Development of novel imaging tools for selected biomedical applications

Omar E. Olarte

Advisor: Prof. Pablo Loza-Alvarez

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Abstract

In the quest for better and faster images of cellular and subcellular structures, biology-oriented optical microscopes have advanced significantly in the last few decades. Novel microscopy techniques such as non-linear microscopy (NLM), including two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) microscopy, and light-sheet fluorescence microscopy (LSFM) are emerging as alternatives that overcome some the intrinsic limitations of standard microscopy systems. In this thesis I aimed to advance such techniques even more, and combine them with other photonic technologies to provide novel tools that would help to address complex biological questions. This thesis is organized in two main parts. The first part is dedicated to applications involving femtosecond lasers that are employed for precise microsurgery. For that, damage assessment methodologies based on NLM were developed and tested in relevant biomedical models. In the second part, wavefront engineering methods were employed to enhance the imaging capabilities of light-sheet microscopy systems. These novel methodologies were tested as well in relevant biological applications. This thesis is, therefore, organized as follows:

In chapter 1, a brief and comprehensive review of the basic microscopy techniques employed in this thesis is presented, together with the challenges and achievements of this thesis in sequential order.

In chapter 2, a multimodal imaging methodology for the assessment of laser induced collateral damage is presented. This was specifically developed for the control of the damage in femtosecond-laser dissection of single axons within a living *Caenorhabditis elegans* (*C. elegans*). Here, it is shown that collateral damages at the level of the myosin structure of the muscles adjacent to the axon, can be readily detected.

In chapter 3, the optimized multimodal methodology developed in the chapter 2 was employed for minimally invasive dissection of axons of D-type motoneurons in *C elegans*.

Here, a microfluidic chip for *C elegans* immobilization and a detailed protocol was employed to evaluate the axon regeneration of such neurons. The potential of such platform for testing drugs with regeneration-enhancing capabilities is also presented.

In chapter 4, a novel use of TPEF microscopy is presented to characterize and fine tune the laser for photodisruption of excised human crystalline lens samples.

In chapter 5, a thorough description of the implementation of a multimodal Digital Scanned Light-Sheet Microscope (DSLM) able to work in the linear and nonlinear regimes under either Gaussian or Bessel beam excitation schemes, is presented. The enhanced capabilities of the developed system is evaluated using *in vivo C. elegans* samples and multicellular tumor spheroids

In chapter 6, the development of a completely new concept in light sheet-based imaging is presented. This is based on the extension of the depth-of-field of the lens in the emission path of the microscope by using wavefront coding (WFC) techniques. Furthermore, I demonstrate the application of the developed methodology for fast volumetric imaging of living biological specimens and 3D particle tracking.

Chapter 1: Introduction

Major advancements in biological research have been possible by the developing of novel techniques and instrumentation in the fields of optics and photonics. This imposes an enormous challenge that certainly pushes the limits of the optical sciences. Laser development and optical microscopy may have not been the same without the fruitful interaction with biological sciences. Since their introduction in the second half of the twentieth century, lasers have been proven to be versatile and effective tools to non-invasive examination and sub-cellular manipulation of biological samples. The use of lasers to obtain high resolution images of cellular structures is now a daily practice in many biological laboratories around the world. Of special interest for biology is the development of techniques based on the observation of selected fluorescent structures inside the samples. These structures are usually labelled with vital dyes or have attached genetically-encoded proteins that make them fluorescent (in some specimens this is an intrinsic characteristic). In the typical fluorescence (wide-field) microscope the specimen is illuminated with laser light of a specific wavelength which is absorbed by this fluorescent elements, or fluorophores, causing them to emit light of longer wavelengths. This light is then collected using a CCD camera. The main drawback of this configuration is that planes at different depths inside the specimen are excited at the same time. The resulting detected fluorescence includes a large unfocused background component. To eliminate the out-of-focus signals, a confocal configuration is used. A confocal microscope normally uses a high numerical aperture (NA) for creating a diffraction-limited illumination point and uses a pinhole, located in an optically conjugated plane in front of the detector, to eliminate out-of-focus signal. In this way, only the light produced very close to the focal plane can be detected and, in consequence, *optical sectioning* is achieved. In confocal microscopy the focused laser beam, usually in the visible wavelength range, is raster-scanned across the sample to produce an optical section from the selected field of view, this is why this technique is usually termed Confocal Laser Scanning Microscopy (CLSM) [1]. This technique has emerged as a very powerful tool for biological imaging. Its ability to provide high transversal and axial resolution, collect three-dimensional images of relatively thick specimens, together with the user friendliness and versatility of modern

commercial confocal microscopes, has made it the biologist's preferred imaging tool. However, the use of short wavelengths for exciting the fluorescence makes it suffer from fundamental penalties such as phototoxicity/photodamage and low penetration depth due to the strong dependence of scattering coefficients with the wavelength. On the other hand, Nonlinear microscopy (NLM) techniques [2,3], such as two-photon excited fluorescence (TPEF), second harmonic generation (SHG) and third harmonic generation (THG)have resulted in an interesting alternative to CLSM. NLM relies in the use of ultrashort-pulsed lasers which normally emit in the near infrared (NIR) spectral region. The nonlinear contrast is based on second and third-order nonlinear light-matter interactions. Since these nonlinear optical effects are proportional to the second or third power of the fundamental light intensity, they are induced only at the focus of a high numerical aperture (NA) microscope objective. This fact results in the intrinsic elimination of the out-of-focus contributions and results in the intrinsic optical sectioning inherent to nonlinear imaging techniques. Therefore, it is a straightforward task to generate a sharp, three-dimensional image. The excitation beam is simply raster scanned across the focal plane, and the signal generated as a result of the nonlinear excitation is measured and correlated to the beam position in a similar way as CLSM. Imaging using NLM possess several advantages over conventional CLSM: i) it possess intrinsic optical sectioning without the need of any pinhole (and thus all the generated light is used), ii) nonlinear excitation is done normally in the NIR and, as a consequence, larger penetration depths can be achieved, iii) there is a reduced photobleaching as the interaction occurs only at the focal point and iv) NML is amenable for label-free imaging, that is the case of SHG and THG, among others. Nonlinear microscopes provide immense possibilities in biological investigation, but the possibility of counting also with complementary linear imaging techniques is, in many cases, desirable.

Optical microscopes, as any other optical instrument, are limited by a fundamental maximum of resolution determined by the diffraction. This is called the *diffraction limit* of resolution. High-end objectives employed for any of the afore-mentioned microscopy techniques are designed to have very high imaging performance close to the diffraction limit of resolution. However, this is only true when ideal conditions are met, e. g. the sample under observation is thin and transparent. Therefore,

resolution of these microscopes is often compromised by the optical properties of the specimen itself. Spatial variations in the refractive index of the specimen introduce optical aberrations that hinder image quality. This is a particular problem when imaging deep into thick biological specimens. Ultimately, the aberrations restrict the amount of specimen that can be observed by the microscope. This is a serious limitation if one wants to observe cells and their processes in their usual environment, rather than in the unnatural surroundings of a microscope slide. To ameliorate such problems Adaptive Optics (AO) may be used. AO has been broadly applied in astronomy to reduce the effects of changing atmospheric conditions which produce aberrations and deteriorate the image quality of stars and satellites. By employing wavefront sensors (WFS) and deformable mirrors (DM), the distortions produced when distant light sources travel through the turbulent atmosphere can be corrected. This concept can be extrapolated to the problem of aberration compensation in microscopy [4]. There are several approaches to measure and correct sample related wavefront aberrations in fluorescence microscopy. For NLM, correction of the excitation beam will ensure a better focussing and therefore, a more efficient nonlinear process. In practice, measuring and correcting fundamental beam aberrations of microscopes is a difficult task. However, this can be done by finding a well-defined, localized and bright enough source for measuring the aberrations. This is known in AO community as *guide star*. Strategies have been proposed to guide star-like analogous by introducing artificial guide stars (fluorescent micron-sized beads) into the sample [5]. However, this interferes with the sample being an invasive technique. Other alternatives are not based on guide stars and correct the aberrations by optimizing a figure-of-merit defined on the captured images. Both solutions have limited applications and may compromise the viability of delicate/living biological specimens.

My PhD started in this point by trying to find a solution to the challenge of defining a reliable protocol to measure and correct aberrations in the excitation beam of NLM. For this, we presented a novel method taking advantage of the fact that nonlinear excitation (particularly TPEF) occurs only in a highly localized and confined region of the sample. This can in fact be considered a natural reference source which we called *nonlinear guide star*. Using this concept we demonstrated, for the first time,

the use of intrinsically generated guide stars for the purposes of wavefront measurement and correction in AO microscopy. This principle has been demonstrated with both, moderate and increased scattering samples. In both cases we found a TPEF signal improvement.

This work shows an initial example of the use wavefront engineering methods, in this case AO, to attack problems encountered in biological microscopy.

At this point, I realized the big potential that such kind of technology would have to other applications, particularly to laser-based surgery.

Ultrashort-pulsed lasers used for NLM can be employed as well to perform dissections on selected targets on a variety of biological samples. The most significant advantage of using these lasers is the confinement of induced effects to the focal volume owing to the involvement of nonlinear absorption mechanisms. Some of the many applications of femtosecond laser surgery in bio -medical applications are: neuronal axotomy in *C. elegans* [6], wound response of *Drosophila melanogaster* embryos during dorsal closure [7], subcellular nanosurgery in cell cytoskeleton [8], and human eye corneal surgery [9], among others. The physical mechanisms involved in laser ablation of transparent dielectric materials with femtosecond lasers are well known [10]. When ultrashort pulses of NIR light are focused to a tight spot through high NA microscope objective, high peak intensities are achieved at the sample plane with relatively low pulse energies. This generates nonlinear photon (multiphoton) absorption in an otherwise transparent medium and electron ionization within the focal volume. Then, free-electron density increases exponentially due to a process termed as cascade ionization. At a critical density, the resulting free-electron plasma causes optical breakdown and physical disruption of the material. Pulse width and the focusing strength determine the intensity threshold to produce optical breakdown. This suggests that laser parameters can be finely tuned to control the effects of the generated plasma and the subsequent optical breakdown into the irradiated volume.

Following this route, I was devoted to build and optimize a microscopy system capable of high-precision surgery that would be applied to relevant biological and biomedical problems, such as neuron regeneration and crystalline lens softening.

On the other hand, the visualization and quantification of biological processes in difficult samples such as living organisms and tissue models require microscopy methods that can provide 3-dimensional data with high spatial and temporal resolutions over a large field of view. Nevertheless, microscopy methods employed for such difficult tasks should also minimize the phototoxic effects of laser irradiation. For example, In CLSM, every time an optical section is captured, fluorescence in many other axial planes is excited and rejected by the confocal pinhole. This led to an accumulative phototoxicity/photodamage when volumetric imaging is required. Light-sheet fluorescence microscopy (LSFM), also known as selective plane illumination microscopy (SPIM) [11], has been proven to be an interesting alternative to CSLM. In LSFM a thin laminar sheet illuminate sample form the side, thus exciting only a two-dimensional section of the sample. The emitted fluorescence is imaged along an optical axis perpendicular to the illumination plane. This atypical configuration provides intrinsic optical sectioning, with no energy deposition outside the optical section; large fields of view imaging, and enables isotropic resolution [12]. In spite of all this, LSFM still carries the problem of using lasers in the visible wavelengths for excitation, and then is prone to scattering.

Thinking on this, I became aware of the potential of another wavefront engineering technology to modify the point spread function (PSF) of a system but this time to produce thin, long “needles” of light. This “needles”, known as Bessel beams [13], have shown to have interesting properties that make them to keep well formed when propagate inside highly scattering samples. Therefore, I explored the potential of such beams to build a multimodal LSFM, which in combination with femtosecond lasers in the excitation arm, provided a system with high resolution, enhanced contrast and large field-of-view imaging.

Finally, having a 3D microscopy technique that would allow the observation of fast events with high resolutions in a large field of view is of extreme importance for biological applications. Such a technique would allow for a better understanding of complex dynamic processes in living cells, tissues and organisms. Calcium waves of heart and brain cellular cultures, the embryonic zebrafish beating heart and the red blood cells in the developing cardio-vascular are a few of the most challenging

biological processes calling for faster microscopy techniques [14]. LSFM with its many advantages have shown important improvements in terms of speed as compared with regular CLSM. Nevertheless, the need to mechanically scan the sample to retrieve the optical sections sets the limit of the volumetric imaging speed that can be achieved by LSFM.

To deal with this, I have explored the use of a similar wavefront engineering approach applied to the collection path of the previously developed light-sheet microscope. For this, an optical setup inspired in the principles of AO that can effectively provide the system capable to overcome the intrinsic speed limit present in regular LSFM. I will show how this allowed us to obtain fast volumetric imaging of living animals and fast 3D particle tracking.

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