specific secondary Alexa 647 antibody: red), DAF-FM DA (cell-permeable fluorescent probe for the detection of nitric oxide: green) and DAPI (cell permeable fluorescent minor groove-binding probe for DNA, blue); b) immunostained for adenosine A_1 receptors (rabbit primary polyclonal adenosine A_1 receptor antibody and a species specific secondary Alexa 647 antibody: red), DHE (cell-permeable fluorescent probe for the detection of superoxide anion: green) and DAPI (cell permeable fluorescent minor groove-binding probe for DNA, blue). Scale Bar = 20 μm. White arrows indicate that NO (labeled with DAF-FM DA) or superoxide anion (labeled with DHE) in the vicinity of adenosine A_1 receptors.

3. Imaging receptors with LSCM: quantitative analysis

Notwithstanding the importance of qualitative informations, reliable quantitative data related with the expression of receptors (or other type of proteins) can be precious and open possibilities, combined with functional data, to new perspectives of study, contributing for an integrative view of receptors/proteins, both in physiology and pharmacology.

Quantification is crucial for the understanding of physiopathological roles of receptors, particularly in disease states where it has been reported that specific receptors types are sub or overexpressed[1, 20-23]. Until recently, few studies have addressed the extraction of quantitative information on the expression/presence of receptors[24-26], mostly due to methodological difficulties. In this sense, there are several image analysis software commercial or freely available (such as Metamorph, PAQI, Image J) allowing automated or semiautomated image analysis to quantify one or more receptors/protein types through particle counting, area determination and/or intensity measurement. Nevertheless, the quantification algorithm will depend on the specificities of the immunohistochemical protocols used and the aims of the study. Therefore, adjustments in the quantification procedures may be required, leading to procedures customization, to obtain valid segmentation profiles of the object of interest. Only through an adequate and criterious validation process it is possible to ensure that the methodology application is unbiased, credible and reliable[27]. Image processing can require optimization, adjustments, the use of filters, histograms, segmentation/thresholding and stack projections.

Another important aspect that should be considered is the time (duration) of the observation since this leads to fluorophores consumption which is critical when the parameter to be quantified is fluorescence intensity. Fluorescence intensity and bleaching of fluorophores can be influenced by several factors, namely mounting medium, power of excitatory light, etc. Other aspect that should be taken into consideration in LSCM is photobleaching since[28] the higher power and the focused beam induce enhanced fading of the specimen. Strategies to minimize these problems include changing experimental conditions related with fluorophores, such as increasing fluorophores concentration, use of antifade agents in mounting media or use of fluorophores resistant to bleaching.

The quality of the original data is crucial for measurement accuracy and depends on the resolution of the microscope and the amount of light detected. Digital images quality can be assessed using scatter graphs comparing the correlation of an image of a single fluorophore with a second image of the some fluorophore. Pearson's correlation coefficient can be used as a quantitative measure of quality to correct the measured colocalization of two flurophores. Depending of the type of study other correlation measurements may be needed, and are compiled in the JACoP plugin for Image J. Additionally, a proper preparation of images for coefficients calculations, and correct interpretation of obtained results will determine the sucess of a quantitative study[29, 30].

Quantification algorithms can be increasingly complex according to the study aims, namely if the researcher wants to segment staining and threshold adjacent but not co-localized objects of interest (proteins, signaling molecules, receptors, etc). This latter aspect is directly related with the LSCM resolution (see above), but may also depend on the study features, i. e, if the researcher want to evaluate the colocalization profiles or molecules trafficking.

Table 1. Description of the algorithm steps for the quantification of receptors, nerves and of the interception of receptors and nerves

Step 1 (analysis of sympathetic nerves) and step 2 (analysis of adenosine A_1 receptors)
1. Ensure that image is properly prepared, acquired, and processed.
2. Open Fluorescence images of sympathetic nerves (such as image from Figure 1A, TH) or of adenosine A_1 receptors (such as image from Figure 1A, A1)
3. Determining/Setting the threshold intensity reference background/noise (for all images)
4. Obtaining binary image that marks sympathetic nerves or adenosine A_1 receptors
5. Obtaining new image containing only the demarcated areas of sympathetic nerves or adenosine A_1 receptors and corrected intensity background
6. Sympathetic nerve or adenosine A_1 receptor binary images analysis and determination of the surface area of the sympathetic nerves or surface area of attachment of the adenosine A_1 receptors and the fluorescence intensity on the surface of the sympathetic nerves or adenosine A_1 receptors, corrected for background.
Step 3 (analysis of the receptors on the sympathetic nerves (overlay))
1. Measurement of the surface area and strength of adenosine A_1 receptors on the nerves
2. Results: Fluorescence images of adenosine A_1 receptors; dividing binary images of sympathetic nerves; dividing binary image adenosine A_1 receptor.
3. Obtaining image that marks the adenosine A_1 receptors on sympathetic nerves
4. Obtaining image containing only the demarcated areas of adenosine A_1 receptors on sympathetic nerves and corrected intensity background
5. The latter is analysed for determination of the surface area of attachment of the adenosine A_1 receptors on the sympathetic nerves and intensity of fluorescence of the adenosine A_1 receptors on sympathetic nerves, corrected for background

The complexities described above have impaired quantitative studies concerning receptors to be widespread. If we take images depicted in Figure 1A as an example, quantification procedures require an algorithm that includes instructions for the measurement of the surface area and intensity of sympathetic nerve terminals, receptors and receptors on the sympathetic nerve terminals. This algorithm allows to accurately segment and threshold adjacent but not co-localized proteins, tyrosine hydroxylase and adenosine A_1 receptors (Table 1) and was designed to run in PAQI platform (CEMUP, Porto, Portugal).

After applying this algorithm, the appropriate calculation coefficients were performed and results are (in pixels/μm^2): total sympathetic nerve fluorescence intensity ($5.6x10^5$), total adenosine A_1 receptors fluorescence intensity ($6.1x10^5$), adenosine A_1 receptors overlaid with sympathetic nerves ($4.1\ x10^5$). These data, clearly evidenced that from all receptors quantified 67% were present in sympathetic nerve terminals and 33% were found in non-neuronal cells, confirming qualitative data.

Concerning disease states comparison between values obtained from normal *versus* pathological state can reveal the role of a specific receptor in the disease in study. Moreover, quantitative results concerning adenosine A_1 receptors located in non-neuronal cells may evidence a pathophysiological role attributable to these cells. Depending on the cell type involved (fibroblasts, immune or glial cells) more specific conclusions can be drawn.

Apart from the quantitative analysis of LSCM imaging other ongoing developing fluorescence methods allow to extract quantitative measurements, namely the use of fluorescent dyes that bind to effector molecules such as Ca^{2+} (Quin-2, Fura-2, Indo-1 and Fluo-3), NO (DAF-2; DAF-FM), ROS (DHE), Na^+ (SBFI), K^+ (PBFI), etc. An important advance achieved in the last decade was the engineering of fluorescent protein and its use as a tag since this approach allowed to better understand the function and location of several proteins/receptors improving intracelullar multidimensional analysis. Indeed, two families of fluorescent proteins are currently available, the green fluorescent protein (GFP), originating from the jelly fish *Aequorea victoria* and the reef coral fluorescent protein (RCFP), originating from reef corals.

The use of fluorescence fusion proteins can be suitable, for instance, for studies where their expression is transient or expression levels change overtime (for details see [6]). These quantitative studies can be performed statically or in real time imaging. In this sense, there are plugins for Image J of upmost usefulness for this purpose. In colocalization studies when the two fluorophores colocalization may fluctuate in number or as function of time, for example, the Image J plugin "FRET and Colocalization Analyser" can be used. Moreover, many fluorescent agonists and antagonist are available for the study of receptors[31], but they need to conserve its efficacy and affinity in order to be useful for the study of the dynamic processes that include activation, translocation and cell function of receptors, so far difficult to address.

4. Conclusions

Until recently, most, if not all, pharmacological studies have been based on the development of agonist or antagonists and the targeting of the signalling cascades triggered by membrane receptor modulation. Nevertheless, several studies have raised the possibility that receptors might be located in cellular compartments other than the plasma membrane. This perspective can increase the pharmacological understanding of drugs and lead to the development of new therapeutic approaches, particularly if their physico-chemical properties enable them to cross the plasma membrane. Indeed, in the last 15 years several authors have investigated the ocurrence of receptors located inside the cell, both in cytoplasmatic organelles[32, 33] and in the nuclei[34-36], helping in the understanding of the subsequent signalling events triggered by their activation.

Receptors should be viewed as dynamic proteins in constant traffic within the plasma membrane or among other organelle membranes where they can be stimulated by exogenous or endogenous ligands. Receptor activity is modulated by fine tunning and their steady state is balanced by a mechanism involving receptor internalization, through endocytosis. Then, receptors are routed to endosomes and they can be either recycled into its "active state" into membranes for repeated receptor activation [33] or be degradated inside lysosomes, which lead to the termination of receptor signalling mediated events[32, 37-39].

On the other hand, there is increasing evidence of the presence and role of G protein coupled receptors such as endothelin-1, angiotensin II, bradykinin and neuropeptide Y receptors in nuclear membrane of many cell types[35]. Therefore, the subcellular location and physiological functions of receptors are closely related and justify the importance to precisely determine the distribution of different ligands and receptors inside intracellular structures, their trafficking, dimerization or other types of receptor-protein interactions.

The combination of new techniques for fluorescent labeling and staining with advance fluorescence microscopy has offered new opportunities concerning the analysis of cellular dynamic behavior of cellular molecules, such as receptors. These methodologies renewed experimental approaches to study receptors location and/or dynamics, allowing intracellular visualization and quantification of signalling events triggered by receptor activation, through continuous determination of ions (calcium, sodium, potassium, etc) as well as their spatial and temporal concentration variations in

single cells or in cell compartments/organelles. It is accepted that the use of dye-tagged ligands coupled to real 3D confocal microscopy is the best methodology for the research and development of new drugs and it can be performed *in vitro,* in different cell types, or *in vivo* using tissue sections of treated animals[40, 41]. A even more recent refinement of this technique consists in the cytosolic microinjection of dye-tagged ligands before real 3D confocal microscopy analysis.

In summary, available sensitive microscopy equipment, development of fluorescent antibodies, tags and probes including for agonists and antagonists, and powerful analytical computer-assisted methods is allowing increasing research advances concerning location, traffic and function of receptors in different experimental conditions including real-time scale. These advances can greatly help researchers to better understand intracellular events and receptor physiopathological roles impacting our knowledge of health and disease.

Acknowledgments: The support by Fundo Europeu de Desenvolvimento Regional (FEDER) through the Program of Operational Competitiveness Factors–(COMPETE) is gratefully acknowledged. JBS support by Fundação para a Ciência e Tecnologia (FCT) for her PhD grant [SFRH/BD//2009] is also gratefully acknowledged. Authors are also gratefull to Dr Carlos Sá for his technical support.

References

[1] Zeng C, Vangveravong S, Xu J, Chang KC, Hotchkiss RS, Wheeler KT, Shen D, Zhuang ZP, Kung HF, Mach RH. Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy. Cancer research. 2007;67:6708-16
[2] Daly CJ, Parmryd I, McGrath JC. Visualization and analysis of vascular receptors using confocal laser scanning microscopy and fluorescent ligands. Methods in molecular biology. 2012;897:95-107
[3] Belanger LF. Autoradiography of in vitro activated sections of tissue as a tool in histochemistry, in relation to metabolic disorders and cancer. Laboratory investigation; a journal of technical methods and pathology. 1959;8:139-46; discussion 46-8
[4] Winteringham FP, Harrison A, Hammond JH. Autoradiography of water-soluble tracers in histological sections. Nature. 1950;165:149, illust
[5] Young WS, 3rd, Kuhar MJ. A new method for receptor autoradiography: [3h]opioid receptors in rat brain. Brain research. 1979;179:255-70
[6] Castillo-Badillo JA, Cabrera-Wrooman A, Garcia-Sainz JA. Visualizing g protein-coupled receptors in action through confocal microscopy techniques. Archives of medical research. 2014
[7] Hori Y, Kikuchi K. Protein labeling with fluorogenic probes for no-wash live-cell imaging of proteins. Current opinion in chemical biology. 2013;17:644-50
[8] Kobilka B, Gether U, Seifert R, Lin S, Ghanouni P. Characterization of ligand-induced conformational states in the beta 2 adrenergic receptor. Journal of receptor and signal transduction research. 1999;19:293-300
[9] Calizo RC, Scarlata S. Discrepancy between fluorescence correlation spectroscopy and fluorescence recovery after photobleaching diffusion measurements of g-protein-coupled receptors. Analytical biochemistry. 2013;440:40-8
[10] Paddock SW. Principles and practices of laser scanning confocal microscopy. Molecular biotechnology. 2000;16:127-49
[11] Capek M, Janacek J, Kubinova L. Methods for compensation of the light attenuation with depth of images captured by a confocal microscope. Microscopy research and technique. 2006;69:624-35
[12] Bozard BR, Ganapathy PS, Duplantier J, Mysona B, Ha Y, Roon P, Smith R, Goldman ID, Prasad P, Martin PM, Ganapathy V, Smith SB. Molecular and biochemical characterization of folate transport proteins in retinal muller cells. Investigative ophthalmology & visual science. 2010;51:3226-35
[13] Yuan GS, Pan GT, Wu FM, Han CG, Huang HG, Hu M, Sheng L, Chen J, Liu YX. [subcellular localization and tissues expression profile of hgpcrc: An orphan g protein-coupled receptor]. Sheng wu gong cheng xue bao = Chinese journal of biotechnology. 2005;21:365-9
[14] Arribas SM, Daly CJ, McGrath IC. Measurements of vascular remodeling by confocal microscopy. Methods in enzymology. 1999;307:246-73
[15] Arribas SM, Gonzalez JM, Briones AM, Somoza B, Daly CJ, Vila E, Gonzalez MC, McGrath JC. Confocal myography for the study of hypertensive vascular remodelling. Clinical hemorheology and microcirculation. 2007;37:205-10
[16] Arribas SM, Daly CJ, Gonzalez MC, McGrath JC. Imaging the vascular wall using confocal microscopy. The Journal of physiology. 2007;584:5-9
[17] Majesky MW, Dong XR, Hoglund V, Mahoney WM, Jr., Daum G. The adventitia: A dynamic interface containing resident progenitor cells. Arteriosclerosis, thrombosis, and vascular biology. 2011;31:1530-9
[18] Thakur S, Du J, Hourani S, Ledent C, Li JM. Inactivation of adenosine a2a receptor attenuates basal and angiotensin ii-induced ros production by nox2 in endothelial cells. The Journal of biological chemistry. 2010;285:40104-13
[19] Ray CJ, Marshall JM. The cellular mechanisms by which adenosine evokes release of nitric oxide from rat aortic endothelium. The Journal of physiology. 2006;570:85-96
[20] Li S, Huang S, Peng SB. Overexpression of g protein-coupled receptors in cancer cells: Involvement in tumor progression. International journal of oncology. 2005;27:1329-39
[21] Ni A, Yin H, Agata J, Yang Z, Chao L, Chao J. Overexpression of kinin b1 receptors induces hypertensive response to des-arg9-bradykinin and susceptibility to inflammation. The Journal of biological chemistry. 2003;278:219-25

[22] Dominguez-Avila N, Ruiz-Castaneda G, Gonzalez-Ramirez J, Fernandez-Jaramillo N, Escoto J, Sanchez-Munoz F, Marquez-Velasco R, Bojalil R, Espinosa-Cervantes R, Sanchez F. Over, and underexpression of endothelin 1 and tgf-beta family ligands and receptors in lung tissue of broilers with pulmonary hypertension. BioMed research international. 2013;2013:190382
[23] Di Fabio F, Alvarado C, Majdan A, Gologan A, Voda L, Mitmaker E, Beitel LK, Gordon PH, Trifiro M. Underexpression of mineralocorticoid receptor in colorectal carcinomas and association with vegfr-2 overexpression. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2007;11:1521-8
[24] Lenkei Z, Beaudet A, Chartrel N, De Mota N, Irinopoulou T, Braun B, Vaudry H, Llorens-Cortes C. A highly sensitive quantitative cytosensor technique for the identification of receptor ligands in tissue extracts. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2000;48:1553-64
[25] Miserey-Lenkei S, Lenkei Z, Parnot C, Corvol P, Clauser E. A functional enhanced green fluorescent protein (egfp)-tagged angiotensin ii at(1a) receptor recruits the endogenous galphaq/11 protein to the membrane and induces its specific internalization independently of receptor-g protein coupling in hek-293 cells. Molecular endocrinology. 2001;15:294-307
[26] Abadir PM, Periasamy A, Carey RM, Siragy HM. Angiotensin ii type 2 receptor-bradykinin b2 receptor functional heterodimerization. Hypertension. 2006;48:316-22
[27] Diniz C. Microscopic image analysis using computer-assisted methodology to quantify immunostained receptors. In: Microscopy: Science, Technology, Applications and Education. Eds. Méndez-Vilas A & Díaz J. Formatex Research Center 2010. p.1516-25.
[28] Suzuki T, Matsuzaki T, Hagiwara H, Aoki T, Takata K. Recent advances in fluorescent labeling techniques for fluorescence microscopy. Acta histochemica et cytochemica. 2007;40:131-7
[29] Zinchuk V, Grossenbacher-Zinchuk O. Quantitative colocalization analysis of confocal fluorescence microscopy images. Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]. 2011;Chapter 4:Unit4 19
[30] Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. Journal of microscopy. 2006;224:213-32
[31] Daly CJ, McGrath JC. Fluorescent ligands, antibodies, and proteins for the study of receptors. Pharmacology & therapeutics. 2003;100:101-18
[32] Katzmann DJ, Babst M, Emr SD. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, escrt-i. Cell. 2001;106:145-55
[33] Doherty GJ, McMahon HT. Mechanisms of endocytosis. Annual review of biochemistry. 2009;78:857-902
[34] Bkaily G, Avedanian L, Jacques D. Nuclear membrane receptors and channels as targets for drug development in cardiovascular diseases. Canadian journal of physiology and pharmacology. 2009;87:108-19
[35] Bkaily G, Avedanian L, Al-Khoury J, Ahmarani L, Perreault C, Jacques D. Receptors and ionic transporters in nuclear membranes: New targets for therapeutical pharmacological interventions. Canadian journal of physiology and pharmacology. 2012;90:953-65
[36] Bkaily G, Al-Khoury J, Jacques D. Nuclear membranes gpcrs: Implication in cardiovascular health and diseases. Current vascular pharmacology. 2014
[37] Sorkin A, von Zastrow M. Endocytosis and signalling: Intertwining molecular networks. Nature reviews. Molecular cell biology. 2009;10:609-22
[38] Haglund K, Dikic I. The role of ubiquitylation in receptor endocytosis and endosomal sorting. Journal of cell science. 2012;125:265-75
[39] Raiborg C, Stenmark H. The escrt machinery in endosomal sorting of ubiquitylated membrane proteins. Nature. 2009;458:445-52
[40] Avedanian L, Jacques D, Bkaily G. Presence of tubular and reticular structures in the nucleus of human vascular smooth muscle cells. Journal of molecular and cellular cardiology. 2011;50:175-86
[41] Chahine M, Bkaily G, Nader M, Al-Khoury J, Jacques D, Beier N, Scholz W. Nhe-1-dependent intracellular sodium overload in hypertrophic hereditary cardiomyopathy: Prevention by nhe-1 inhibitor. Journal of molecular and cellular cardiology. 2005;38:571-82