Multimodal microscopy for rapid diagnosis of pancreatic cancer.

por

Maria del Carmen Grados Luyando

Tesis sometida como requisito parcial para obtener el grado de

MAESTRO EN CIENCIAS EN LA ESPECIALIDAD DE ÓPTICA

en el

Instituto Nacional de Astrofísica, Óptica y Electrónica.
Octubre de 2014
Tonantzintla, Puebla

Supervisada por:

Dr. Julio César Ramírez San Juan
INAOE
5.1 Measurements 38
5.2 Results and Analysis 41

CHAPTER VI 47

CONCLUSION 47

CHART OF FIGURES 49

BIBLIOGRAPHY 51
Summary

Multimodal confocal mosaic images scan image wide surgical margins (approximately 1 cm) with sub-cellular resolution and mimic the appearance of conventional hematoxylin and eosin histopathology (H&E). The goal of this work is to combine three confocal imaging modalities: acridine orange (AO) fluorescence for labeling nuclei, eosin fluorescence (Eo) for labeling cytoplasm, and endogenous reflectance (R) for marking collagen and keratin. Absorption contrast is achieved by alternating the excitation wavelength: 488 nm (AO fluorescence) and 532 nm (Eo fluorescence). Scattering contrast is achieved by the reflectance at either or both of the wavelengths. Superposition and false-coloring of the images from these three modalities mimics H&E, enabling detection of pancreatic cell carcinomas. The sum of images Eo + R is false-colored pink to mimic the appearance of eosin, while the AO mosaic is false-colored purple to mimic the appearance of hematoxylin in H&E. In this study, mosaics of pancreatic cell carcinomas were processed. The results suggest that confocal mosaics could be used for pancreatic cancer diagnosis in the operating room, although the staining process with Eosin may be optimized as the reflectance mode.
Introduction

Previous reports have been made in the area of confocal microscopy with the end of generating quick images of different kinds of tissue, like skin tissue [1] or breast tissue [2], which are diagnostic for cancer. New modalities for rapid noninvasive optical imaging of skin cancers include Raman Spectroscopy [3], fluorescence polarization, multiphoton, fluorescence lifetime, fluorescence imaging, optical coherence tomography, and reflectance confocal microscopy [4]. Confocal microscopy is the most clinical practical technology, with high resolution and sectioning comparable with that of conventional histology.

The confocal microscope may be use for a rapid diagnostic in the operating room without waiting for the pathologist, who may take around 25 minutes to several days for a “quick” diagnosis [5]. Previous studies have shown good images of the confocal microscope for making diagnostic in skin [6]. It is necessary to prove that this device is capable of making good images for diagnostic in a couple of minutes for any kind of tissue.

Rapid micrographic pathology represents an opportunity for confocal microscopy of whole tissue excisions. By stitching square raster-scanned fields of view together, a 12 x 12 mm mosaic can be produced in 9 min [7], which is similar to a 2x histological view of a Mohs excision. A recently developed “strip mosaicing”
technique is even faster, producing a 10 x10 mm mosaic in about 3 min [8]. In strip mosaicing, the slow-axis scanning galvanometer is fixed on the optical axis while the fast polygon scans a stationary line in the tissue. The sample is physically scanned perpendicular to the stationary line in the focal plane to acquire long rectangular images are stitched together in less time than square images [8]. The long rectangular images conveniently have no field curvature in their long (mechanically scanned) axis. These are rapid techniques compared to histology. Adapting the technique to guide excision of different cancer types would save more tissue and possibly enable new surgeries.

In this research we will use a confocal microscope as a tool for the diagnosis of pancreatic cancer, it is one of the common cancer types that are diagnosed with the greatest frequency in México and in the United States, excluding nonmelanoma skin cancers. [9]

Cancer is the third cause of dead in México and according to the International Union Against Cancer, every year more than 128,000 Mexicans are diagnosed with it. Since 2008, is the first cause of dead in the world. [10] “Pancreatic cancer is the fourth cause of dead in México for oncologic problems; it is very aggressive and by the time the diagnosis is done most of the times is too late”, Carlos Chan, president of the Hepatic Pancreatic Biliary Mexican Association (AMHPB). [11]

The American Cancer Society’s most recent estimates for pancreatic cancer in the United States are for 2014:

About 46,420 people (23,530 men and 22,890 women) will be diagnosed with pancreatic cancer.

About 39,590 people (20,170 men and 19,420 women) will die of pancreatic cancer.

Rates of pancreatic cancer have been increasing slightly over the past decade or so. Pancreatic cancer accounts for about 3% of all cancers in the US, and accounts for about 7% of cancer deaths. [12]

The purpose of this work is to find out if the confocal microscope is able to make images of pancreatic cancer good enough for making a diagnosis and quick enough for being profitable in the hospital.
In Chapter II a brief introduction about the kinds of tissue that exist and what is their role in the body. After that we will be able to better understand what is a neoplasia and an anaplasia. The histology (healthy tissue) of the pancreas is described in this chapter as the pathology (not healthy tissue) of it.

Chapter III describes how a confocal microscope works, how is the image formed and the different resolutions. The functioning of the fluorescence microscope is also described here.

The experimental arrangement and the methodology of the experiment for the preparation of the sample is described in chapter IV.

In chapter V the process of measuring and the results are presented, the analysis of them is right after each image describing what it was found.

The general conclusions of this work are presented in chapter VI.
The tissue

Introduction

A brief introduction about the different kinds of tissue is presented in this chapter, and their role in the body. The concepts of anaplasia and neoplasia are described for a better understanding of the pancreatic carcinoma.

2.1 Different kinds of tissue

The cell is the basic building block that makes up all the various organs of the body. Between cells and organs are important intermediaries: the tissues that are formed by grouping cells to collectively develop a special function. In the formation of the organs, two or more tissues are involved.

Although more than 250 cell types are involved in forming the body of mammals, there are only four main tissue types: epithelial tissue, connective tissue (including cartilage, blood and bone tissue), muscle tissue and nervous tissue. [13]
2.1.1 Epithelial Tissue

The epithelium is a tissue composed of adjacent cells without separate intercellular substances and includes all membranes composed of cells lining the exterior of the organism and the inner surfaces. There are different types of epithelium Fig. 1: simple and stratified squamous epithelium, simple and stratified cuboidal epithelium, simple columnar epithelium, pseudo stratified and stratified. [13]

![Types of Epithelium](image)

Fig. 1 Different types of epithelium. [14]

2.1.2 Connective Tissue

The connective tissue is also called support tissue as it represents the skeleton holding other tissues and organs. As the connective tissue forms a coherent mass between the blood vascular system and all epithelia, any exchange of substances should be carried through the connective tissue, so it can be considered as the internal environment of the organism. The connective tissue fibers are divided into 3 types, collagen fibers, reticular or fibroblastic and elastic. [13] These are shown in Fig. 2.
2.1.3 Muscle Tissue

Oriented active movement is characteristic of higher life forms and multicellular organisms have developed highly specialized cells, muscle cells. Muscle cells are elongate, oriented in the direction of longitudinal axis movement, frequently so obvious that they used to be called fibers. In the body of vertebrates there are 3 types of muscles well differentiated by structure and function: smooth muscle, skeletal muscle and cardiac muscle. [13]
2.1.4 Nervous Tissue

Normal functions of the body depend on receiving stimuli from the external and internal environment, and the generation of the integrated reactions directed in response. This activity requires a coordinating link between stimulus and response, between the receptor organ and the effector organ. The nervous system includes all the nervous tissue of the body and its main role is communication and is traditionally divided into the central nervous system and peripheral nervous system. [13]

![Image of central nervous system and peripheral nervous system](image)

**Fig. 4 The central nervous system and the peripheral nervous system in the human body. [17]**

2.2 What is a Neoplasia?

Neoplasia literally means the process of “new growth”, and a new growth is called a neoplasm. The term tumor was originally applied to the swelling caused by inflammation. Neoplasms also may induce swellings, but by long precedent, the non-neoplastic usage of tumor has passed into limbo; thus, the term is now equated with neoplasm. Cancer is the common term for all malignant tumors.

“A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persist in the same excessive manner after cessation of the stimuli which evoked the change”.
2.2.1 Malignant Neoplasms

Malignant neoplasms of epithelial cell origin, derived from any of the three germ layers, are called carcinomas. The natural history of most malignant tumors can be divided into four phases: 1) malignant change in the target cell, referred to as transformation; 2) growth of the transformed cells; 3) local invasion; and 4) distant metastases. [13]

2.3 Differentiation and anaplasia

Differentiation refers to the extent to which neoplastic cells resemble comparable normal cells, both morphologically and functionally; lack of differentiation is called anaplasia.

Anaplasia is marked by a number of morphologic changes:
1.- Pleomorphism. Both the cells and the nuclei characteristically display variation in size and shape.
2.- Abnormal nuclear morphology. Characteristically the nuclei contain an abundance of DNA and are extremely dark staining. The nuclei are disproportionately large for the cell, and the nucleus-to-cytoplasm ratio may approach 1:1 instead of the normal 1:4 or 1:6.
3.- Mitoses. As compared with benign tumors and some well-differentiated malignant neoplasms, undifferentiated tumors usually possess large number of mitoses, reflecting the higher proliferative activity of the parenchymal cells (any cell that is a functional element of an organ, such as a hepatocyte) [18]. The presence of mitoses, however, does not necessarily indicate that a tumor is malignant or that the tissue is neoplastic.
4.- Loss of polarity. In addition to the cytologic abnormalities, the orientation of anaplastic cells is markedly disturbed. Sheets or large masses of tumor cells grow in an anarchic, disorganized fashion.
5.- Other changes. Another feature of anaplasia is the formation of tumor giant cells, some possessing only a single huge polymorphic nucleus and others having two or more nuclei.
When dysplastic changes (dysplastic changes, are atypical changes in the nuclei of cells (the inside of the cell that contains DNA), the cytoplasm (the portion of the cell surrounding the nuclei), or in the growth pattern of cells) [19] are marked and involve the entire thickness of the epithelium, but the lesion remains confined to the normal tissue, it is considered a preinvasive neoplasm and is referred it as carcinoma in situ.

In general the benign tumors are well differentiated. Despite the exceptions, the more rapidly growing and the more anaplastic a tumor, the less likely it is that there will be specialized functional activity. The cells in benign tumors are almost always well differentiated and resemble their normal cells of origin; the cells in cancer are more or less differentiated, but some loss of differentiation is always present.

How long does it take to produce a clinically overt tumor mass? It can be readily calculated that the original transformed cell (approximately 10 µm in diameter) must undergo at least 30 population doublings to produce $10^9$ cells (weighing approximately 4.2 g as a 1 cm-diameter sphere), which is an easily palpable cancer. Nevertheless, this calculation highlights an extremely important concept about tumor growth: by the time a solid tumor is clinically detected, it has already completed a major portion of its life cycle. This is a major impediment in the treatment of cancer, and underscores the need to develop diagnostic markers for detection of early cancers.

The rate of growth of a tumor is determined by three main factors: the doubling time of tumor cells, the fraction of tumor cells that are in the replicative pool, and the rate at which cells are shed and lost in the growing lesion.

Then, how long does it take for a tumor to become detectable? If every one of the daughter cells remained actively dividing and no cells were shed or lost, we could anticipate the answer to be 90 days (30 days x 3 days to double). In reality, the latent period before which a tumor becomes clinically detectable is unpredictable but typically much longer than 90 days, up to many years for most solid tumors, emphasizing once again that human cancers are diagnosed only after they are fairly advanced in their life cycle. [13]
2.4 The Pancreatic Tissue

Although the organ gets its name from the Greek *pankreas*, meaning “all flesh”, the pancreas is in fact, a complex lobulated organ with distinct exocrine and endocrine components.

The exocrine portion of the gland, which produces digestive enzymes, constitutes, 80% to 85% of the pancreas. The endocrine portion is composed of about 1 million clusters of cells, the isles of Langerhans. The islet cells secrete insulin, glucagon, and somatostatin and constitute only 1% to 2% of the organ. [20]

The most significant disorders of the pancreas are diabetes, neoplasms and pancreatitis.

Fig. 5 The endocrine and exocrine pancreas with the different hormones that secretes. [21]

2.4.1 Exocrine pancreas

Normal tissue

The exocrine pancreas is composed of acinar cells, which produce the enzymes needed for digestion, and a series of ductules and ducts to convey the secretions to the duodenum. Acinar cells are pyramidally shaped epithelial cells that are radially
oriented around a central lumen. The basal portion of acinar cells is deeply basophilic and contains abundant endoplasmic reticulum. Acinar cells also contain a well-developed supra nuclear Golgi complex that is part of an apically oriented secretory pathway that forms membrane-bound zymogen granules containing the digestive enzymes. Fig. 6

**Fig. 6** A low magnification image of equine pancreas (H&E stain) showing a large interlobular duct in association with a pancreatic artery (A) and vein (V). An intralobular duct (D) is seen on the right side.

Pathology
Disease of the exocrine pancreas include cystic fibrosis, congenital anomalies, acute and chronic pancreatitis, and neoplasms. [13]

Neoplasms
A broad spectrum of exocrine neoplasms can arise in the pancreas. They may be cystic or solid; Some are benign, while others are among the most lethal of all malignancies. [13]

Pancreatic Carcinoma
Infiltrating ductal adenocarcinoma of the pancreas, more commonly known as “pancreatic cancer” (Fig. 7), is the fourth leading cause of cancer death in the
United States, preceded only by lung colon, and breast cancers. Pancreatic cancer has one of the highest mortality rates of any cancer. It is estimated that in 2004, approximately 30,000 Americans will be diagnosed with pancreatic cancer, and virtually all of them will die from disease. The 5 year survival rate is a dismal, less that 5%.

Approximately 60% of cancers of the pancreas arise in the head of the gland, 15% in the body, and 5% in the tail; in 20% the neoplasm diffusely involves the entire gland. Carcinomas of the pancreas are usually hard, stellate, gray-white, poorly defined masses. Two features are characteristic of pancreatic cancer: it is highly invasive, and it elicits an intense non-neopastic host reaction composed of fibroblasts, lymphocytes and extracellular matrix.

The appearance is usually that of a moderately to poorly differentiated adenocarcinoma forming abortive tubular structures or cell clusters and exhibiting an aggressive deeply infiltrative growth pattern. Dense stromal fibrosis accompanies tumor invasion, and there is a proclivity for perineural invasion within and beyond the organ. The malignant glands are usually lined by anaplastic cuboidal-to-columnar epithelial cells. Well-differentiated tumors are the exception. [13]

**Fig. 7** H&E of the pancreatic ductal adenocarcinoma (the most common type of pancreatic cancer)
If we compare Fig. 6 with Fig. 7 we are able to see that the first one has well differentiated structure, meanwhile the second is poorly differentiated, with a lot of cell clusters.
Confocal Microscopy

Introduction

The basic principles of confocal microscopy are reviewed in this chapter, the advantages and disadvantages of this device and some applications are presented. The concepts of resolution and how the image is formed are discussed and the principle of fluorescence microscopy.

3.1 Confocal microscopy

It is probably fair to say that the development and wide commercial availability of the confocal microscope have been one of the most significant advances in light microscopy in the recent past. The main reason for the popularity of this instrument derives from its ability to permit the structure of thick specimens of biological tissue to be investigated in three dimensions by using a scanning approach together with a novel (confocal) optical system.

The principle of confocal imaging was patented by Marvin Minsky in 1957 and aims to overcome some limitations in traditional wide-field fluorescence microscopes.

The traditional wide-field conventional microscope is a parallel processing system that images the entire object field simultaneously. This is quite a severe
requirement for the optical components, but we can relax this requirement if we no longer try to image the whole object at once. The limit of this relaxation is to detect light from one point at a time within the field of view. In this case, the optics need only to provide a good signal from one point. The price to pay is that the detection point must be scanned over the field of view in order to build up an image. The answer to the question whether this price is worth paying will, to some extent, depend on the application in question.

The confocal principle in epi-fluorescence laser scanning microscopy is presented in Fig. 8 [22]. Coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture.

The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole and forms extended Airy disks in the aperture plane. Because only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not contribute significantly to the resulting image.

In laser scanning confocal microscopy, the image of an extended specimen is generated by scanning the focused beam across a defined area in a raster pattern controlled by two high-speed oscillating mirrors driven by galvanometer motors. One of the mirrors moves the beam from left to right along the \( x \) lateral axis, while the other translates the beam in the \( y \) direction. After each single scan along the \( x \) axis, the beam is rapidly transported back to the starting point and shifted along the \( y \) axis to begin a new scan in a process termed flyback. During the flyback operation, image information is not collected. In this manner, the area of interest on the specimen in a single focal plane is excited by laser illumination from the scanning unit.
As each scan line passes along the specimen in the lateral focal plane, fluorescence emission is collected by the objective and passed back through the confocal optical system. The speed of the scanning mirrors is very slow relative to the speed of light, so the secondary emission follows a light path along the optical axis that is identical to the original excitation beam. Return of fluorescence emission through the galvanometer mirror system is referred to as descanning. After leaving the scanning mirrors, the fluorescence emission passes directly through the dichromatic mirror and is focused at the detector pinhole aperture. Unlike the raster scanning pattern of excitation light passing over the specimen, fluorescence emission remains in a steady position at the pinhole aperture, but fluctuates with respect to intensity over time as the illumination spot traverses the specimen producing variations in excitation.

Fig. 8 Confocal microscopy arrangement [22]
Some of the advantages of a confocal microscope are:

- Optical Sectioning ability - can image cells/tissues internally
- 3D reconstruction - subcellular location of labeling
- Excellent resolution - close to the theoretical limit of 0.1 to 0.2 µm
- Specific wavelengths of light used - greatly improves multiple labeling
- Very high sensitivity - capable of collecting single molecule fluorescence
- Digital images - easy manipulation and merging images
- Computer controlled - complex settings can be programmed and recalled.

Disadvantages of confocal microscopy are limited primarily to the limited number of excitation wavelengths available with common lasers (referred to as laser lines), which occur over very narrow bands and are expensive to produce in the ultraviolet region. In contrast, conventional widefield microscopes use mercury or xenon based arc-discharge lamps to provide a full range of excitation wavelengths in the ultraviolet, visible, and near-infrared spectral regions. Another downside is the harmful nature of high-intensity laser irradiation to living cells and tissues (an issue that has recently been addressed by multiphoton and Nipkow disk confocal imaging). Finally, the high cost of purchasing and operating multi-user confocal microscope systems, which can range up to an order of magnitude higher than comparable widefield microscopes, often limits their implementation in smaller laboratories. This problem can be easily overcome by cost-shared microscope systems that service one or more departments in a core facility. The recent introduction of personal confocal systems has competitively driven down the price of low-end confocal microscopes and increased the number of individual users. [22]

There are many design aspects to a confocal microscope that make these microscopes much more versatile than a conventional fluorescence microscope. Although the confocal microscope is often thought of as an instrument that can create 3D images of live cells, the large number of operational features available on these instruments actually allow many creative methods of examining not just the structural details, but also the dynamics of physiological and developmental processes in living cells and tissues.

The principle of confocal microscopy is that the out of focus light is removed from the image by the use of a suitably positioned pinhole. This not only creates images of exceptional resolution, but also allows one to collect optical slices of the object, and to use this slices to create a 3D representation of the sample.
3.2 How is the image formed?

Scanning a diffraction-limited point of excitation light across the sample in a raster formation creates the image in a confocal microscope. Two scanning mirrors are used, one to scan at high-speed in the “x” direction, and the other to scan somewhat slower in the “y” direction. The irradiating laser light is used to excite a suitable fluorophore, applied or naturally occurring within the sample. A small amount of the fluorescent light emitted from the sample passes back through the objective lens is separated from the light of a second fluorophore or unwanted reflected excitation light by the use of suitable partially reflecting dichroic mirrors.

The “in focus” light, after passing through the confocal microscope pinhole is detected by a very sensitive light detector called a photomultiplier tube (PTM). The analogue signal from the photomultiplier tube is converted to a digital form that contains both information on the position of the laser in the image and the amount of light coming from the sample.

The image is displayed on a computer screen as a shaded grey image that can be suitably coloured, and the intensity levels adjusted, later, for presentation.

3.3 How is out of focus light removed?

One of the great difficulties with conventional epi-fluorescence microscopy is that the out of focus light in the image can greatly detract from the quality of the image. Single cells or very thin tissue samples can normally be imaged by conventional fluorescence microscopy with relative ease, although as we have seen above even thin samples may be much better visualized by confocal microscopy. However, the confocal microscope really comes into force when you need to image multicellular samples, such as complex tissues.

The thin optical slice of confocal microscopy is obtained simply by eliminating the light rays that originate from out of focus positions, or more correctly, other focal planes, within the sample. Light from a single focal plane is collected by focusing the fluorescent light through a small aperture or confocal pinhole. Light rays from other focal planes are not correctly aligned with the confocal iris, and are thus eliminated from the image. This ability to remove out of focus light has the effect of producing an image that consist of information from only a very thin focal plane (a thin z section) and is often referred to as an optical slice. A series of these “z”
sections can be combined in a number of ways that allow one to visualize the 3D structure of the original sample.

3.4 Resolution

All optical microscopes, including conventional wide field, confocal and two photon instruments are limited in the resolution that they can achieve by a series of fundamental physical factors. In a perfect optical system, resolution is restricted by the numerical aperture of optical components and the wavelength of light, both incident (excitation) and detected (emission). The concept of resolution is inseparable from contrast, and is defined as the minimum separation between two points that results in a certain level of contrast between them. [22]

The relationship between contrast and resolution with regard to the ability to distinguish two closely spaced specimen features implies that resolution cannot be defined without reference to contrast, and it is this interdependence that has led to considerable ambiguity involving the term resolution and the factors that influence it in microscopy. Recent advances in fluorescent protein technology have led to an enormous increase in studies of dynamic processes in living cells and tissues. Such specimens are optically thick and inhomogeneous, resulting in a far-from-ideal imaging situation in the microscope. Other factors, such as cell viability and sensitivity to thermal damage and photobleaching, place limits on the light intensity and duration of exposure, consequently limiting the attainable resolution. Given that the available time scale may be dictated by these factors and by the necessity to record rapid dynamic events in living cells, it must be accepted that the quality of images will not be as high as those obtained by fixed and stained specimens. The most reasonable resolution goal for imaging in a given experimental situation is that the microscope provides the best resolution possible within the constraints imposed by the experiment.

3.4.1 Lateral resolution

The foundation of light microscopy was established, a century ago by, Ernst Abbe (1873.1884). He demonstrated how the diffraction of light by the specimen and by the objective lens determined image resolution, defined the conditions needed to design a lens whose resolution was diffraction limited, and established the role of objective lens and condenser numerical apertures on image resolution.
When two objects are widely separated, the images are easily distinguished on the basis of the variation of image intensity. When sufficiently separated, the intensity change in the area between the objects is the maximum possible, cycling from the peak intensity to zero and returning to the maximum value at the center of the second point. At decreased distance of separation in object space, the intensity distribution functions of the two points, in the image plane, begin to overlap and the resulting image may appear to be that of a single larger or brighter object or feature rather than being recognizable as two objects. If resolution is defined, in general terms, as the minimum separation distance at which the two objects can be sufficiently distinguished, then resolution, is related to the width of the intensity peaks. Microscope resolution is directly related, therefore, to the full width at half maximum (FWHM) of the instrument’s intensity point spread function in the component directions.

Some ambiguity in use of the term resolution results from the variability in defining the degree of separation between features and the point spread functions that are sufficient to allow them to be distinguished as two objects rather than one. In general, minute features of interest in microscopy specimens produce point images that overlap to some extent, displaying two peaks separated by a gap. The greater the gap between the peaks, the easier it is to distinguish or resolve the two objects. By specifying the depth of the dip in intensity between two overlapping point spread functions, the ambiguity in evaluating resolution can be removed, and a quantitative aspect introduce.

The relationship between contrast and separation distance for two point-like objects is referred to as the contrast/distance function or contrast transfer function. Resolution can be defined as the separation distance at which two objects are imaged with a certain contrast value. It is obvious that when zero contrast exists, the points are not resolved; the so-called Sparrow criterion defines the resolution of an optical system as being equivalent to the contrast cut-off distance. It is common, however, to specify that greater contrast is necessary to adequately distinguish two closely spaced points visually, and the well-known Rayleigh criterion for resolution states that two points are resolved when the first minimum (zero crossing) of one Airy disk is aligned with the central maximum of the second Airy disk. Under optimum imaging conditions, the Rayleigh criterion separation distance corresponds to a contrast value of 26.4 percent. Although any contrast value greater than zero can be specified in defining resolution, the 26-
percent contrast of the Rayleigh criterion is considered reasonable in typical fluorescence microscopy applications, and is the basis for the common expression defining lateral resolution according to the following equation, in which the point separation \( r \) in the image plane is the distance between the central maximum and the first minimum in the Airy disk:

\[
r_{\text{lateral}} = \frac{1.22 \lambda}{(2 \times \text{NA})} = \frac{0.6 \lambda}{\text{NA}}
\]

(3.1)

where \( \lambda \) is the emitted light wavelength and \( \text{NA} \) is the numerical aperture of the objective.

Resolution in the microscope is directly related to the FWHM dimensions of the microscope's point spread function, and it is common to measure this value experimentally in order to avoid the difficulty in attempting to identify intensity maxima in the Airy disk. Measurements of resolution utilizing the FWHM values of the point spread function are somewhat smaller than those calculated employing the Rayleigh criterion. Furthermore, in confocal fluorescence configurations, pointwise illumination scanning and pointwise detection are employed, so that only the fluorophores in the shared volume of the illumination and detection point spread functions are able to be detected. The intensity point spread function in the confocal case is, therefore, the product of the independent illumination intensity and detection intensity point spread functions. For confocal fluorescence, the lateral (and axial) extent of the point spread function is reduced by about 30 percent compared to that in the widefield microscope. Because of the narrower intensity point spread function, the separation of points required to produce acceptable contrast in the confocal microscope is reduced to a distance approximated by:

\[
r_{\text{lateral}} = \frac{0.4 \lambda}{\text{NA}}
\]

(3.2)
If the illumination and fluorescence emission wavelengths are approximately the same, the size of the Airy disk in the confocal fluorescence microscope is the square Airy disk in the widefield microscope. Consequently, the contrast cut-off distance is reduced in the confocal arrangement, and equivalent contrast can be achieved at a shorter distance compared to the widefield illumination configuration. Regardless of the instrument configuration, the lateral resolution displays a proportional relationship to wavelength, and is inversely proportional to the objective lens numerical aperture.

### 3.4.2 Axial resolution

A variety of equations are presented in the literature, which pertain to different models for calculating axial resolution for various microscope configurations. The ones most applicable to fluorescence emission are similar in form to the expressions evaluating depth of field, and demonstrate that axial resolution is proportional to the wavelength and refractive index of the specimen medium, and inversely proportional to the square of the numerical aperture. Consequently, the numerical aperture of the microscope objective has a much greater effect on axial resolution than does the emission wavelength. One equation commonly used to describe axial resolution for the confocal configuration is given below, with \( h \) representing the index of refraction, and the other variables as specified previously:

\[
 r_{axial} = \frac{1.4 \lambda \times n}{NA^2} \tag{3.3}
\]

Although the confocal microscope configuration exhibits only a modest improvement in measured axial resolution over that of the widefield microscope, the true advantage of the confocal approach is in the optical sectioning capability in thick specimens, which results in a dramatic improvement in effective axial resolution over conventional techniques.
3.5 Fluorescence Microscopy

3.5.1 Fluorescence Process

Fluorescence is the result of a three-stage process that occurs in certain molecules called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to respond to a specific stimulus or to localize within a specific region of a biological specimen. The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in Fig. 9. [23]

![Jablonski diagram of fluorescence process](image)

Fig. 9 Jablonski diagram of the fluorescence process pointing the three different stages 1, 2, 3 described in the text.

Stage 1: Excitation

A photon of energy $h\nu_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state ($S_1'$). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

Stage 2: Excited-State Lifetime

The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes
have two important consequences. First, the energy of $S_1'$ is partially dissipated, yielding a relaxed singlet excited state ($S_1$) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (Stage 1) return to the ground state ($S_0$) by fluorescence emission. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

**Stage 3: Fluorescence Emission**

A photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state $S_0$. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength than the excitation photon $h\nu_{EX}$. The difference in energy or wavelength represented by $(h\nu_{EX} - h\nu_{EM})$ is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength. [23]

**3.5.2 Microscopy**

The fluorescence microscopy has proved to be a very useful and powerful tool in many branches of biology and life science. It has to be emphasized, that the image formation properties of fluorescence microscopes are completely different from those of brightfield instruments. This results from the nature of the fluorescence generation mechanism.

First lets see the conventional microscope of Fig. 10 a), and imagine that suitable filters are present such that only the fluorescence radiation is detected. We can immediately see that the resolution results essentially from the primary, incident radiation and not from the longer wavelength fluorescence: the collector lens essentially collects fluorescence radiation onto a detector. In a conventional, non-scanning microscope, Fig. 10 b), this is not the case. Here, the primary radiation excites the fluorescence, which is then imaged by the objective lens. Thus, in this case, the resolution results essentially from the longer wavelength fluorescence radiation. This leads us to make the very important statement that we expect
scanning microscopes to be able to produce superior fluorescence images. If we combine these advantages with those concerning dosage and bleaching, together with the optical sectioning of confocal microscopy, we can understand why the technique is so useful. [24]

Fig. 10 Optical arrangement of two different kind of microscopes. a) Conventional microscope. b) A form of conventional scanning microscope.

If we assume that the fluorescence in the object destroys the coherence of the illuminating radiation and produces an incoherent fluorescent field proportional to the intensity of the incident radiation, \(I(u,v)\), then we may write the effective intensity point spread function, which describes image formation in the incoherent confocal fluorescence microscope as:

\[
I(u,v)I \left( \frac{v}{\beta}, \beta \right)
\]

where the optical coordinates \(u\) and \(v\) are defined relative to the primary radiation and \(\beta = \frac{\lambda^2}{\lambda_1}\) is the ratio of the fluorescence to the primary wavelength. We note that \(v = \left(\frac{2\pi}{\lambda}\right)r \sin \alpha\), where \(r\) denotes the actual radial distance and \(u\) is a normalized axial coordinate related to real axial distance, \(z\), \(u = \frac{8\pi}{\lambda} [n z \sin^2(\alpha/2)]\), and \(\alpha\) is shown in Fig. 11.
Fig. 11 The origin of the optical sectioning or depth discrimination property of the confocal optical system. [25]

That suggests that the imaging performance depends on the value of $\beta$. [26]
CHAPTER IV

Experimental arrangement and sample preparation

Introduction

The experimental arrangement is described in this chapter, explaining how does it work and every component of it. Also is shown an actual picture of the microscope. In this chapter is described the process to prepare the sample, the different dyes used and what do they do in the tissue, the protocols used in this experiment are presented step by step.

4.1 Experimental Arrangement

The experimental confocal microscope Fig. 12 used in this study was the Vivascope from Lucid Inc. It was modified by Dan Gareau at Oregon Health and Science University to utilizes 2 laser light sources, at 532 nm wavelength green laser (Lasermate GMLN532-50AC) and at 488 nm blue laser (Blue Sky Research). The objective lens features 30X magnification to provide a 430 µm field of view. With a numerical aperture NA of 0.9, the calculated axial section thickness is: \( \Delta z = \frac{1.4\lambda n}{NA^2} = 1.1 \) µm and the lateral resolution is: \( \Delta x = \frac{0.46\lambda}{NA} = 0.25 \) µm, which is adequate for imaging nuclear morphology.
Two laser beams were aligned to hit the scanning mirror, then they pass through 2 dichroic mirrors that let pass all the green and blue laser light. The two beams arrive to the spinning polygonal mirror, which scans in the “x” axis. A pair of lenses works as a telescope. A galvanometer scans in the “y” axis. A pair of “relay” lenses transfers the scanned beams to the objective lens, sending collimated light into the rear port objective lens, which causes a focused point in the sample plane that is scanned laterally in x and y. The reflection and the fluorescence from the sample go backwards along in the same path just described, the signals travel together all the way back until the dichroic mirrors. Once the signals reach that point, the fluorescence signal hits the dichroic mirror and is send it to the photomultiplier tube (PTM), meanwhile the reflectance signal passes through the first dichroic mirror hitting the second dichroic mirror and is send it to the avalanche photodiode (APD). An actual picture of the microscope is shown in Fig. 13.

Fig. 12 Optical design of the confocal microscope with the following components: Diode pumped solid state 532nm green laser, argon-ion laser 488 nm, , spinning polygon (P), galvanometric scan mirror (G), relay telescopes (T), and objective lens (Obj). A dichroic beamsplitter (B/S₁) steers fluorescent emission toward a photomultiplier tube (PTM). A polarizing beamsplitter (B/S₂) steers reflected light toward an avalanche photodiode (APD).
4.2 Sample preparation

Samples of pancreatic cancer where stained using protocols already established in previous papers [1] also they were used to create a new protocol described here. The pancreatic tumors were generated by taking patient tissue from the operation room (OR) whipple or ramps procedures and implanting the tumors subcutaneously in a Nod SCID gamma (immune compromised) mouse. The tumors were allowed to grow and then harvested. Tumors used were from multiple passages, meaning that the tumors had been excised and re-implanted into new mice.
The tissue was first stained using the protocol found in [1], and then settled in a cover glass of thickness of 1mm. The protocol consisted in 5 steps:

1. 30-s rinse in 95% ethanol for dehydration

2. 30-s soak in stock Eo solution (identical to that used in the gold standard histopathology)

3. 30-s rinse in isotonic phosphate-buffered saline solution

4. 30-s soak in AO solution (1 mM, in phosphate buffered saline at pH 6.0 controlled by addition of HCl), and

5. 5-s rinse in isotonic phosphate-buffered saline solution.

The second protocol consisted of 8 steps:

1.- 30-s rinse in Acridine Orange

2.- 15-s rinse in water

3.- 15-sec soak in Water/Ethanol (50/50)

4.- 15-s Ethanol (100%)

5.- 30-s soak in stock Eo solution

6.-15-s Ethanol (100%)

7.- 15-s soak in Water/Ethanol (50/50)

8.- 15-s rinse in water

The third protocol involved staining the tissue with a concentration of 1mM of AO during 40 seconds.
The next table explains the labeling of the different parts of the tissue by the dyes and their excitation wavelengths.

Table 1

<table>
<thead>
<tr>
<th>Tissue component</th>
<th>Confocal mode</th>
<th>$\lambda$ Illumination</th>
<th>Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>Fluorescence</td>
<td>532 nm</td>
<td>Eosin</td>
</tr>
<tr>
<td>Nuclei</td>
<td>Fluorescence</td>
<td>488 nm</td>
<td>Acridine Orange</td>
</tr>
<tr>
<td>Collagen/Keratin</td>
<td>Reflectance</td>
<td>488 nm</td>
<td>No stain</td>
</tr>
</tbody>
</table>

After the staining protocol, and setting the tissue on a 1mm thick glass slide, it was covered by a ~1 mm wide piece of agarose gel (80% water, 20% agarose) and pressed by a glass ~130 mm thick coverslip to sandwich the tumor sample. Pressing the tissue ensured a flat surface in the cover glass, making the scanning easier and yielding better images.

For the first measurement the tissue is illuminated just with the blue laser. The blue laser as shown in Table 1 highlights nuclei and collagen. The use of just one laser at the time is due to the photo bleaching nature of the dyes, if the green laser was on at the same time as the blue laser AO will photobleach in seconds.

With the blue laser and focused in the plane of the same surface the confocal microscope scanned the tissue using the fluorescence channel. After the scanning, the green laser was turned on, and using the same channel, the fluorescence from the Eosin was imaged. Then the channel was changed to the reflectance mode and the reflectance scan was taken, which imaged the collagen and keratin.

Only the excised tissue surface is examined in Mohs histology, to determine the lateral extent of tumor. Thus, for imaging, staining with acridine orange was necessary only superficially through a few cell layers. A cell layer in skin is about 10 $\mu$m thick, and the Mohs surgeon usually examines 3 to 5 frozen histology sections, each being 5 to 6-mm thin. The confocal microscope imaged to a maximum depth of 30 $\mu$m, which corresponded to 3 cell layers. This depth is also approximately the maximum to which real-time confocal imaging is possible in dermal tissue with very low milliwatt power blue 488-nm illumination.

Rapid staining depends on the diffusion of the fluorescent dye into the excised tissue. The uptake kinetics of any particular dye depends on molecular weight and
tissue conditions such as pressure in interstitial spaces. The average time \( t \) [s] for diffusion across a distance \( x \) cm may be determined from the diffusion coefficient \( D \) \( \text{cm}^2/\text{s} \) as:

\[
t = \frac{x^2}{2D}
\]  

(4.1)

The diffusion coefficient varies with molecular weight \( M_r \) in tumor and normal tissue by the following power law:

\[
D = a(M_r)^b
\]

(4.2)

Experiments by Nugent and Jain [27] determined the coefficients to be \( a=10^6 \), \( b=-2.96 \) for normal tissue, and \( a=2.51 \times 10^{-2} \), \( b=-1.14 \) for tumor VX2 carcinoma, rabbit. For acridine orange, the diffusion coefficient is then calculated to be \( 15.2 \times 10^{-3} \) \( \text{cm}^2/\text{s} \) in normal tissue and \( 24.5 \times 10^{-3} \) \( \text{cm}^2/\text{s} \) in tumor, assuming similar order-of-magnitude parameters for normal skin and BCC tumors. Using \( x=30 \, \mu\text{m} \) in Eq. 4.1, the average diffusion time is 0.6 ms for tumor and 0.37 s for normal tissue.
Measurements, Results and Analysis

Introduction

In this chapter the process of measuring (how to start running the microscope and how to set the tissue) are presented, the results founded for each protocol previously described and what do the images founded can be interpreted are shown.

5.1 Measurements

During the process of imaging, the polygon scans in the x-axis; meanwhile the galvanometer scans in the y-axis. The whole process consists of staining a fresh biopsy and imaging it. The process of staining may be similar for some kinds of tissue, but definitely will differ.

The speed of spinning of the polygonal mirror makes this microscope faster than other microscopes that have 2 galvanometers, instead of 1 galvanometer and 1 polygonal mirror.

The light sources of the microscope were 2 lasers, emitting at 470 nm with approximately 53 mW of power, and at 532 nm with approximately 52 mW of power, both coherent and with linear polarization.
The scanning microscope comes with a software (Vivascan); the computer program is opened by clicking on an icon on the computer screen, and soon the sound of the polygonal mirror spinning says that the microscope its ready to work.

To check that the microscope was working correctly, a piece of paper with fluorescent ink highlighter was set on the glass and pressed, in previous studies, water as an immersion medium was often substituted with water-based gels such as Aquasonic 100 ultrasound gel and Suave® hair gel. These gels have refractive indices of 1.36 and 1.34, respectively, that are reasonably close to that 1.33 of water. Furthermore, the gel has a higher degree of viscosity and can therefore be placed on the inverted objective lens. By comparison to water, the use of these gels particularly, Suave® appeared not to degrade the image quality of the superficial tissue layer. The immersion gel is placed between the objective lens and the coverglass window. [28].

Pressing the buttons in the computer graphic user interface (GUI) adjusted the depth of focusing, allowing one to search for the tissue surface. Also other buttons controlled the lateral (right, left, up and down) movements. Another control switched from the reflectance channel to the fluorescence channel. The images captured are shown for the fluorescence channel in Fig. 14 and for the reflectance channel in Fig. 15, where the fibers of the paper are clearly seen.

Once this is done, the intensities of the lasers are manually attenuated by a pair of neutral density filter wheels. Then a tissue sample prepared as is described in chapter 3, is placed in the microscope for imaging. The photobleaching time is really short, around 1 minute, so the scanning in the fluorescence channel of the Acridine Orange has to be the first one, after that the scanning in the reflectance channel is done.
**Fig. 14** Figures from the piece of paper with highlighter, being illuminated with a green laser and scanned in the fluorescence channel. Making zoom from a) to c) to show that the paper fibers are clearly seen.

**Fig. 15** Figures from the piece of paper with highlighter, being illuminated with a green laser and scanned in the reflectance channel. Making zoom from a) to c).

Multiple images were captured as the field of view is translated by the microscope, by moving the sample stage with micrometers. These images were then combined into a larger mosaic image (software by Dan Gareau. In a matlab program) the fluorescence capture is false colored in purple and the reflectance is false colored in pink for mimic H&E. Once this is done the two images are put together in a final mosaic image.
5.2 Results and Analysis

The imaging of pancreatic carcinoma, use different variations on the staining protocol. In the experiments, the best results used only the AO staining and not using Eosin. Using the protocols described previously in materials and methods, the images found are show below.

First protocol.

Pancreatic tumor of a mouse pH 7, 1mM.

![Images from a pancreatic tumor stained with the main protocol, a) image from the entire piece of tissue, b) Zoom into one of the zones with more nuclei, c) in a circle the only nuclei founded.](image)

**Fig. 16** Images from a pancreatic tumor stained with the main protocol, a) image from the entire piece of tissue, b) Zoom into one of the zones with more nuclei, c) in a circle the only nuclei founded.

In Fig. 16 a piece of pancreatic tumor of a mouse was stained with the first protocol presented previously in a concentration of 1mM of acridine orange. If the concentration of the AO is too high it made an inhomogeneous solution, not good for the staining procedure. If the concentration was too low, not enough stain entered the cell. It is difficult to see any connective tissue from the reflectance channel, in Fig. 16 c) is shown the zoom of the zone with more nuclei, but the details of the nuclei are not enough for a diagnostic assessment, we expected to see more nuclei, cytoplasm and connective tissue, good enough for distinguish each part of the pancreatic configuration.
Pancreatic tumor of a mouse, pH 7, 1.2 mM.

Fig. 17 Pancreatic tumor of a mouse main stain protocol with 1.2 mM of concentration in AO. a) Image from the entire piece of tissue, b) zoom into the part with more nuclei, c) nuclei founded marked in circles and connective tissue pointed with arrow.

Fig. 18 Pancreatic tumor of a mouse main stain protocol with 1.2 mM of concentration in AO. a) Image from the entire piece of tissue, b) zoom into the part with circles marked pointed with arrows.
In Fig. 17 and Fig. 18 the mouse tumor was stained with the first protocol and with a concentration of 1.2 mM of acridine orange, to check if the concentration in the previous experiment wasn’t enough, in Fig. 17 more nuclei are visible than in the previous try. The reflectance is still being a problem and is not enough to distinguish the histological structure of the pancreas. Fig. 18 doesn’t have much more nuclei than Fig. 17, also in the figure we can see some kind of circles are visible, which probably are part of the structure of the pancreas.

Second protocol.
Pancreatic tissue pH 7, 1.2 mM

![Pancreatic tumor](image)

**Fig. 19** Pancreatic tumor of a mouse stained with protocol 2. a) Image of the whole piece of tissue, b) zoom into the only part with visible nuclei pointed with arrows; star showing circles made by the glass and the cone of the objective microscope.

Fig. 19 shows the pancreatic tumor of a mouse stained with the second protocol and concentration of 1.2 mM due to the better results with these concentrations in the previous experiment. The figure just shows a little part with nuclei, but nothing really significant for our purpose, even though these nuclei are more defined and with more color than in previous images. The black star in Fig. 19 b) points to part
of the circle made by the glass and the cone of the objective microscope. In this particular part we are able to see something like water, this is water from the tissue, more probably stain.

![Image of pancreatic tumor](image.png)

**Fig. 20** Pancreatic tumor of a mouse, only connective tissue is visible.

In Fig. 20 we weren’t able to see any nuclei however the connective tissue is visible, at the end of these and several more tries, we notice that something was happening between the two staining, the next pictures were stained with another protocol to see if there was a problem.
Third protocol.
Pancreatic tissue with AO 40 sec, pH 6, 1.2 mM.

Fig. 21 Pancreatic tumor of a mouse stained just using AO for 40 seconds. a) Image of the whole piece of tissue, b) zoom into one of the zones with more nuclei, c) nuclei of the pancreas.

Fig 15, is one of the first images where the presence of nuclei is noticeable, never the less the lack of reflectance in this image doesn’t allows us to have a good idea about the structure of the pancreatic tissue, without that information is not possible for a pathologist to make a good diagnostic. We can see nuclei, but we are not able to see in which part of the structure they are.
a)                                        b)                                        c)

**Fig. 22** Pancreatic tumor of a mouse stained just using Ao for 40 seconds. a) Image of the whole piece of tissue, b) zoom into one of the zones with more nuclei, c) nuclei of the pancreas.

The third protocol was the one that give us better results. In Fig. 22 we are able to see a piece of tumor from a mouse, clearly we can see nuclei all around the tissue, sometimes even forming circles, those would give information from the structure of the pancreas, here we are not able to see any good connective tissue or cytoplasm marked, which is a problem for a diagnostic. The nuclear-to-cytoplasmic ratio is one of the features that the pathologists are looking for when they are searching for cancer in tissue. The problem is that in this protocol we didn’t use Eosin which is the stain that marks the cytoplasm. The 2 pink parts marked with black stars in Fig. 22 c) are air bubbles made between the tissue and the glass. The goal every time that tissue is going to be scanned is to press it enough against the glass to avoid any air bubbles.
Conclusion

At the end, in the last images, the staining of the nuclei told us that this microscope may be able to make images good enough for making diagnostic of pancreatic cancer, also the times of staining and scanning are really good comparing with the pathologist time, it took around 9 minutes for the whole process to be finished, since the cutting of the part, the staining, the imaging, and the false coloring of the tissue. The reflectance channel is one of the biggest problems, but when it had a good alignment, the connective tissue was seen. The study of the protocol by a specialist and the ensure of the alignment of all the parts of the microscope during the process of scanning, would give better results.

The microscope has micrometer screws in the base where the tissue will be placed; these for ensure that the base is completely flat with respect to the microscope objective and that the scanning would be in the same surface of the tissue, nevertheless these screws are really sensitive to vibrations, and when the scanning is taking place all the frame moves really brusquely causing loosely screws. So every certain time the base has to be adjusted, something that is not an easy work or fast.

The reflectance channel; the fluorescence channel has 2 screws for the alignment of the signal though the pinhole and to the detector, however the reflectance channel doesn’t, the detector is over a frame and for adjusting the signal to the detector the movements are too rough. This is one of the biggest problems I think; because of this the reflectance images are not good enough, and they are an important part of
the final image, without them is not possible for the pathologist to make a good diagnostic.

During the scanning process and the movement of the frame where the tissue is placed, it may be and inconvenient if the tissue is to fresh, for example healthy tissue of the pancreas even after the protocol still having larges amount of water in it, so when is time for making an image and I pressed to the glass with a cover glass or just with the circles of agarose the tissue used to move, because of the pressure and for the movement of the frame during the scanning, so what I did was to print in the 3D printer a cylinder with a hole in the bottom and another cylinder without hole which fitted in the first one. I made some circles of agarose and put them in the bottom of the cylinder with the hole and then I pressed it with the second cylinder, the agarose going out from the cylinder pressed the tissue against the glass, fixing the cylinder to the glass with tape the tissue wasn’t able to move, also I think it would be good to developed a better method for this.

For me as a physicist the part or chemistry was an obstacle, I had the advise for this part from Dr. Gibbs who is an immunohistochemistry at Oregon Health and Science University, however I believe that the full time commitment from a immunohistochemistry would make a difference when the staining process is taking place. A complete knowledge in the area may be useful for the good preparation of different kind of tissues. During the process I worked with lungs, pancreas, skin, liver etc. and I was able to see that different tissues need different protocols, maybe not too much one from another, but, every one has different amounts of water in it, and for the full labeling of the parts the tissue needs to be dry enough to permit the entry of the stain.
Chart of Figures

**Fig. 1** Different types of epithelium. [14] ........................................................................................................ 10
**Fig. 2** The three different types of connective tissue. [15] ................................................................................. 11
**Fig. 3** The three different kind of muscle tissue. [16] ...................................................................................... 11
**Fig. 4** The central nervous system and the peripheral nervous system in the human body. [17] .................... 12
**Fig. 5** The endocrine and exocrine pancreas with the different hormones that secretes. [21] .................... 15
**Fig. 6** A low magnification image of equine pancreas (H&E stain) showing a large interlobular duct in association with a pancreatic artery (A) and vein (V). An intralobular duct (D) is seen on the right side. ................................................................. 16
**Fig. 7** H&E of the pancreatic ductal adenocarcinoma (the most common type of pancreatic cancer) ..................... 17
**Fig. 8** Confocal microscopy arrangement [21] ..................................................................................................... 21
**Fig. 9** Jablonski diagram of the fluorescence process pointing the three different stages 1,2,3 described in the text. ............................................................................................................................................... 28
**Fig. 10** Optical arrangement of two different kind of microscopes. a) Conventional microscope. b) A form of conventional scanning microscope. ........................................................................................................ 30
**Fig. 11** The origin of the optical sectioning or depth discrimination property of the confocal optical system. [24] ........................................................................................................................................... 31
**Fig. 12** Optical design of the confocal microscope with the following components: Diode pumped solid state 532nm green laser, argon-ion laser 488 nm, , spinning polygon (P), galvanometric scan mirror (G), relay telescopes (T), and objective lens (OBJ). A dichroic beamsplitter (B / S1) steers fluorescent emission toward a photomultiplier tube (PTM). A polarizing beamsplitter (B/S2) steers reflected light toward an avalanche photodiode (APD). ........................................................................................................... 33
**Fig. 13** Picture of the confocal microscope, A) top view, B) side view.................................................................. 34
**Fig. 14** Figures from the piece of paper with highlighter, being illuminated with a green laser and scanned in the fluorescence channel. Making zoom from A) to C) to show that the paper fibers are clearly seen. ........................................................................................................... 40
**Fig. 15** Figures from the piece of paper with highlighter, being illuminated with a green laser and scanned in the reflectance channel. Making zoom from A) to C) .................................................................................. 40
**Fig. 16** Images from a pancreatic tumor stained with the main protocol, A) image from the entire piece of tissue, B) zoom into one of the zones with more nuclei, C) in a circle the only nuclei founded. .................................................................................. 41
**Fig. 17** Pancreatic tumor of a mouse main stain protocol with 1.2 mM of concentration in AO. A) image from the entire piece of tissue, B) zoom into the part with more nuclei, C) nuclei founded marked in circles and connective tissue pointed with arrow. ........................................................................................................... 42
Fig. 18 Pancreatic tumor of a mouse main stain protocol with 1.2 mM of concentration in AO. A) Image from the entire piece of tissue, B) zoom into the part with circles marked pointed with arrows. ................................................................. 42

Fig. 19 Pancreatic tumor of a mouse stained with protocol 2. A) Image of the whole piece of tissue, B) zoom into the only part with visible nuclei pointed with arrows; star showing circles made by the glass and the cone of the objective microscope. 43

Fig. 20 Pancreatic tumor of a mouse, only connective tissue is visible................................................. 44

Fig. 21 Pancreatic tumor of a mouse stained just using AO for 40 seconds. A) Image of the whole piece of tissue, B) zoom into one of the zones with more nuclei, C) nuclei of the pancreas. ............................................................................................................ 45

Fig. 22 Pancreatic tumor of a mouse stained just using AO for 40 seconds. A) Image of the whole piece of tissue, B) zoom into one of the zones with more nuclei, C) nuclei of the pancreas................................................................. 46


http://www.cancer.gov/cancertopics/types/commoncancers


http://www.jornada.unam.mx/2013/06/18/ciencias/a02n1cie

http://www.cancer.org/cancer/pancreaticcancer/detailedguide/pancreatic-cancer-key-statistics


[17] Look for diagnosis. [Online].
    http://www.lookfordiagnosis.com/mesh_info.php?term=Central+Nervous+System(=1
    dictionary.thefreedictionary.com/parenchymal+cell
    http://www.barrettsinfo.com/content/3c_what_is_dysplasia.cfm
    Panamericana, 2009.
    http://www.olympusfluoview.com/theory/confocalintro.html
[23] Live Technologies. [Online].
    handbook/introduction-to-fluorescence-techniques.html
    ]
    ]
    reflectance mosaicing of basal cell carcinomas in Mohs surgical skin excisions, ,"
[29] James B. Pawley, Handbook of biological confocal microscopy. Madison, Wisconsin:
    Plenum Press.
    Academic/Plenum Publishers.