

CONFOCAL MICROSCOPY: FROM FUNDAMENTAL OPTICS TO INNOVATIVE APPLICATIONS

C. Saldaña¹, V. Morales-Tlalpan² and V.M. Castaño^{3,4,*}

¹Departamento de Biomedicina. Facultad de Medicina. Universidad Autónoma de Querétaro. México

²Hospital Regional de Alta Especialidad del Bajío. León Guanajuato. México

³Universidad Autónoma de Querétaro, Campus Cerro de las Campanas, Querétaro. México

⁴On sabbatical leave from Centro de Física Aplicada y Tecnología Avanzada. UNAM. Mexico.

*corresponding author (castano@fata.unam.mx)

Confocal microscopy is a relatively new technique, which has attracted a good deal of attention in the last decade or so, mainly due to its applications in biomedicine. The first confocal microscope was patented in 1957 by Minsk and, within the following decade, equivalent designs were available. The seventies witnessed an impressive growth in the science of confocal microscopy, thanks to the development of lasers, computer technology and image processing software. In the mid-eighties, papers related to biological approaches utilizing confocal microscopy began to appear. This chapter aims to highlight, to the newcomer to this area, the potential of confocal microscopy to eliminate out of focus planes and thus the blurring of the specimens observed, emphasizing the ability of this method to perform three-dimensional reconstructions, thus generating a new area in 3D microscopy. Using fluorescent illumination also opens the attractive perspective to study organisms *in vivo*, through the so-called confocal laser scanning fluorescence microscopy. Nowadays, confocal microscopy setups can also include time and spatial series analysis and even allow to combine them together with enough separation of the emission spectrum as to characterize living specimens with high resolution, both spatially and timewise.

Over the past 15 years then, confocal microscopy has represented a true revolution in biomedical research, as a powerful and sophisticated tool for biology, molecular biology, cytogenetic, physiology and, more recently, in physics and materials science.

Accordingly, this chapter reviews basic aspects of confocal microscopy, by first presenting a general description of the imaging principles, then, some details of the evolution of confocal microscopy, from the beginnings to today's technology available. Likewise, the reader will hopefully taste some of the potential of this technique for biological and biomedical applications, as well as an introduction to the whole new world of nanomaterials, as viewed through a confocal microscope.

Keywords Confocal Microscopy, Nanotechnology, Fluorochrome,

1. Confocal Imaging theory

The so-called Abbe theory [1] of image formation begins by considering an object illuminated by a grid through an objective. Thus, the corresponding diffraction pattern of the grid will be observed at the back focal plane of the objective. The diffraction patterns obtained by the grid are combined to form an image on the image plane, as schematically depicted in Figure 1.

Roughly speaking, this is the theoretical basic description of the image formation, according to Fourier optics, through which one can realize that two Fourier transforms are involved, corresponding to the effect of the objective lens and the propagation to the plane of the image [2]. In this relation of images (or Fourier transforms, if one can speak mathematically), the resolution basically depends on the size of the grating of the grid, which, through optical manipulation, can also allow to produce phase contrast and dark field imaging.

However, there is a dispute in the case when an object is not homogeneously illuminated, as in the case of the so-called Köhler illumination [3]. This is usually sorted out through the use of Rayleigh's criterion, which allows improving incoherent imaging [4]. Such criterion fundamentally states that an image depends on the numerical aperture of the condenser and the objective lenses. In such a way, the formation of coherent and incoherent images is experimentally controlled by small numerical apertures of the condenser and large numerical apertures in the objective planes.

2. The confocal microscopy, from the beginnings to the modern instruments

Confocal microscopy has nowadays become an important tool in several scientific areas, including biology, biomedicine and, more recently, materials science [5-16]. The standard procedure involves the use of molecules with a fluorescent capability, known as fluorophores or fluorochromes. Fluorochromes arrays have been used to identify cells, subcellular structures and cellular microdomains, focusing principally on live specimens, thus revealing a high

level of specificity in the detection of single fluorescent molecule [17, 18]. Similarly, through the use of multi-labeled specimens, different probes can simultaneously identify several target molecules, both on fixed specimens as well as in living cells and tissues. [19]. However, it must be said that confocal microscopy has a spatial resolving power limited by the diffraction characteristics of the fluorescent molecules employed.

As briefly mentioned above, the origins of the confocal microscope date back to the fifties, when a Harvard PhD student, Marvin Minsky, intended to solve the complex network of neural connections through the use of microscopy images [20, 21]. Minsky aimed to avoid the use of stains tissue and thus try to detect the events of synaptic transmission in vivo. The experiments and observations of Minsky remained marginal due to the low power of the lamps available at the time and the lack of a robust system to analyze the images. Minsky finally managed to patent his design in 1961.

In the late 1960's, David Egger and Mojmir Petro used a Nikow disk to examine sections of brain and ganglion cells without using dyes [22]. Egger succeeded, in 1973, in obtaining the first image of a cell, establishing the principles of what today is known as confocal laser scanning microscopy [23]. The global scientific interest on confocal microscopy emerged during the seventies and eighties, with the rapid development of computer systems, laser technology and algorithms for digital image manipulation [24].

By the late seventies, Minsky's patent had expired, leading to a new era of confocal microscopes. Fred G. Brakenhoff, simultaneously developed a scanning confocal microscope [25], while Colin Sheppard was contributing to the formation of images [26]. These technological advances were used by Tony Wilson, Brad Amos and John White, and applied to the visualization of biological samples using fluorescent probes [27, 28]. From these observations, a series of publications revealed the potential of confocal microscopy. This pioneering stage of confocal microscopy ended in 1987 with the first commercial systems

During the nineties, technological advances in optics, lasers and electronics allowed more powerful, stable and better definition sweeps in two and three dimensions. This was further optimized by dichroic mirrors, fiber optics, high-performance dielectric films and photodetectors [5]. On the other hand, fluorochromes with improved quantum efficiencies [17] were available in the market. All these advances were, of course, supported by the improvement of computing power and greater storage capacity.

Finally, at the turn of the new century, the success of confocal microscopy is explained by the ease with which it can obtain high-resolution images from preparations for conventional fluorescence microscopy, either for fixed or living cells and tissues. Confocal microscopy allows preparations with a thickness greater than two micrometers, which usually produce a high degree of fluorescence, through a substantial improvement in axial (z), lateral resolution (X, Y) and temporal resolution (t). Today's confocal systems allow combinations of the three levels (X, Y, t, or X, Y, Z, t), with a strong decrease of photon noise outside the focal plane [29-30]. However, and despite the fact that confocal microscopy offers higher resolution than wide-field microscopy, it has not been able to offer a resolution close to that of electron microscopy, which has somehow limited the use in other exciting applications.

Modern confocal microscopy can be considered as a completely integrated electronic system, where the optical microscopy is linked to one or more electronic detectors, a computer and several laser systems of various wavelengths, coupled to a beam scanning assembly. At the same time, it offers several advantages over conventional widefield microscopy, including control depth, elimination or reduction of background information away from the focal plane and the capability to collect series of optical sections from thick specimens.

3. Biological applications

The advantage of confocal microscopy is the ability to produce optical sections through the specimens with thickness ranging 1-50 micrometer or even more. The series of images are collected by increments in the fine focus mechanism, using a step motor, to perform sequential image acquisitions in each step. Each image is restricted to a unique plane and the noise from others planes in the specimen can be thus discriminated. Contrast and definition are improved due to the reduction in background noise. The optical slides decrease artifacts induced during specimen manipulation and processing with fluorescent probes. [32, 33].

The combination of confocal microscopy and fluorescent molecules, allow visualizing a wide range of alive and fixed specimens, including quantitative confocal microscopy, immunolabelling, imaging in live cells, ion dynamics, cell division, apoptosis, necrosis, membrane potential, endocytosis in several conditions, multiple labeling, FRAP, FLIP, FRET, FISH, line scanning, exocytosis, membrane fluidity, protein trafficking, signal transduction, and enzymatic activity, just to mention few of the applications reported in the literature [to review see 32,34, 35].

A great variety of molecules can be made into fluorescent probes. These probes started out as molecular dyes known to bind to specific subcellular structures or molecules of interest [32, 2]. In recent years, a large number of non-fluorescent probes have been made into fluorescents probes by the addition of suitable molecular groups. Inside the cells, tissues and selected organism, the structure, organization and dynamics of the diverse organelles, specific proteins and protein interaction can be detected by using specific probes [2].

Many fluorescent probes are available from diverse companies [33] and are designed to recognized specific biological macromolecule (for example, a protein, nucleic acid), or to localize within a specific structural region, such as the cytoskeleton (Phalloidin-Rodamine) [36], mitochondria (Mitotracker: Green, Orange and Red, 3,3'-

dihexyloxycarbocyanine iodide (DiOC₆(3)), Golgi apparatus (BODIPY-Brefeldin) [36, 37], endoplasmic reticulum [37], and nucleus (DAPI, TO-PRO, SYTOX, Hoechst 33342) [37, 38]. Other fluorescent molecules are employed to monitor dynamic processes and localized environmental variables, including concentrations of ions (calcium, sodium and potassium) [37, 38, 39], pH, reactive oxygen species [38], and membrane potential [2, 32].

Additionally, advances in immunology and molecular biology have produced a wide spectrum of secondary antibodies and provided insight into the molecular design of fluorescent molecules targeted at specific regions within macromolecular complexes [36]. The fluorescent probe technology and biomedical sciences were significantly revolutionized by the green fluorescent protein (GFP) from jellyfish and the development of mutant spectral variants blue (EBFP), cyan (ECFP), yellow (EYFP), which have opened the use of non-invasive fluorescence multicolor investigations of subcellular protein localization, intermolecular interactions, and trafficking using living cell cultures [2, 31]. Recently, the development of nanoprobe, nanosensor and nanoparticles has provided a new avenue for research in CM. These molecules have been developed for imaging and dynamic monitoring in vivo of the molecular or ionic components, constructs, forces and dynamics, all in real time, during biological/chemical/physical processes. With their biocompatible small size and inert matrix, the nano molecules have been successfully applied for non-invasive real-time measurements of analyses and fields in cells and rodents, with spatial, temporal, physical and chemical resolution [37, 39].

4. Advantages for biomedical research

Confocal microscopy has not only resulted in a great deal of interest for the study of fixed cell/tissue. Also, other important techniques have been developed to determine molecular dynamics, fluorescence recovery after photobleaching (FRAP), for measuring connectivity of cellular compartments, fluorescent loss in photobleaching (FLIP), used to determine molecular proximity, fluorescent resonance energy transfer (FRET) for gene mapping, fluorescence in situ hybridisation FISH; to perform dynamic processes in msec-sec line scanning, quantitative confocal microscopy, immunolabelling, imaging in live cells as: ion dynamics, cell division, apoptosis, necrosis, membrane potential, endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction, and enzymatic activity [2, 31-41].

Confocal microscopy has been also employed to perform a precise and rapid diagnostic of bacterial biofilms. These are complex, mono- or poly-microbial communities, adhered to biotic or abiotic surfaces. This adaptation has been regarded by some as a survival strategy. The formation of biofilms is mediated by mechanical, biochemical and genetical factors. The biofilms enhance the virulence of the pathogen and play a potential role in various infections, such as dental caries, cystic fibrosis, osteonecrosis, urinary tract infection and eye infections [41].

Generally speaking, many of the original academic uses of confocal microscopy, meant for biological studies, are finding more and more applications in biomedicine, either as a powerful tool for diagnosis or for determining mechanisms, both biological and physico-chemical, for a growing variety of diseases.

5. Materials science and the nanoworld from a confocal standpoint

Interestingly, in spite that Materials Science has been one of the more active areas of R&D in the last century (most materials used daily today were invented in the recent 80 years!), the experts in this area have just began to recognize it as a valuable analytical tool [42- 44], although confocal microscopy, as explained above, is well established in the life sciences. This is rather surprising since one of the aims of Materials Science is to explore the structure-properties relationships, being microscopy in general, one of the most common techniques employed by materials scientists worldwide. Moreover, electron microscopes, for one example, owe most of their impressive development to the requirements of materials science-related industries [45-47].

As opposed to the bio area, in materials science the Light scattered by the specimen is not only regarded as a way to produce images, but also as an analytical opportunity, by using, for example, a coupled Raman spectrometer, which allows to gather chemical information from a well defined volume of the samples, smaller than 1 μm^3 .

The enormous variety of microscopy techniques available to modern materials scientists open up the opportunity of combining confocal microscopy with atomic force microscopes, for example, thus counting with a very powerful combination [48-50].

Uses of confocal microscopy in materials science and engineering include the inspection of metallic, polymeric or ceramic surfaces, for the solar, car manufacturing and printing industries, among others, as well as the precise measurement of micro-roughness and layer thickness in micro- and nanotechnology for a wide range of applications, from electronics to bioengineering. It is worth mentioning that confocal microscopy is finding uses not only in the academia, but as a valuable instrument for quality control in industry, as well [51- 54].

In principle, it could seem that confocal microscopy can play a little role on imaging systems few nanometers in size. However, confocal microscopy and nanotechnology, together, represent a very promising area for R&D, not only for the aforementioned use of nanosystems as novel chromophores and nanoactuators, but also through the use of ultrafast

spectroscopy combined with 3D imaging and manufacturing [55], among other exciting alternatives currently under way.

6. Perspectives and concluding remarks

In spite of the enormous activity of confocal-related R&D, the technique is still in its beginnings, in terms of all the theoretical, experimental and technological work remaining to be explored. Indeed, the very optics behind a confocal microscopy represents an attractive area for investigating limits of resolution, alternative imaging methods, improved illumination systems, etc. The very rapid growth of commercially-available nanotechnology products also mean an attractive opportunity for novel chromophores, in situ treatments through chemical or physical interaction of the beam with metal or ceramic nanoparticles, etc. Finally, the application of this fascinating tool to nearly all areas of technology development today, has, as perhaps the only limiting factor, our own imagination.

7. References

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Foot figure

Figure 1a: Schematic diagram of optical pathway in laser scanning confocal microscopy. In the right panels representative experiment performed with CM. 1B: *Candida albicans* stained with calcineurin-AM (Acosta L and Castaño VM); 1C: cancer-ovarian tissue r visualized with functionalized gold-nanoparticles (with permission of De la Rosa H and Salas P); 1D: Mitochondrial network in MCF-7 was visualized with Mitotracker red (Saldaña C), and 1F: subcellular distribution of endomembranas in MCF-7 (Saldaña C and Castaño VM). The scale bar in the images (white lines) indicates 10 μ m.



