Universidad Autónoma de San Luis Potosí
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Design of portable and fast response chemiluminescence and fluorescence devices for assisting the diagnosis of skin diseases

A THESIS

Submitted in partial fulfillment of the requirements to obtain the degree of:

Doctor in mechanical engineering
with final orientation in mechatronics and mechanical systems

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P R E S E N T E.

En atención a su solicitud de Temario, presentada por los **Dres. Juan Antonio Cárdenas Galindo y Walfre Franco**, Asesor y Co-asesor de la Tesis que desarrollará Usted, con el objeto de obtener el Grado de **Doctora en Ingeniería Mecánica con Orientación Terminal en Mecatrónica y Sistemas Mecánicos**, me es grato comunicarle que en la Sesión del H. Consejo Técnico Consultivo celebrada el día 16 de enero del presente, fue aprobado el Temario propuesto:

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"Diseño de dispositivos portátiles y de respuesta rápida de quimioluminiscencia y fluorescencia para asistencia al diagnóstico de enfermedades de la piel"

Introducción
1. Enfermedades de la piel y sus métodos de diagnóstico.
2. Diseño de un sistema portable basado en quimioluminiscencia para la detección de inflamación en micro-muestras de piel.
3. Diseño de microscopio de fluorescencia basado en un teléfono celular para la detección de patógenos.

Conclusiones.
Referencias.

"MODOS ET CUNCTARUM RERUM MENSURAS AUDEBO"

A T E N T A M E N T E

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Abstract

In general skin diseases are characterized by the presence of two main factors: inflammation, and pathogens. There is no specific test for inflammation detection, several assays can guide the dermatologist to an inflammation diagnostic, but generally they are time consuming, and the use of expensive and sophisticated equipment is needed. Attending to this need, in this work, the design and development of a minimally invasive point-of-care (POC) system for detection of inflammation is proposed. The operation principle of the system is chemiluminescence, which is light emission as a result of a chemical reaction. This emission can be associated to the presence of immune cells called neutrophils, that get activated when an inflammatory event occurs. Therefore, the magnitude of the chemiluminescence signal is related to the amount of neutrophil activation, which makes possible the detection of different inflammation levels. In order to validate the designed system, experiments with in-vivo samples presenting different levels of inflammation were performed, and the results were compared with histology analysis. The final results showed that the system was able to detect different levels of inflammation by performing a simple and fast assay (about 15 minutes), in comparison to the conventional techniques that could take even days to get a result. Due to the versatility of the designed system, it could also be used for the detection of the presence of different cells related to several diseases, just with the modification of the chemical components needed to produce a chemiluminescence reaction. Still experimentation an validation in human skin are needed, but the system presented in this work shows potential to be used as a tool for the diagnosis of skin inflammation and other diseases.

There are several techniques for the detection of the presence of pathogens, as polymerase chain reaction (PCR), immunology-based methods, fluorescence microscopy, and cultures among others, but all of them require specialized training and sophisticated, expensive and bulky equipment, which is a huge limitation for low resource settings and developing regions. Fluorescence microscopy is one of the most used imaging techniques for detection of pathogens, but it carries all the limitations mentioned, due to these reasons a new variant of this technique was introduced time ago, it is called smartphone-based fluorescence microscopy. Smartphone-based fluorescence microscopy tries to replace a confocal microscope of about $8,000 usd with a

smartphone, a 3D printed attachment, and a LED for sample illumination, with the price of lower resolution and presence of aberrations, but even with those limitations it can be used in applications that do not require high resolution, as positive/negative assays. Most of the works reported in the literature about smartphone-based fluorescence microscopy have several limitations, even though they are portable, they are still hard to reproduce, expensive, low resolution, and most of the time they are designed for specific applications. Attending to these limitations, in the present work is proposed the design and development of a smartphone-based fluorescence microscopy that can be used for several applications. It consists of a iPod touch 6th generation, a camera lens of the same iPod touch model, a 3D printed attachment, a set of LEDs for sample excitation, and a battery to power both smartphone and LED. The designed system showed a resolution of 6.9 μm, it was tested with fluorescent micro-particles and biological samples, its performance was compared with a commercial fluorescence microscope for validation. The results show that the system could potentially be used for positive/negative assays, in several applications with different excitation/emission wavelengths.

The main aim of this work is to assist the diagnosis of skin diseases in low resource settings, where the gold methods for detection of inflammation and pathogens are not available, by designing and developing low cost, fast response, and portable tools based on light emission. These tools are designed with the purpose of providing preliminary results that could serve as an early alarm of possible diseases and future conventional assays needed to generate a precise diagnostic.

Even though more experimentation is still needed, both fluorescence and chemiluminescence systems could potentially be used in clinics for the detection of inflammation and pathogens to assist the diagnosis of skin diseases.

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Abbreviations and symbols

ANOVA	Analysis of Variance.
CMOS	Complementary Metal-Oxide-Semiconductor.
CFW	Calcofluor White.
CRP	C-Reactive Protein.
DALY	Disability-Adjusted Life Years.
DCs	Dendritic Cells.
DCT	Diagnostic Communication Technology.
DM	Dichroic Mirror.
ESR	Erythrocyte Sedimentation Rate.
ELISA	Enzyme-Linked Immunosorbent Assay.
FISH	Fluorescence In Situ Hybridization.
FITC	Fluorescein Isothiocyanate.
GBD	Global Burden of Disease.
H&E	Hematoxylin and Eosin.
HD	High Definition.
HIV	Human Immunodeficiency Viruses.
HRP	Horseradish Peroxidase.
ICAMs	Intracellular Adhesion Molecules.
IVM	Intravital Microscopy.
LCs	Langerhans Cells.
LC-PCR	Light Cycler Polymerase Chain Reaction.
LED	Light Emitting Diode.
LysM	Lysozyme M.
MEMS	Micro-Electro-Mechanical Systems.
MPNPCR	Most Probable Number Polymerase Chain Reaction.
NADPH	Nicotinamide adenine dinucleotide phosphate.
NETs	Neutrophil Extracellular Traps.
NTD	Neglected Tropical Diseases.
PA	Photoacoustic.

PAMPs	Pathogen-Associated Molecular Patterns.
PCR	Polymerase Chain Reaction.
PCR-ELISA	Polymerase Chain Reaction - Enzyme-Linked Immunosorbent Assay.
PDL	Pulsed Dye Laser.
PMA	Phorbol Myristate Acetate.
PMNs	Polymorphonuclear Leujocytes.
PMT	Photomultiplier tube.
POC	Point-of-Care.
PRRs	Pattern Recognition Receptors.
RBCs	Red Blood Cells.
RLUs	Relative Light Units.
ROS	Reactive Oxygen Species.
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction.
SHAs	Sandwich Hybridization Assays.
SOD	Superoxide Dismutase.
SPE	Serum Protein Eletrophoresis.
SPR	Surface Plasmon Resonance.
STED	Stimulated Emission Depletion.
TB	Tuberculosis.
TIRF	Total Internal Reflection.
TLRs	Toll-Like Receptors.
YLD	Years Lived with Disability.
ϕ_C	Chemical yield.
ϕ_{CL}	Overall quantum yield.
ϕ_{Ex}	Excited molecules.
ϕ_F	Fluorescence quantum yield.
c	Speed of light.
E	Photon energy.
h	Planck's constant.
v	Frequency of the wave.
λ	Wavelength.

Introduction

The skin is the largest organ of the human body and represents a complex barrier structure composed of surface keratinocytes, inter-keratinocyte substances, dermis and sub-dermal structures. The cutaneous structures serve a variety functions that preserve homeostasis of the body, including cooling and electrolyte balance, formation and processing of vitamins and hormones, and physical and thermal protection of underlying muscle, bone and internal organs [2, 20, 21]. It represents a tremendous interface with the environment, processing both allergens, irritants and pathogens through a broad-based immunological system consisting of exclusively cutaneous immune cells, circulating immune cells and intrinsic production of anti-pathogenic substances [20, 22]. There are diseases unique to the skin and changes in it that are responses to systemic diseases, including HIV and neglected tropical diseases NTD, such as elephantiasis and other lymphedema causing diseases [21, 23].

Skin diseases are profoundly impacted by genetic factors, population aging, new treatment modalities, environmental exposures, and often vary from region to region within a country [24, 23, 25]. They cause significant non-fatal disability, especially in resource-poor regions. An investigation revealed that skin diseases are the fourth leading cause of morbidity worldwide, and the most common cause of human illness [26, 25].

One method of understanding the epidemiological burden of skin disease is through the Global Burden of Disease GBD. The Global Burden of Disease GBD project is based at the Institutes of Health Metrics. It provides disability and mortality estimates for a broad range of diseases, injuries, and risk factors. Disability burden is calculated using disability-adjusted life years (DALY), and years lived with disability YLD, which both account for life years lost due to disease and allow for comparison across conditions [23].

In 2013, skin conditions contributed 1.79% to the total global burden of disease measured in DALYs across 306 diseases and injuries. When comparing absolute DALYs/YLDs, skin and subcutaneous disorders were the 4th leading cause of non-fatal disease burden [26]. 15 skin disease categories were assessed: dermatitis, psoriasis, cellulitis, pyoderma, scabies, fungal skin diseases, viral skin diseases, acne vulgaris, alopecia areata, pruritus, urticarial, decubitus ulcer, malignant skin

melanoma, and keratinocyte carcinoma (including basal and squamous carcinomas), and other skin conditions. Dermatitis resulted the greatest burden of the skin conditions, costing 9.3 million DALYs [26].

More than 3000 dermatological conditions have been identified in the literature [27, 28]. Skin diseases in the population are universal, common, and can cause significant economic burden [29]. In 2013, it was estimated that one in four people of all ages (85 million individuals) in the United States were treated for at least one dermatological disease, resulting in direct healthcare costs of $75 billion USD and indirect costs of $11 billion USD [29]. Therefore, dermatological diseases are a public health problem due to the economic burden, impact in quality of life, and decrease in productivity and life expectancy [28].

In Mexico (2015), a total of 9,230,968 hospital discharges were registered nationwide, of which 170,917 (1.85%) reported a dermatological disease as the main diagnosis [24]. 109 dermatological pathologies were identified as the main diagnosis of hospital discharge. Among the pathologies by most prevalent groups, 32.08% of the cases corresponded to skin infections, 27.80% to non-cancerous skin growths, 7.88% to nonmelanoma skin cancer, 5.69% to viral warts due to human papilomavirus and molluscum, and 3.97% to granulomatous disorders of the skin and subcutaneous tissue, these cases correspond to 77.42% of all hospital discharges reported for dermatological conditions [24]. The total cost estimation for treatment of skin diseases in Mexico (2015) is $67,741,542.89 USD, just considering the public healthcare [24].

In order to reduce the global burden of skin disease and helping to provide dermatological care to underserved regions in a cost-effective manner, the design and implementation of innovative diagnostic and treatment tools or techniques is required, and it is the main aim of this work.

In this work, the design of two point-of-care tools for assisting the diagnosis of skin diseases is presented. One of them for the detection of inflammation using chemical reactions. The other one for the detection of pathogens through fluorescence. Both systems are able to be implemented in low-resource settings, easy to use and reproduce, fast response, and portable.

For the inflammation detection system, the basic concepts about inflammation as activation mechanism, involved chemical reactions and cells, recent diagnostic tools and techniques, limitations, areas of opportunity, and challenges were studied in order to propose a practical and minimally invasive way to detect it. The detection of inflammation using chemiluminescence reactions was proposed based on the neutrophil respiratory burst phenomena in skin samples. A micro-biopsy tool was design in order to extract tissue samples avoiding scarring. A system for the measurement of light emitted from chemiluminescence reactions with low cost components was designed, built, and tested with common reactions reported in the

literature, isolated neutrophils, isolated neutrophils in skin, and with real cases of skin inflammation. The results showed that is possible to detect inflammation in skin micro-samples through chemiluminescence in a minimally invasive way, showing the possibility to test the prototype in patients for its future implementation in clinics.

Regarding the pathogen detection system, the basic concepts about fluorescence were studied, such as the fluorescence principle, and the operation and components of a fluorescence microscope. Based on a literature review, the design of a phone-based fluorescence microscope was proposed in order to implement the fluorescence analysis in low-resource settings. Using low cost components, a prototype was designed, built and tested using fluorescence micro-particles and biological samples, the performance of the prototype was compared with a commercial fluorescence microscope. The results showed that the prototype is able to detect fluorescence with 6.9μm resolution, in comparison to 1.9μm from the commercial microscope.

Both prototypes were designed following the methodology of the design process (Illustrated in Figure 2.1)

Hypothesis

As mentioned, the diagnosis of skin diseases can be divided in two main factors; the presence of inflammation and pathogens. The techniques for their detection need to be improved, since they depend mostly on the physician experience, and sometimes require long time to get a diagnosis. In this work, two tools for assisting the diagnosis of skin diseases are presented, one is based on chemiluminescence and the other one on fluorescence. The goal of this work is to analyze if the designed tools are able to improve or assist the diagnosis of skin diseases in low-resource settings.

In the case of the system for assisting the detection of inflammation, is well known that a kind of cells (neutrophils) are presented in tissue during the inflammatory response, when those cells get activated they produce oxygen species than can be used to generate chemiluminescence reactions. The hypothesis is that, if a tissue sample is extracted and mixed with chemiluminescence reagents, and light emission is measured, it could be an indicative of presence of inflammation in the tissue sample.

In the case of the system for assisting the detection of pathogens, is well known that fluorescence microscopy is the gold method for pathogen detection, but unfortunately it requires expensive and sophisticated equipment and training. Due to these reasons, it is not available for developing communities. The hypothesis for this system is that, a low cost smartphone-based fluorescence microscope can be built

with simple components to assist the detection of pathogens in differential assays, where high resolution is not required.

Objective

The main objective of this work is to design portable and fast response tools, based on chemiluminescence and fluorescence with the aim of assisting the diagnosis of skin diseases.

Specific objectives

1. Design, construction and test of a tool for minimally invasive skin sampling.
2. Design, construction and test of a system for the detection of chemiluminescence for revealing the presence of neutrophils in skin samples.
3. Design, construction and test of a fluorescence detector based on an iPod® touch by using low cost components.

This work was divided as follows: In the Chapter 1, the main skin diseases and their diagnostic methods and tools are described, in the Chapter 2 a detailed description of the design of a system for detection of inflammation is presented, the Chapter 3 shows the design and characterization of a system for detection of pathogens through fluorescence, and finally conclusions, discussion and future work are presented.

Chapter 1

Skin diseases and their diagnostic methods

1.1 Skin biology

The skin is the heaviest organ of the human body, on average accounting for 10% of the body mass and covering nearly 2 m^2 of the body surface area. It defines the boundary between the body and its surroundings, thus allowing vital bodily functions to occur within a controlled physiological environment [2].

The skin is one of the largest organs of the body and is continuously exposed to a variety of external stimuli, such as bacteria, viruses, fungi, ultraviolet light, chemicals, dryness, haptens, and protein antigens. Thus, the skin is an important barrier between the living organism and its environment to maintain homeostasis. Defending physically against external stimuli, the skin is also an immunological defense [1]. Some of this defensive activity occurs through the immune system. In the skin, Langerhans cells (LCs), dermal dendritic cells (DCs), endothelial cells, keratinocytes, mast cells, basophils, and other cells all participate under certain circumstances (Figure 1.2).

Human skin is a stratified epithelium, each tissue layer consisting of different cell types that perform distinct functions. It can be divided into the overlying epidermis, dermis and underlying hypodermis (or subcutis) as shown in Figure 1.1. The epidermis can further be subdivided, from the outside to the inside, into the stratum corneum (horny layer), stratum granulosum (granular layer), stratum spinosum (prickle cell layer) and stratum basale (basal layer also called stratum germinativum) [30, 31]. The stratum basale and stratum spinosum are collectively known as the Malpighian layer. An additional layer, the stratum lucidum (clear layer) can be observed on parts of the body with thickened skin, such as the palm and sole of the foot. However, the tratum lucidum is often not considered a distinct epidermal

layer but the lower part of the stratum corneum. In addition, there are appendageal features including hair follicles and sweat ducts that traverse various skin layers [32, 1].

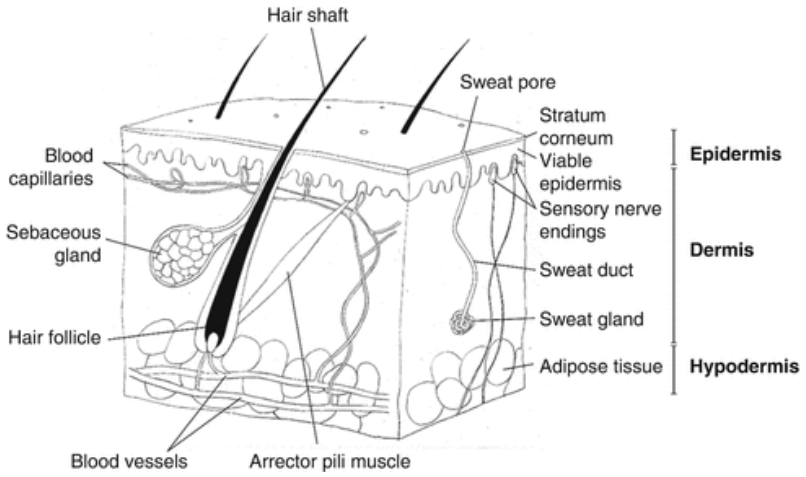


Figure 1.1: The composition of the skin [1].

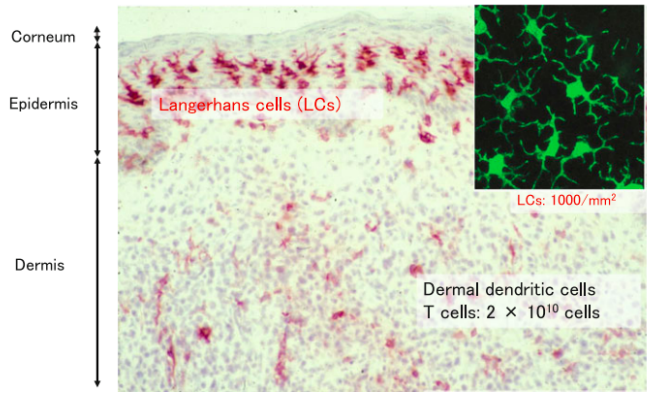


Figure 1.2: Immune cells found in the skin [2].

The stratum corneum is the outermost layer of the skin. It is typically 10-20 μm thick and composed of 10-15 layers of corneocytes [33, 34]. Corneocytes are nonliving cells derived from terminally differentiated keratinocytes that have originated from the deeper layers of the epidermis [32]. Morphologically, corneocytes are flattened and elongated, measuring about 0.2 μm thick and 40-60 μm wide [35]. Corneocytes have a cornified envelope in place of a plasma membrane, which is surrounded by a lipid coat. They lack nuclei and cytoplasmic organelles but filled with keratin filaments and are interspersed in a lipid-enriched extracellular matrix that also contains protein/peptide components [36]. This organisation of the stratum corneum is commonly referred to as the "brick and mortar" model [3, 37], where the corneocytes are likened to bricks and the extracellular matrix analogous to the mortar in a brick wall as shown in Figure 1.3. Corneocytes are connected by corneodesmosomes and

are continuously shed from the skin surface by desquamation. The stratum corneum directly faces the external environment; therefore it functions as a barrier against this external environment, protecting internal cells and tissues from external insults while maintaining normal cellular functions. In addition to having protective barrier functions, the stratum corneum serves as a sensor of external conditions [38].

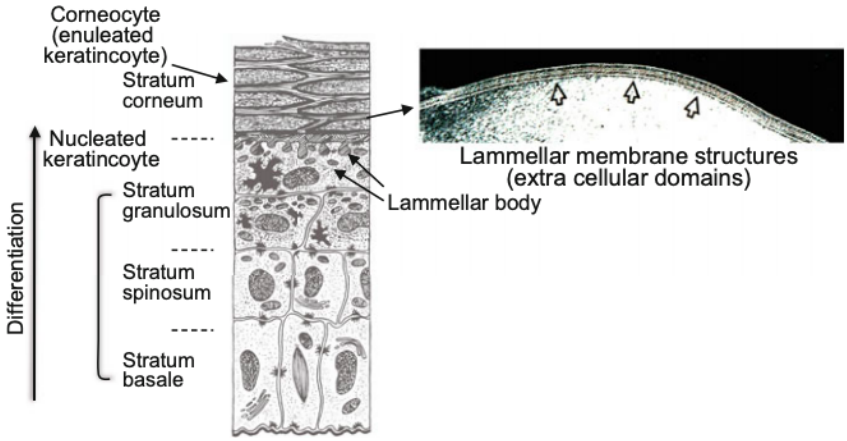


Figure 1.3: Epidermis composition [3].

Excluding the stratum corneum, the rest of the epidermis is composed of nucleated cells and therefore collectively referred to as the viable epidermis. The viable epidermis is typically 50-100 μm thick [39] and devoid of blood capillaries and sensory nerve endings. It is composed primarily of keratinocytes (95%), with the remainder being Langerhans cells, melanocytes and Merkel cells. Keratinocytes arise from the stratum basale and undergo progressive differentiation whilst migrating towards the stratum corneum. Keratinocyte differentiation is characterised by increasing keratinisation (formation of intracellular networks of keratin fibres), the formation of the lamellar bodies that secrete stratum corneum lipids and the loss of intracellular organelles and nuclei. The process culminates in the formation of corneocytes in the stratum corneum. Keratinocyte differentiation serves to maintain the stratum corneum by replenishing stratum corneum lipids and corneocytes lost via desquamation [32].

The dermis, typically $\geq$ 1 mm thick [40], comprises the bulk of the skin and is responsible for its elasticity and strength. It is composed principally of fibroblasts in an extracellular matrix of structural proteins, mainly collagen and elastin. It also contains a range of immune cells including macrophages and dermal dendritic cells. The dermis can be subdivided into the upper papillary dermis and the lower reticular dermis, which can be distinguished microscopically from each other by the thinner and looser packing of collagen fibres in the papillary dermis. The papillary dermis contains papillae that interdigitate with the basal layer of the epidermis at the dermo-epidermal junction. The dermis contains hair follicles, swat glands, sebaceous

glands, sensory nerve endings, lymphatic vessels and blood capillaries which extend to the dermal side of the dermo-epidermal junction. This allows nutrient and oxygen delivery to, as well as waste removal from, the avascular epidermis to occur by diffusion across the dermo-epidermal junction [32].

The hypodermis is the innermost layer of the skin. However, its absence is notable in some lean skin, such as that on the eyelid. The hypodermis is composed mainly of subcutaneous fat. Embedded in this skin layer are larger lymphatic and blood vessels.

The primary function of the skin is to separate the internal physiological environment of the body from the external non-physiological environment [34]. The skin barrier is physical, chemical and immunological in nature. The physical barrier is provided primarily by the stratum corneum. This physical barrier is responsible for regulating not only the ingress of exogenous materials but also preventing excessive water loss from the body. The chemical barrier is known as the "acid mantle". The skin owes its chemical barrier function to the acidic (pH 4-6) nature of the skin surface which protects the body in two ways. Firstly, it confers selective antimicrobial properties to the skin by maintaining the natural skin microflora, which live optimally in an acidic environment, whilst arresting the growth of pathogenic microorganisms which thrive in alkaline environments. Secondly, it helps to maintain the integrity of the stratum corneum barrier since many skin enzymes pivotal to stratum corneum lipid homeostasis have a pH optima within this pH range [41, 42]. Sebaceous glands in the skin, which secrete sebum, perform a similar function. Following its secretion to the skin surface, sebum forms a greasy film on the skin, which waterproofs the skin to maintain hydration and suppleness. Sebum also contains antimicrobial constituents.

The skin is also an immune-competent organ. A range of immune cells including Langerhans cells, dermal dendritic cells and marophages are found in the skin [43]. These cells conduct immune surveillance and defend the body against newly encountered antigens. This is an important role of the skin considering a compromised skin barrier is a common route of pathogen entry into the body. There is also an increasing body of evidence that supports a role for some skin dendritic cell subsets in inducing immune tolerance [44], which is equally important for maintaining immune homeostasis.

Moreover, the skin has an important role in thermoregulation, allowing thermal energy to be dissipated or conserved. Thermoreceptors in the skin detect heat and cold; they provide sensory input to the hypothalamus, which then invokes a range of thermoregulatory mechanisms to achieve temperature homeostasis. Adipose tissue in the hypodermis insulates the body from cold and prevents excessive heat loss from the body. Body hairs on the skin provide additional insulation by trapping a thin

layer of air on the skin surface. This effect is maximised by the erecting of hairs, via constriction of the arrector pili muscle. Perspiration secreted through sweat pores on the skin surface helps to reduce body temperature by dissipating heat from the body through the evaporation of water in sweat. Blood vessels in the skin dilate or constrict to adjust the blood flow and heat loss across the large skin surface area. These thermoregulatory mechanisms work to help maintain a constant core body temperature of about 37C.

Apart from heat and cold, sensory nerve endings in the dermis detect touch, vibrations and pain. These sensations are critical to other functions of the body, such as locomotion and coordination. The ability to sense pain alerts us of danger and is crucial to survival.

Furthermore, the skin carries out important metabolic functions. Adipocytes in the hypodermis store excess energy in the form of subcutaneous fat, which can be monilised rapidly during energy deprivation. The epidermis is the primary site if vitamin D synthesis in the body [45]. The process, photolysed by ultraviolet irradiation, produces a precursor for vitamin D in the stratum spinosum and stratum basale, which is then converted into vitamin D by keratinocytes.

The skin additionally serves an excretory function, as minerals and other organic wastes are relleased through the skin dissolved in sweat. The hypodermis also provides mechanical protection to inner organs by cushioning the body against physical shock.

As already mentioned, the skin has several functions that are vital for the optimum performance of the human organism. Those functions get altered with the presence of skin diseases, in order to reduce those deficiencies or alterations, effective diagnostic techniques and treatments for skin diseases are needed. Some of the most common skin diseases as well as their causes are mentioned in the following section.

1.2 Skin diseases

Skin is the primary interface between the body and the environment, the spectrum of insults to which skin is susceptible includes disorders caused by chemical and microbial agents, thermal and electromagnetic radiation, and mechanical trauma. The most damaging consequence of the disruption of skin is invasion by pathogenic microorganisms, and the need for an effective means of protection against this challenge has been a fundamental force behind the evolution of the immune system. The translation of insults into cutaneous inflammation (innate immunity) and the recruitment of memory T lumphocytes that have clonally expanded in response to antigens encountered at the cutaneous interface with the environment (acquired immunity) are both required for successful cutaneous immune surveillance. In other

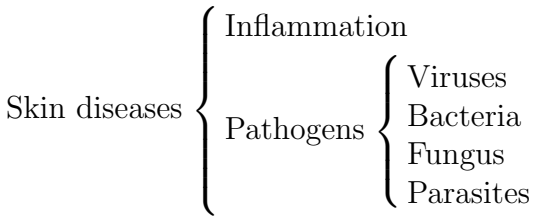


Figure 1.4: Features of skin diseases.

words, skin diseases are always accompanied by the presence of pathogens and inflammation, as showed in Figure 1.4.

Skin diseases or disorders vary greatly in symptoms and severity. They can be temporary or permanent, and may be painless of painful. Some have situational causes, while others may be genetic. Some skin conditions are minor, and others can be life threatening. While most skin diseases are minor, other can indicate a more serious issue.

Some of the most common skin diseases are: skin cancer, psoriasis, dermatitis and dermatophytosis. Most of the time they are hard to detect because of the presence of inflammation, and even harder in low resource settings, until now, there is no an specific assay for inflammation diagnosis. These skin diseases, their consequences and the importance of their early diagnosis are explained below.

1.2.1 Skin cancer

Skin cancer is the most common of all cancers; basal cell and squamous cell cancers affect more than one million Americans each year (a number that is raising rapidly). Some other people are diagnose with melanoma. But is also the easiest cancer to cure if diagnosed and treated early. Prolonged exposure to intermittent overexposure to sunlight is the primary cause of skin cancers. In fact, about 90% of all skin cancer is related to sun exposure, and most skin cancers are found on parts of the body exposed to sunlight [46]. There are three basic types of skin cancer: basal cell, squamous cell, and melanoma. Basal cell carcinoma usually appears as a small, shiny bump on sun-exposed areas, such as the face, neck, chest, upper back, and hands, primarily in fair-skinned people (specially those who burn easily). The lesions gradually grow and may crust, bleed, or ulcerate, although they usually do not spread. Local destruction of the skin and underlying tissues may be considerable if this type of cancer is left untreated. Squamous cell carcinoma usually appears as a red, scaly patch. It grows slowly, occasionally becoming a nodule and frequently becoming crusted and eroded. Bleeding is common. Basal cell and squamous cell cancers are almost certainly related to cumulative sun exposure, occurring mostly

on exposed places. Unlike basal cell carcinoma, squamous cell cancers grow and may spread (metastasize) [46, 47].

Malignant melanoma is the third type of cancer, the most deadly of the three. Melanoma is the most common cancer among people aged 25 to 29. Melanomas are usually small brown, black, or multicolored patches, plaques, or nodules with an irregular outline. They may crust on the surface or bleed, and many of them appear in preexisting moles. Melanoma is much more dangerous than other forms of skin cancer because of its tendency to spread rapidly to vital internal organs as the lungs, liver, and brain [46, 48].

Most skin cancers (even malignant melanoma) can be cured if discovered early enough, which is why attention to symptoms and regular self-examination is highly recommended. When cancers of skin are discovered early, there are a variety of treatment possibilities, depending on the type of tumor, size, location, and other factors affecting the patient's general health [49]. A biopsy is often studied before a definitive therapy is selected. Malignant melanoma causes 75% of all deaths from skin cancer [46].

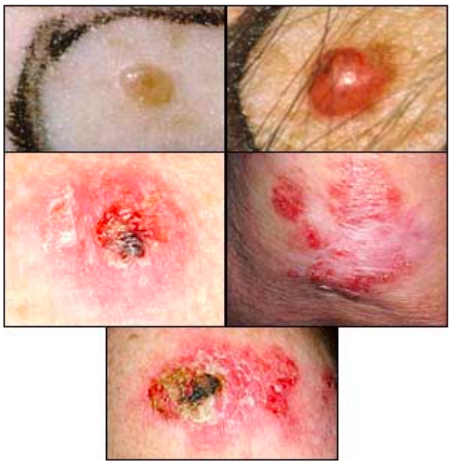


Figure 1.5: Pictures of different cases of skin cancer [4].

1.2.2 Psoriasis

Psoriasis is a T-cell mediated chronic inflammatory disorder of the skin seen in about 3.5% of the population. Psoriasis is a lifelong inflammatory disorder of the skin that can vary widely in its presentation but is characterized by the common features of erythema, skin thickening and scaling [50, 47]. It causes to build up rapidly on the surface of the skin. The extra skin cells from scales and red patches are itchy and sometimes painful [47]. The diagnosis is usually made on clinical grounds by any well-trained clinician. Despite considerable research into its etiology, there are still no definitive genetic or biochemical markers for psoriasis, and it continues to

be diagnosed primarily base on skin manifestations [50, 47]. The main goal of the treatment is to stop the skin cells from growing so quickly [47].

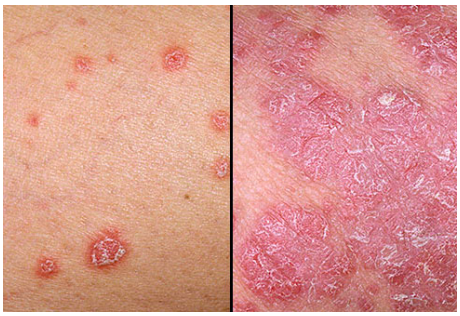


Figure 1.6: Picture of a case of psoriasis [4].

1.2.3 Allergic contact dermatitis

Allergic contact dermatitis, also known as contact hypersensitivity, is a T-cell-dependent skin disease with the kinetics of a delayed-type hypersensitivity response. This disorder is even more prevalent than psoriasis, and although it is rarely life-threatening, the costs to society of occupation-related allergic contact dermatitis are high. In this disorder, the offending antigen is introduced epicutaneously through intact skin [47, 51].

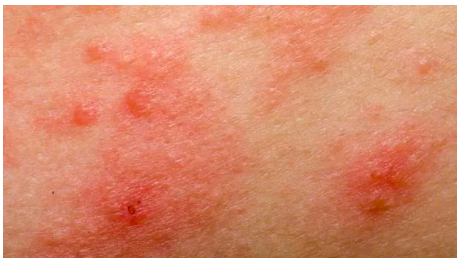


Figure 1.7: Picture of a case of dermatitis [4].

1.2.4 Dermatophytosis

Superficial fungal infections are common skin diseases, affecting millions of people worldwide. These infections occur in both healthy and immunocompromised patients and etiologic agents consist of dermatophytes, yeasts and nondermatophyte molds. Dermatophytes are responsible for most superficial fungal infections and the estimated lifetime risk of acquiring a dermatophyte infection is between 10 -20% [52].

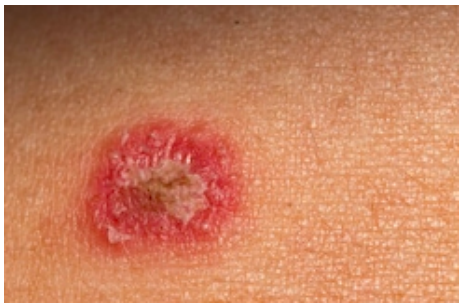


Figure 1.8: Picture of a case of dermatophytosis [4].

1.3 Diagnostic of skin diseases

The skin is the only organ of the body which is exposed to the external environment. The rashes on the skin can be seen and the prognosis of treatment can be assessed by the naked eye. Many people think that skin diseases are easy to diagnose because the rashes are visible.

The key to successful treatment lies in the accurate diagnosis. To come to an accurate diagnosis the doctor should examine not only the rash, but also the entire skin and the other systems when necessary [53, 54]. The physician should have knowledge of the anatomy and physiology of the skin, the disease, its distribution and characteristics, and should be well verse with the basic dermatologic terminology [54].

1.3.1 Common diagnostic routine

The doctor or dermatologist analysis starts with "history taking" which consists of series of questions to the patient. It should include the present history, past history, family history, drug history and personal history. It is important to ask a few questions of the rash, depending upon its site and type of rash. Some of the questions asked are: Where did the rash occur, is it acute or chronic?, Is the rash associated with symptoms such as itching, burning, pain etc?., is the morphology of the rash the same as when it occurred or has it changed?, has the rash spread from its initial site? among others [53, 54].

A drug history is also important in dermatology. Some rashes are due to hypersensitivity to drugs. Some drugs produce rashes similar to a disease. Drug history can give a clue to the diagnosis of the rash, and withdrawal of the drug would bring about a cure. A history of previous drugs taken for the disease should be noted; there is no point in giving the same medications that the patient has received before without any improvement [46].

Examination of the skin should be done in broad daylight. A magnifying lens, a tongue depressor and a glass slide/spatula should be present while examining the skin. A light source should be available when sunlight is not sufficient. Examination of the skin includes examination of the skin, nails, scalp and the oral cavity and the genito-urinay area when necessary [53, 54, 46].

Some of the important factors to examine are: Rash morphology/Type, presence of nodules, ulcers, erosions, fissures, scars, crusts, gangrene, excoriations or vesicles. Morphological pattern of the rash, are the lesions linear, annular, discoid or nummular, serpiginous, grouped, discrete, confluent or reticulate. Colour of the rash, can give a clue to the diagnosis. Shape, surface, regularity or irregularity of the rash. Inflammation, hyper-pigmentation or hypo-pigmentation in the surrounding areas. Distribution of the rash (localized or generalized). Site of the rash. The rash should be palpated to see if it is soft, firm, hard, attached to the skin or mobile [53, 54, 46].

Some tools are needed in order to improve the diagnosis procedure:

- Adequate sunlight is essential for the examination of the skin. If sunlight is not sufficient then an alternative light source should be available [46].

- Magnifying lens [46].

- Wood's lamp, is a tool that uses trans-illumination (light) to detect bacterial or fungal skin infections. It ca also detect skin pigment disorders such as vitiligo and other irregularities. This procedure can also be used to determine corneal abrasion (scratch) on the surface of the eye. This test is also known as "the black light test" or "the ultraviolet light test" [46].

- Diascopy test, is a blanchability examination performed by applying pressure with a finger or glass slide and observing color changes. It is used to determine whether a lesion is vascular (inflammatory or congenital), non-vascular (nevus), or hemorrhagic (petechia or purpura) [46].

- Dermatoscopy or epiluminescence microscopy, it allows for inspection of skin lesions unobstructed by skim surface reflections. The dermatoscope consists of a magnifier, a light source (polarized or non-polarised), a transparent plate and a sometimes liquid medium between the instrument and the skin. This tool is useful to distinguish benign from malignant (cancerous) lesions, especially in the diagnosis of melanoma [46].

- Prick testing, is a method for medical diagnosis of allergies that attempts to provoke a small, controlled, allergic response. This type of testing uses needles (lancets) that barely penetrate the skin surface, avoiding bleeding or

major pain. This test checks for immediate allergic reactions to as many as 40 different substances at once [46].

- Patch testing, it is a method to determine whether a specific substance causes allergic inflammation of a patient's skin. It helps to identify which substances may be causing a delayed-type allergic reaction in a patient, and may identify allergens not identifies by blood testing or skin prick testing. Ir is intended to produce a local allergic reaction on a small area of the patient's back, where the diluted chemicals were planted [46].

- Tzanck test, is scraping of an ulcer base to look for Tzanck cells, it is sometimes also called "the chickenpox skin test" and the "herpes skin test". It requires to stain the sample with Giemsa, methylene blue or Wright's stain, and it is analyzed using microscopic examination with immersion oil lens [46].

- Immunofluorescence assay, is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific bio-molecule targets within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyze the distribution of proteins, glycans and small biological and non-biological molecules. This assays are very useful in diagnostic of autoimmune disorders, such as autoimmune bullous disorders, autoimmune collagen disorders and vasculitis [46].

- Skin biopsy, is a procedure of removing a small sample of skin to have it tested [46].

- Blood and urine examination, blood chemistry and serological tests [46].

As it was already mentioned, inflammation and presence of pathogens play an important role in skin diseases diagnosis, both features are described then.

1.4 Inflammation

The human body is subjected to constant insults that stimulate the inflammatory response. Once homeostasis is disturbed, and in response to certain stimuli, our body may launch a process that is defined as inflammation, manifested with cardinal macroscopic signs (redness, swelling, fever, and pain) and underlined by microvascular, cellular, and molecular events that culminate in leukocyte accumulation (granulocytes at first) in affected tissue areas (from infection, tissue damage,

etc). During early phases of inflammation, tissue resident cells sense the inflammatory stimulus and launch the release of soluble inflammatory mediators, including chemokines, cytokines, free radicals, vasoactive amines, and eicosanoids. The activation of sensors is focused on the production of pro-inflammatory mediators, such as receptors toll-like, followed by the activation of their respective signaling pathways. A prototypical pro-inflammatory signaling pathway is centered on the activation of the nuclear factor kappa B(NF-κB) transcription factor, which in turn, triggers the expression of pro-inflammatory and anti-apoptotic genes. These events are accompanied by micro-vascular changes and up-regulation of adhesion molecules by circulating leukocytes and endothelial cells, resulting in exudation of liquid and proteins and influx of granulocytes (generally neutrophils or eosinophils, depending on the stimulus) from the blood into the affected tissue [55].

The inflammatory process can be didactically divided into three main phases: 1) onset, when pro-inflammatory soluble mediators initiate the inflammatory cascade; 2) resolution, when another set of mediators dictate events that terminate the inflammatory process; and 3) the more recently described post-resolution phase, when the affected tissue develops adaptive immunity and regains a status of "adapted homeostasis". If successful, the inflammatory response tends to progress from the onset to the post-resolution phase through a coordinated series of molecular and cellular events that lead to the restoration of tissue structure, organ function, and "adapted homeostasis" [56, 57].

Inflammation is a beneficial host response to foreign challenge of tissue injury that leads ultimately to the restoration of tissue structure and function. It is a reaction of the micro-circulation that is characterized by the movement of serum proteins and leukocytes from the blood to the extra-vascular tissue. This movement is regulated by the sequential release of vasoactive and chemoactic mediators, which contribute to the cardinal signs of inflammation (heat, redness, swelling, pain and loss of tissue function). Local vasodilation increases regional blood flow to the inflamed area and, together with an increase in microvascular permeability, results in the loss of fluid and plasma proteins into the tissues. Concomitantly, there is an up-regulation of adhesion molecule expression on endothelial cells and the release of chemotactic factors from the inflamed site, which facilitate the adherence of circulating cells to the vascular endothelium and their migration into the affected area. These tightly regulated events result in a predominance of polymorphonuclear leukocytes (PMNs) in the inflamed area at the onset of the lesion, which are later gradually replaced by mononuclear cells (mainly monocytes, which differentiate into macrophages). These phagocytic cells ingest foreign material and cell debris. They also release hydrolytic and proteolytic enzymes, and generate reactive oxygen

species that eliminate and digest invading organisms. Finally, the injurious stimulus is cleared and normal tissue structure is restored [56, 58].

1.4.1 Inflammation detection

There is no specific test that can diagnose inflammation or conditions that cause it. Instead, based on the symptoms, the doctor may give tests to the patient to make a diagnosis. The most common is the blood test, there are a few markers that help to diagnose inflammation in the body. However, these markers are nonspecific, meaning that abnormal levels can show that something is wrong, but not what is wrong. Some of these markers are serum protein electrophoresis (SPE), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), plasma viscosity, etc [7].

SPE is considered the best way to confirm inflammation. It measures certain proteins in the liquid part of the blood to identify any issues. Too much or too little of these proteins can point to inflammation and markers for other conditions.

CRP is naturally produced in the liver in response to inflammation. A high level of CRP in the blood can occur due to several inflammatory conditions. While this test is very sensitive for inflammation, it does not help to differentiate between acute and chronic inflammation, since CRP will be elevated during both. High levels of CRP, combined with other symptoms can help the doctor to make a diagnosis.

The ESR test is sometimes called sedimentation rate test. This test indirectly measures inflammation by measuring the rate at which red blood cells sink in a tube of blood. This test is rarely performed alone, as it does not help to point specific causes of inflammation. It can help the doctor to identify that inflammation is occurring, or to monitor the condition of the patient.

Yanbing et al [59] propose an assay using quantum dots for the detection of inflammation biomarkers. They use different quantum dots in order to detect different fluorescence responses. And the fluorescence response is related to the presence of specific inflammation biomarkers. The technique was tested in vitro, and it requires a long period of time for sample preparation (hours). Is well known that quantum dots are having an important impact for the diagnosis of several diseases, but they are expensive and require high resolution systems to visualize their fluorescence response, which limits their application in low resource settings.

There are some new proposed methods for inflammation detection. Jiamin et al [60] present a technique for precise quantification of the production of reactive oxygen species released during the inflammatory response, they use Photoacoustic (PA) imaging to visualize the oxidation process of a nanoplatform comprising Ag shell couated Pd-tipped gold nanorods. Their technique was validated in a mice model, the results showed that is possible to differentiate inflammation compared

with normal tissue. Their limitation is that a PA set up is hard to reproduce, and requires sophisticated and expensive components, and the feasibility of this technique depends on the tissue penetration depth that can be reached.

Doremalen et al [61] propose a technique for inflammation detection in diabetic foot by using infrared cameras. They use image processing to combine pictures obtained from 3 infrared cameras, to get a thermal map of the foot. Based on the temperature difference between sections, they determine the possible presence of ulcers. The limitation of this technique is that temperature differences are not specific to inflammation presence [5], and also the complexity of the proposed system limits its use in low resource settings.

Zongxi et al [62] designed a bandage with the capability to monitor skin inflammation based on oxygen sensing. The bandage is built with certain phosphors and dyes for the oxygen detection. When those phosphors get excited by a light source light emission takes place. Based on the behavior of the light emission inflammation is detected. The analysis of that signal requires the use of a special cameras and filters, which increases the cost of the technique and reduces its application in low resource settings. In this study was found that skin sections with more redness showed higher level of inflammation, which is not necessarily the same for all inflammatory diseases. It has the huge advantage of being non invasive, but it detects oxygenation just on the surface of the skin or short depth.

1.4.2 Importance of inflammation

Inflammation is a very important factor in skin diseases, or diseases in general, because it is present in many cases. Unfortunately, as it was already mentioned, there is no a quantitative way to measure or detect inflammation in skin. The diagnosis and treatment depend mostly on the experience and analysis of the dermatologist.

Figure 1.9 is a practical example of the importance of inflammation, it shows six cases of skin diseases, all of them have several similarities and not all show inflammation. Is easy to notice that is hard to distinguish between diseases, and the presence of inflammation is not obvious. Most of the time, skin redness can be related to inflammation, but this is not necessarily correct in all the cases. This characteristic increases the possibility of misdiagnosis and hence wrong treatment, which could cause even death.

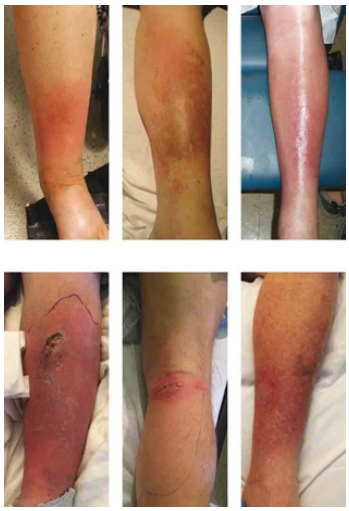


Figure 1.9: Skin diseases with similar appearance [5].

Also, as it was mentioned in section 1.4, inflammation is a vital part of the immune system's response to injury and infection, the body's way of signaling the immune system to heal and repair damaged tissue, as well as defend itself against foreign invaders, such as viruses and bacteria. Without inflammation as a physiological response, wounds would fester, and infections could become deadly. However, if the inflammatory process goes on for too long or if the inflammatory response occurs in places where it is not needed, it can become problematic. Due to all these characteristics, a quantitative way to measure inflammation is needed, in order to get information about the efficiency of the immune system, and to facilitate the diagnosis of skin diseases.

1.4.3 Inflammatory response

The inflammatory response consists of an innate system of cellular and humoral responses following injury (such as after heat or cold exposure, ischemia/reperfusion, blunt trauma, etc.), in which the body attempts to restore the tissue to its preinjury state [7]. In the acute inflammatory response, there is a complex orchestration of events involving leakage of water, salt, and proteins from the vascular compartment; activation of endothelial cells; adhesive interactions between leukocytes and the vascular endothelium; recruitment of leukocytes; activation of tissue macrophages; activation of platelets and their aggregation; activation of the complement; clotting and fibrinpolytic systems; and release of proteases and oxidants from phagocytic cells, all of which may assist in coping with the state of injury. Whether due to physical or chemical causes, infectious organisms, or any member of other reasons that damage tissues, the earliest in vivo hallmark of the acute inflammatory response is the adhesion of neutrophils (polymorphonuclear leukocytes, PMNs) to the vascular endothelium ("margination") [7, 57]. The chronic inflammatory response is defined

according to the nature of the inflammatory cells appearing in tissues. The definition of chronic inflammation is not related to the duration of the inflammatory response. Reversal or resolution of the inflammatory response implies that leukocytes will be removed either via lymohatics or by apoptosis (programmed cell suicide) and that the ongoing acute inflammatory response is terminated. As a consequence, during resolution increased vascular permeability is reversed due to the closure of the open light junction and PMN emigration from the blood compartment ceases. In both the vascular and extravascular compartments, fibrin deposits are removed by pathways that lead to activation of plasminogen (to plasmin), which degrades fibrin. Cell debris and red blood cells (RBCs) in the extravascular compartment are removed by phagocytosis involving tissue macrophages. there are many situations in which the acute inflammatory response becomes excessive or prolonged, leading to serious damage of tissues and organs [7, 57].

1.4.4 Neutrophils

Polymorphonuclear leukocytes or granulocytes are hematopoietically derived phagocytes characterized by multilobed nuclei and the presence of multiple, distinct granules within their cytoplasm. Three different polymorphonuclear leukocytes are distinguished according to their granular staining properties: neutrophils (polymorphonuclear neutrophils or PMNs), basophils, and eosinophils. Neutrophils granules stain preferentially with neutral dyes, whereas basophil granules stain with basic dyes, and eosinophilic granules stain with acidic colorants such as eosin. These three types of leukocytes differ not only in their tinctorial properties, but also in their functions and roles during the inflammatory process. They constitute key effector cells in innate immunity, and the frontline of host defense in response to foreign antigens and microorganisms [6].

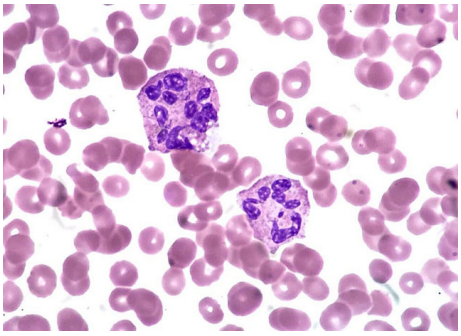


Figure 1.10: Neutrophils in peripheral blood smear. Neutrophils can be distinguished by its segmented nuclei (purple dots) [6].

Roughly 10^{11} neutrophils are released into the bloodstream daily. Neutrophil half-life in blood is 6 -8 hours and, under normal circumstances, neutrophils constitute roughly 60% of all leukocytes in human peripheral blood.

In the absence of inflammatory stimuli, neutrophil populations are maintained within a relatively narrow range. Although incompletely understood, the mechanisms controlling neutrophil homeostasis regulate both neutrophil production and clearance. In the bone marrow, as well as in the spleen and liver, damaged and aging neutrophils are cleared by tissue macrophages. Reduction of neutrophil populations is also effected through indication of apoptosis. For example, aged neutrophils undergo programmed cell death when exposed to tumor necrosis).

Neutrophil activation

The ability of bloodstream neutrophils to adhere to endothelium is an essential step required to concentrate them and direct them to sites of inflammation. Membranes on both neutrophils and endothelial cells have the capacity to express multiple families of interacting adhesion molecules. These include selectins, integrins, and intercellular adhesion molecules (ICAMs), as well as sialylated glycoproteins. Under noninflammatory conditions, neutrophils and other leukocytes travel primarily through the center of the blood vessel lumen, where flow is fastest. In response to pro-inflammatory signals, both the neutrophils and the blood vessels undergo a series of changes. As a consequence of vascular dilatation with endothelial cells. A process of rolling adhesion ensues, in which P- and L- selectins on neutrophils, and E-selectins on endothelia, interact with sialyl-Lewis moieties (s-Le^x) on their respective partners. These interactions are reversible and transient, and prepare the cells for a tighter binding step follow. In response to CXCL8 (released by tissue macrophages and transported actively through the venular endothelial cells), complement or formyl peptide fragments generated at the inflammatory site, integrins (predominantly LFA-1 and CR3) already expressed on neutrophil surfaces are stimulated undergo conformational changes that render them adhesive for their cognate receptors.

Movement of neutrophils out of the circulatory system, or diapedesis, requires first the interaction between molecules of CD31 (expressed by PMNs and also by the intercellular junctions of endothelial cells) allowing the cell to pass through the endothelium. The secretion of a broad range of MMPs degrades the basement membrane and permits the neutrophil passage through the cellular matrix. By mechanisms not fully established, the basement membrane appears to reseal behind the exited neutrophil. Once in the interstitial comparment, neutrophils migrate along the chemotactic gradient toward the site of injury or infection. The mechanism of chemotaxis involves, in part, directed localization of chemoattractant receptors

to the leading edge of the neutrophil ("headlight phenomenon") and cytoskeletal rearrangements to permit unidirectional motion.

Once recruited to the inflammatory site, neutrophils directly recognize, phagocytose, and destroy foreign pathogens. Phagocytosis, a specialized form of endocytosis, is the cellular process of engulfing particles by the cell membrane to form an internal phagosome. Initiation of phagocytosis can be achieved by neutrophil recognition of pathogen-associated molecular patterns, or PAMPs, small repetitive molecular motifs that are found on bacteria and/or viruses but non on mammalian cells. PAMPs are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). Indeed, it has been demonstrated that human neutrophils are able to express all TLRs except for TLR3. Upon TLR stimulation, neutrophil shape is altered and phagocytosis is increased.

Neutrophil phagocytosis is greatly facilitated by opsonization, or the coating of bacterial or other targets with immunoglobulins and complement C3b fragments. Antibody-coated phatogens are recognized by both complement and immunoglobulin Fc receptors o the neutrophil surface. Neutrophils possess receptors to the IgC isotypes IgG_1 and IgG_3. Opsonization of bacteria by immunoglobulins plays a particularly important role in the response of neutrophils to bacteria with polysaccharide capsules, since their capsules help them evade direct phagocytosis.

Neutrophil mechanisms of pathogen destruction are multiple, and involve granule fusion, toxic oxygen radical production, activation of latent proteolytic enzymes, and the activity of antibacterial proteins. The phagosome undergoes fusion with neutrophil granules to form phagolysosome, the protected space in which pathogen degradation occurs.

Oxygen-dependent degradation of pathogens requires a NADPH oxidase system and a respiratory burst: a rapid production and release of reactive oxygen species (ROS) such as NO, superoxide anion, and hydrogen peroxide, all of which are toxic to bacteria. The NADPH oxidase enzyme is a membrane-bound multimeric complex that assembles from membrane and cytoplasmic proteins upon cell stimulation. The NADPH oxidase system converts oxygen molecules (O_2) into superoxide anions (O_2^-). A second enzyme, superoxide dismutase (SOD) then converts O_2^- into hydrogen (H_2O_2), which can killo microorganisms. H_2O_2 can be further converted by myeloperoxidase into hypochlorous acid (HOCl, chlorine bleach), which also has potent antibacterial activity. Whereas the membrane components of NADPH oxidase are localized to specific granules, myeloperoxidase is localized to rimary granules; thus, the production of HOCl cannot occur until both granule classes fuse into the phagolysosome.

Oxygen-independent degradation also depends on the fusion of granules into the phagolysosome. Primary, specific, and gelatinase granules contain proteolytic en-

zymes such as lysozyme and metalloproteinases, as well as defensins and cationic proteins with intrinsic antimicrobial properties required for the destruction of invading microorganisms.

Most recently, a unique, nonphagocytic mechanism of bacterial killing has been identified in neutrophils. Neutrophil extracellular traps (NETs) are extracellular neutrophil structures composed of chromatin and granule proteins that bind and kill microorganisms. In settings of extreme stimulation, and in the presence of stress from ROS, neutrophils may undergo a novel form of cell death characterized by cell membrane breakdown and the release of NETs. These NETs can bind neutralize extracellular pathogens. Because this process requires the sacrifice of the neutrophil, the term "beneficial suicide" has been proposed.

Neutrophils are the first cells to be recruited from circulation to the human skin. At these sites, they combat infectious threats via their classic functions, including phagocytosis and degranulation, the production of high concentrations of ROS, and the formation of NETs.

According to what was mentioned in section 1.4, neutrophils are active cells during the inflammatory response and are present in the inflamed site, then if is possible to detect them in a skin sample, inflammation would be diagnosed easily. On the other hand, the presence of pathogens is still needed, some of the conventional techniques for their identification are described next.

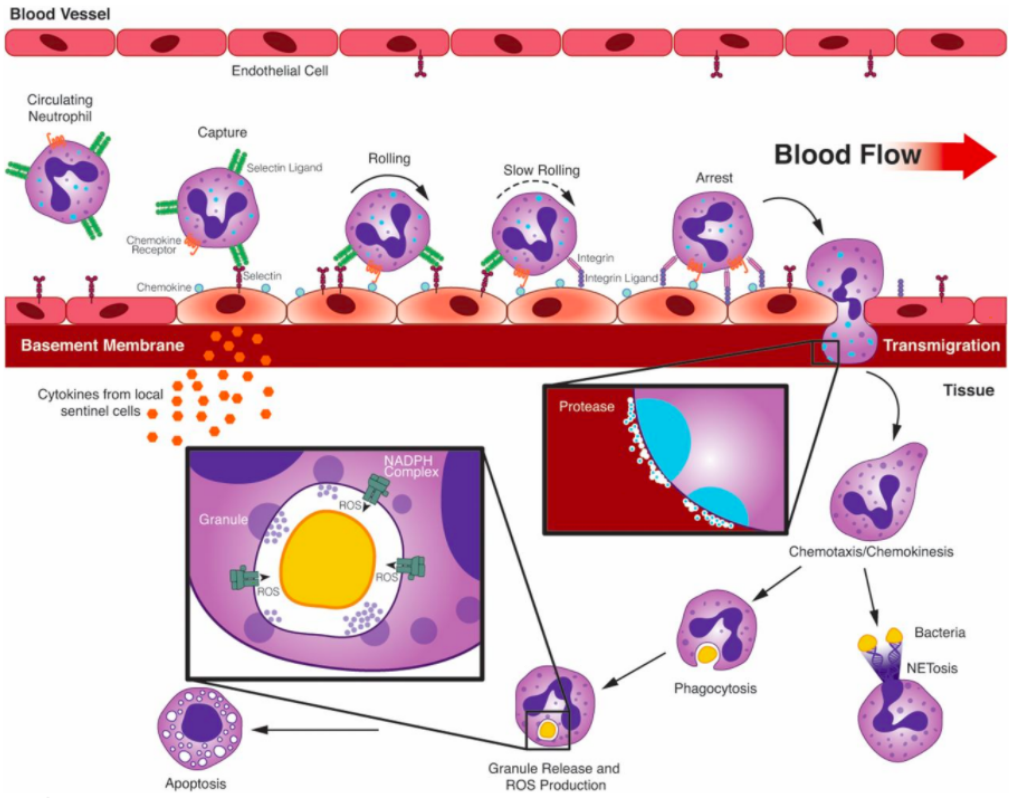


Figure 1.11: Scheme of neutrophil activation [7].

1.5 Pathogen detection

1.5.1 Established methods in pathogen detection

Polymerase chain reaction (PCR), culture and colony counting methods as well as immunology-based methods are the most common tools used for pathogen detection. They involve DNA analysis, counting of bacteria and antigen-antibody interactions, respectively. In spite of disadvantages such as the time required for the analysis or the complexity of their used, they still represent a field where progress is possible. These methods are often combined together to yield more robust results [8].

Polymerase chain reaction

This is a nucleic acid amplification technology. It was developed in the mid 80s (Mullis et al [63]) and it is very widely used in bacterial detection. It is based on the isolation, amplification and quantification of a short DNA sequence including the targeted bacteria's genetic material. Examples of different PCR methods developed for bacterial detection are: (i) real-time PCR (Rodríguez-Lázaro et al [64]), (ii) multiplex PCR (Jofré et al [65]) and (iii) reverse transcriptase coupling PCR (RT-PCR) (Deisingh [66]). There are also methods coupling PCR to other techniques [66, 67].

The PCR is a lot less time-consuming than other techniques, like culturing and planting. It takes from 5 to 24 hours to produce a detection result but this depends on the specific PCR variation used and this does nor include any previous enrichment steps.

Figure 1.12 illustrates the PCR method, consisting in different cycles of denaturation by hear of the extracted and purified DNA, followed by an extension phase using specific primers and a thermostable polymerization enzyme. Then each new doublestranded DNA acts as target for a new cycle and exponential amplification is thus obtained. The presence of the amplified sequence is subsequently detected by gel electrophoresis.

Amongst the different PCR variants, multiplex PCR is very useful as it allows the simultaneous detection of several organisms by introducing different primers to amplify DNA regions coding for specific genes of each bacterial strain targeted. Real-time PCR permits to obtain quicker results without too much manipulation. This technique bases its detection in the fluorescent emission by a specific dye as it attaches itself to the targeted amplicon. Given that fluorescence intensity is proportional to the amount of amplified product [68], it is possible to follow he amplification in real time, thus eliminating laborious post-amplification processing

steps such as gel electrophoresis. Different alternative probes, derivinf from this principle, have been developed recently [69].

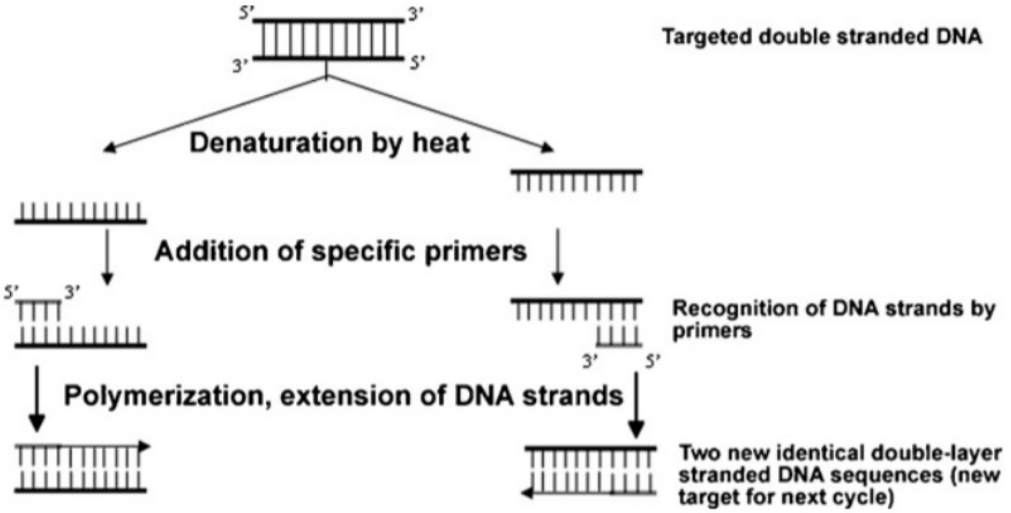


Figure 1.12: Schematic representation of one PCR cycle taking place in thermocycler [8].

One of the limitations of PCR techniques lies in that the user cannot discriminate between viable and non-viable cells because DNA is always present whether the cell is dead or alive. Reverse transcriptase PCR (RT-PCR) was developed in order to detect viable cells only [70]. Several genes specifically present during the bacteria's growth phase can then be detected. This technique gives sensitive results without any time-consuming pre-enrichment step [66].

PCR may also be found coupled to other techniques. Examples are "the most probable number counting method" (MPNPCR) [71], surface plasmon resonance and PCRacoustic wave sensors [66], LightCycler real-time PCR (LC-PCR) and PCR-enzyme-linked immunosorbent assay (PCR-ELISA)), the sandwich hybridization assays (SHAs) [72] or the FISH (fluorescence in situ hybridization) detection test [73].

Culture and colony counting methods

The culturing and plating method is the oldest bacterial detection technique and remains the standard detection method. However, other techniques are necessary because culturing methods are excessively time-consuming. in the case of Campylobacter, 4–9 days are needed to obtain a negative result and between 14 and 16 days for confirmation of a positive result. This is an obvious inconvenience in many applications.

Different selective media are used to detect particular bacteria species. They can contain inhibitors (in order to stop or delay the growth of non-targeted strains) or

particular substrates that only the targeted bacteria can degrade or that confers a particular colour to the growing colonies. Detection is then carried out using optical methods, mainly by ocular inspection.

Immunology-based methods

The field of immunology-based methods for bacteria detection provides very powerful analytical tools for wide range of targets. For example, immunomagnetic separation (IMS), a pre-treatment and/or pre-concentration step, can be used to capture and extract the targeted pathogen from the bacterial suspension by introducing antibody coated magnetic beads in it [74]. IMS can then be combined with almost any detection method (optical, magnetic force microscopy, magnetoresistance, among others) [75, 76].

Other detection methods are only based on immunological techniques; in this case the enzyme-linked immunosorbent assay (ELISA) test is the most established technique nowdays as well as the source of inspiration for many biosensor applications [77]. ELISAs combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. Figure 1.13 illustrates the principles of a typical "sandwich ELISA", which is the most common kind.

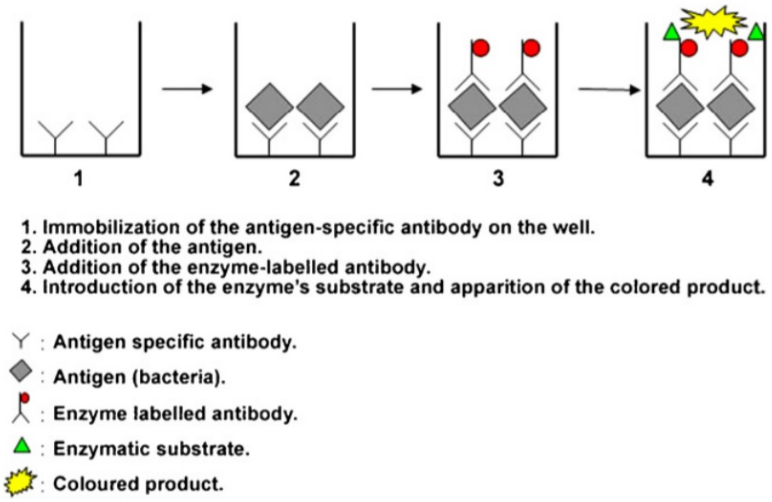


Figure 1.13: Schematic representation of the sandwich-ELISA protocol [8].

1.5.2 Biosensors in pathogen detection

Biosensors have recently been defined as analytical devices incorporating a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products, etc.), a biologically derived material (e.g. recombinant antibodies, engineered proteins, aptamers, etc.) or a biomimic

(e.g. synthetic catalysts, combinatorial ligands and imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical. The present work is focused on optical biosensors.

Optical biosensors

These are probably the most popular in bioanalysis, due to their selectivity and sensitivity. Optical biosensors have been developed for rapid detection of contaminants [78, 79], toxins or drugs [80] and even pathogen bacteria [81]. Recently, fluorescence and surface plasmon resonance, SPR, based methods have gained momentum because of their sensitivity.

Surface plasmon resonance SPR biosensors [82] measure changes in refractive index caused by structural alterations in the vicinity of a thin fill metal surface. Current instruments operates as follows. A glass plate covered by a gold thin film is irradiated from the backside by p-polarised light (from a laser) via hemispherical prism, and the reflectivity is measured as a function of the angle of incidence, θ. The resulting plot is a curve showing a narrow dip. This peak is known as the SPR minimum. The angle position of this minimum is determined by the properties of the gold-solution interface. Hence, adsorption phenomena and even antigen-antibody reaction kinetics can be monitored using this sensitive technique. The main drawbacks of this powerful technique lay in its complexity (specialised staff is required), high cost of equipment and large size of most currently available instruments [83].

Fluorescence detection Fluorescence occurs when a valence electron is excited from its ground state to an excited singlet state. The excitation is produced by the absorption of light of sufficient energy. When the electron returns to its original ground state it emits a photon at lower energy. Another important feature of fluorescence is the little thermal loss and rapid (10 ns) light emission taking place after absorption. The emitted light is at a longer wavelength than the absorbed light since some of the energy is lost due to vibrations, this energy gap is termed Stoke's shift, and it should be large enough to avoid cross talk between excitation and emission signals [84], as shown in figure 3.3, section 3.2.1.

Antibodies may be conjugated to fluorescent compounds, the most common of which is fluorescein isothiocyanate (FITC). There are, however, other fluorescent markers. the use of lanthanides as sources of fluorescence in luminescent assays has very recently been reviewed [85]. Although lanthanides pose several important advantages (good stability, low background luminescence under normal light condi-

tions and large Stoke's shift) compared to more traditional fluorophores, their use is very restricted due to safety reasons.

Fluorescence detection, in contrast to SPR, is also used in combination with established techniques such as PCR and ELISA.

In this case, fluorescence was selected as the easiest way to detect the presence of pathogens in skin samples, due to the time consumed for fluorescence assays.

In order to develop systems for detection of inflammation and pathogens in skin samples for assisting the diagnosis of skin diseases, the design methodology was followed. It described in the following section.

1.6 Conclusions

As mentioned in this chapter, skin diseases show the presence of inflammation and pathogens. Until now the existing methods are not specific to inflammation, and require long time (hours or even days) of performance and special training. For pathogen detection, there are several specific techniques, but they have limitations related to cost, space, and training, and are hard to access in developing regions. Due to these reasons, the design of point-of-care (POC) tools for low resource settings for detection of inflammation and pathogens is needed, and the main aim of this work is to attend that need.

In this work, the design of two systems for detection of inflammation and presence of pathogens was proposed in order to assist the diagnosis of skin diseases. The inflammation system is based on the detection of the presence of neutrophils in skin samples. The pathogen detection system is based on the measurement of fluorescence response of biological samples. In order to develop these systems, the design methodology was followed. Both systems are described in detail in the following chapters.

Chapter 2

Design of a portable system based on chemiluminescence for the detection of inflammation in skin microsamples

As already mentioned, the existence of a new technique or method for a quantitative detection of inflammation is needed in order to improve the diagnosis of skin diseases. To develop that new technique, the design methodology was followed, and it is explained in the following section. The same methodology was used for the design and development of the system for fluorescence detection.

2.1 Design methodology

According to the earlier engineering design methods of the second half of the twentieth century, design is defined as finding a technical solution to a design problem. That problem is formulated as a set of physical, technical, and financial requirements that have to be met, and the solution is typically a description of a material product. The problem is fixed, and the source of the problem - goals or needs, or ideas for products and services - is considered to be a matter of the customer ordering the design, and a matter that lies outside the realm of engineering design. The Figure 2.1 this traditional understanding of engineering design is depicted schematically as a practice. Finding the description of the product that solves the problem, is typically divided into different phases, such as conceptual design, embodiment design, and detail design. Engineering design may involve different iterative steps between these phases, meaning that the findings of a later phase may provide information

that brings the design process back to reconsidering the decisions made in an earlier phase (As shown in Figure 2.1).

One framing of the engineering design process delineates the following stages: problem definition, background research, requirements specification, proposition of possible solutions, choosing a solution, develop the solution, prototyping, test and redesign. Each of this stages are described briefly below.

2.1.1 Problem definition

In this stage, the general need or problem has to be identified, the source of need can be a company, costumer or society. Some helpful questions in this phase are: what is the problem or need?, who has the problem or need?, why is it important to solve?.

2.1.2 Background research

This phase consists on reviewing all the reported information related to the problem that is desire to solve, such as solutions for similar problems, involved phenomena, operation principle of existing technology, techniques that can be applied, among others. All resources can be used; research papers, patents, books, websites, etc.

2.1.3 Requirement specification

Design requirements state the important characteristics that the design or product must meet in order to be successful. One of the best ways to identify the design requirements for the solution is to use the concrete example of a similar, existing product, noting each of its key features. Requirement specification is very important stage, since it helps to limit the design.

2.1.4 Brainstorm, choosing a solution

At this stage, new ideas must be generated based on the background research. All ideas must be discussed and revised in order to choose the most appropriate, which must meet the design requirements.

2.1.5 Developing and prototyping

Once the best idea was chosen, its time to develop it. This is the stage where all the knowledge acquired during the background research must be applied on generating a functional prototype which meets all the requirements to solve the defined problem or to satisfy the initial need.

2.1.6 Test

Once the prototype is ready, it should be tested to verify if its capable to solve the problem or to satisfy the need, obviously by meeting the design requirements.

2.1.7 Redesign

If the prototype meets all the requirements and solves the problem, the design can be validated. If it does not, a redesign process is needed, which means going back to the brainstorm or idea development stages, to generate a new prototype for being tested again.

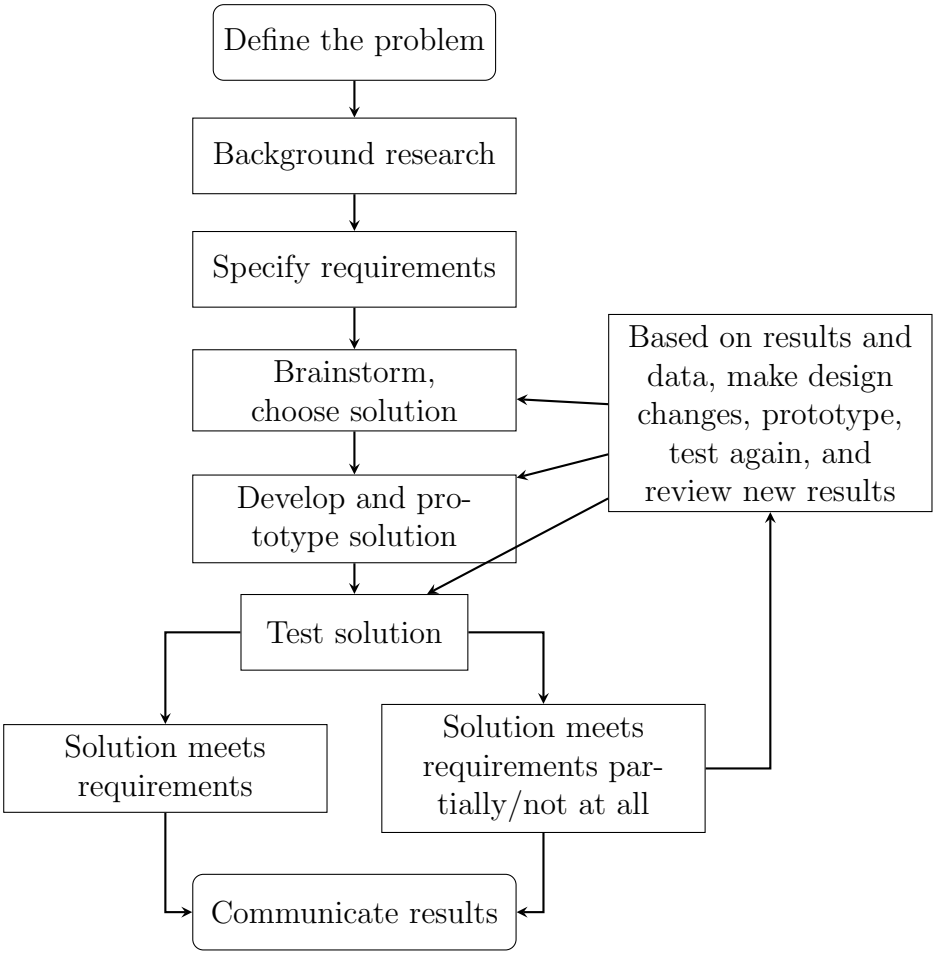


Figure 2.1: Methodology of the design process.

All the mentioned steps were followed for the design of the chemiluminescence detection system. Each phase is described in detail below.

2.2 Problem definition

As mentioned in Chapter 1, skin diseases are mainly dependent on two factors; inflammation and presence of pathogens. For inflammation, there is no specific test for its diagnosis, the most common assay is blood test, but it is time consuming and non specific. According to this, a new, fast, and easy way to detect the presence of inflammation is needed, in order to improve and facilitate the diagnosis of skin diseases.

In this work, according to the information mentioned in section 1.4, the detection of inflammation based on the presence of neutrophils in skin samples is proposed. This is already a technique used in clinics, but until now, it has the huge disadvantages of being invasive, time consuming, and requires specialized equipment. Based on this facts, a new, fast, and cost-effective way to detect the presence of neutrophils is needed.

In order to design a system for the detection of neutrophils in skin samples, the conventional techniques of neutrophil identification were revised, and are presented in the following section, which corresponds to the second step in the design methodology; background research.

2.3 Background research: Neutrophil detection

Many cutaneous diseases are characterized by the presence of neutrophils in the skin, such as bacterial infections, vasculitis, neutrophil dermatoses, and others. Neutrophils are among the first innate immune cells recruited to the site of infection and inflammation. In addition to clinical signs and symptoms, identifying the presence of neutrophils in the skin is a critical part of making an accurate diagnosis. A few techniques for its visualization are described below.

2.3.1 Intravital microscopy

Intravital microscopy (IVM) is an extremely powerful tool that enables imaging several biological processes in live animals (mostly mice) at a high resolution that distinguish between individual cells and tissue. In order to use this technique a surgery has to be performed to implant an imaging window. Which is a section of clear and biocompatible material that allows the light source of the imaging system to reach the organ or section under interest. It also requires the use of fluorophores to target the structures under observation. It has been performed by using various light microscopy techniques, such as widefield fluorescence, laser scanning confocal, multiphoton and spinning disk microscopy [86]. Figure 2.2 shows an scheme of how this procedure is performed.

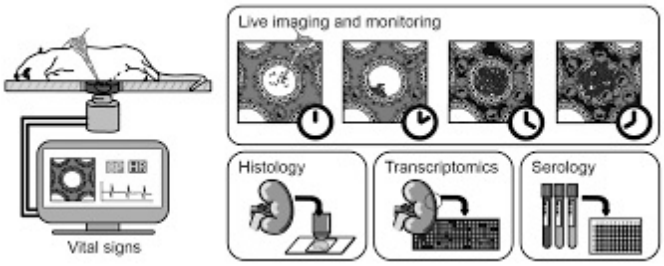


Figure 2.2: Scheme of the intravital microscopy procedure [9].

Intravital imaging has allowed the visualizacion of neutrophils in their native enviroment in both health and disease and provided crucial insights into their mechanisms of action. In the last few years the power of intravital imaging has been considerably extended by the introduction of photoconvertible proteins and intracellular signaling reporter mice [87].

Antibody/Gene/Mouse	Immune cell specificity	Imaging Technique(s)	
Lysozyme M (LysM)	Neutrophil and macrophage	Spinning Disk Multiphoton Confocal	
Ly6G "Catchup"	Neutrophil	Multiphoton Confocal	[87]
Mrp8	Neutrophil	Multiphoton	
Anti-GR1	Neutrophil and macrophage	Multiphoton Confocal	
Anti-Ly6G (1A8)	Neutrophil	Confocal	

2.3.2 Microscope slide staining

Microscope slide staining is a technique used to enable better visualization of cells and cell parts under the microscope. By using different stains, a nucleus or a cell wall are easier to view. Most stains can be used on non-living (fixed) cells, while only some types of stain can be used on living cells. Stains and dyes are frequently used in histology (the study of tissue under the microscope) [88].

Microscope slide staining consists on several steps:

1. Fixation: is the preservation of biological tissues from decay due to autolysis or putrefaction. It terminates any ongoing biochemical reactions and may also increase the mechanical strength or stability of the treated tissues.

2. Mordant: These are chemical agents which allow dyes to stain materials which otherwise are unstainable.

3. Staining: The application of the substances that

4. Permeabilization: This treatment dissolves cell membranes, and allows larger dye molecules into the cell's interior.

5. Mounting: Usually involves attaching the samples to a glass microscope slide- for observation and analysis. In some cases, cells may be grown directly on a slide. For larger pieces of tissue, thin sections (slices) are made using a microtome.

The most common stain for the visualization of neutrophils is hematoxylin and eosin (H&E).

2.3.3 Chemiluminescence

Chemiluminescence is an ultra-weak emission of light generated as result of a chemical reaction. It is a complicated process, generally can be divided into two steps: the excitation stage connected with generation of excited molecule identical with the excited product, and the luminescence stage at which the excited molecule is deactivated to its ground state emitting a quantum of radiation. The first stage is of chemical nature, while the second of physical and combines several processes [89].

The efficiency of light emission is expressed as a quantum yield. The overall quantum yield of a chemiluminescent reaction (Φ_{CL}) is the main parameter which characterizes transformation of chemical energy into radiation, and is defined as the number of photons emitted per number of molecules involved in the reaction. The following equations presents the components of Φ_{CL} [90]:

$$\Phi_{CL} = \Phi_C \Phi_{Ex} \Phi_F \tag{2.1}$$

Where Φ_C means a chemical yield; fractions of molecules going through the main reaction (excluding "dark" side reaction); Φ_{Ex} is the fraction of those molecules which react in the chemiluminescence pathway and become electronically excited; Φ_F is the fluorescence quantum yield of the excited product, i.e. the number of photons emitted, divided by the number of molecules actually excited. These parameters (Φ_C, Φ_{Ex}, Φ_F) making up Φ_{CL} can be estimated independently but with a different level of difficulty [90].

The ultra-weak chemiluminescent reactions often reveal extremely low quantum yields ($\Phi_{CL} = 10^{-3}/10^{-8}$, but also $10^{-9}/10^{-15}$) [90]. In general, the efficient chemiluminescence of chemical compound is a result of oxidation involving molecular oxygen or hydrogen peroxide.

The activation of phagocytes (such as neutrophils) can be detected trough chemiluminescence enhanced by added luminol and lucigenin (as synthetic compounds).

The emission obtained serves as an indicator of the reactive oxygen metabolites, peroxidase, oxidase, oxygenase process, and lipid peroxidation in cells.

Chemiluminescence signals are measured using luminometers. All luminometers contain photodiodes or photomultiplier tubes to detect chemiluminescence signals. These detectors are either placed below or to the side of the sample tubes to capture the signals. Basically they are designed to measure the signals as photons or electric current when the photons strike the photomultiplier tube. The results are expressed as or millivolts/sec, counted photons/min (cpm), or RLUs (Relative Light Units) [91]. Luminometers are specialized equipment, their cost varies from $2,500 to $10,000 dollars, generally have several limitations (sample shape and size, complicated software, no portability, etc.). The figure 2.3 shows an example of one of the most common luminometers in the labs.

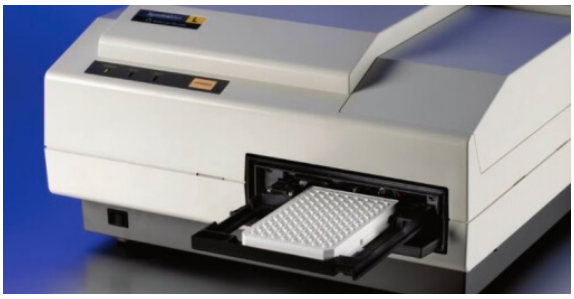


Figure 2.3: Example of luminometer most used in laboratories [10].

The type of signal expected from a chemiluminescence assay is shown in figure 2.4. It is a time dependent signal, generally the beginning of the reaction follows a linear behavior and is shorter in time compared with the decay. Depending on the chemical reaction the signal could last even more than 60 minutes.

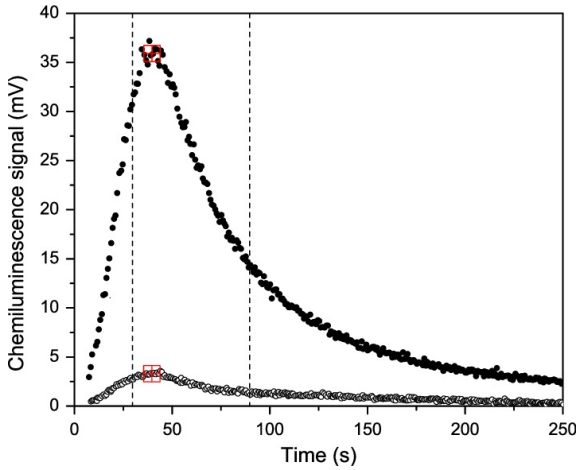


Figure 2.4: Example of a chemiluminescence signal [11].

Currently, neutrophil identification in the skin requires a skin biopsy and histopathology, which are costly and slow. In order to improve clinical care and reduce costs, a

rapid method for identifying neutrophils in the skin is needed. Due to these reasons, a chemiluminescence based, fast, minimally invasive and simple device for evaluating inflammation at the point-of-care is developed in this work.

Now that the operation principle was chosen, the specifications for the design need to be set, which corresponds to third step of the design methodology.

2.4 Requirements specification

As mentioned before, the main aim of this work is to improve or facilitate the diagnosis of skin diseases by designing point-of-care tools. The following design requirements were set basing on the fact that those tools or devices are expected to be used in clinics by physicians.

- Minimally invasive. Most of the convectional techniques for inflammation diagnosis require the extraction of biopsies, which is a painful procedure and produces scarring in the sampling area, for this reason, a minimally invasive new procedure is strongly required.
- Fast response. For this case, is required that the inflammation test does not exceed 15 minutes.
- Easy to use. The system is expected to be used by physicians, so minimal training should be needed to be operated, also the components should be easy to replace in case of damage.
- Portability. The system should be easy to carry
- Cost-effective. Since the system is expected to be used in low-resource settings, the low cost is desired.
- Simple assembly. In order to reduce the operation time, and to keep simplicity in the design, the device should not have several parts to assembly.
- Sterile. Since the device will be used with tissue samples, with possible presence of pathogens, the system should be sterilized, or at least the container where the tissue sample are going to be placed.
- Low maintenance. Since the system is expected to be used in low-resource settings, requires components of low maintenance, that should not be replaced frequently to increase the productive life of the device.

2.5 Choosing a solution: Neutrophil detection using chemiluminescence for inflammation measurement

As mentioned in section 1.4.4, during the process of phagocytosis, leukocytes undergo remarkable alterations in oxidative metabolism[92]; for instance, the generation of chemiluminescence [93]. Respiratory burst activity is essential for chemiluminescence, normal resting cells do not emit light. In general, the intensity levels of light are low, obviating the need of a large number of cells and a highly sensitive detection system.

The compound luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is commonly used for amplifying the chemiluminescence response. Luminol-enhanced chemiluminescence is a common method for studying neutrophils because it allows the use of much lower numbers of cells and has been shown to measure chemiluminescence in samples of whole blood [94]. The exact reaction is not known, but luminol is presumably oxidized by some reactive species of oxygen generated during the respiratory burst, although luminol can also be oxidized by several O_2 intermediates, such as O_2, $HO^{\cdot}$, H_2O_2, and $^1O_2$ (singlet oxygen)[95]. The oxidation of luminol results in the production of an excited aminophthalate anion that relaxes to the ground state with the production of light at all visible wavelengths[92]. Luminol-enhanced light emission by neutrophils is thus a nonspecific manifestation of the respiratory burst.

Neutrophil chemiluminescence has been explored before as a diagnostic tool: Boveris et al. [96] utilized the spontaneous and hydroperoxide-induced chemiluminescence of in situ and perfused rat liver for continuous monitoring of organ metabolism in vivo; Iwata et al. [97, 98] measured the chemiluminescence response due to the influx of inflammatory cells, before and for 90 min following phorbol myristate acetate (PMA) and ethanol perfusion, in rat liver in situ under ischemic conditions; and, Cadenas et al. [99] described the kinetics of macrophage light emission and the dependence on O_2 tension of the external medium. In humans, Ou-Yang et al. used in vivo chemiluminescence as a non-invasive method to assess oxidative stress in the skin[100].

In this work, a method for detection of neutrophils in tissue samples using chemiluminescence is proposed. As it was already mentioned, chemiluminescence is the emission of light as a result of a chemical reaction as shown in expression (2.2). According to section 1.4.4 a considerable amount of oxidative species are produce during neutrophil activation, when this products react with luminol chemiluminescence takes place. In this particular case, the reagent $[A]$ represents the oxygen

species released during the human neutrophil respiratory burst, the reagent $[B]$ is luminol, and the result of this chemical reaction is products and light:

$$[A] + [B] \longrightarrow [Products] + Light \tag{2.2}$$

The proposed method consist of the extraction of tissue samples, followed by the addition of some chemical components to generate the chemiluminescence reaction. The light produced by the chemical reaction is related to the presence of neutrophils in the sample and hence inflammation. Each phase of this procedure is described below.

2.5.1 Tissue samples extraction

The skin biopsy is one of the most essential techniques in dermatology for diagnosis of skin conditions through histopathological assessment. The procedure involves the removal of suspicious skin lesions followed by examination under microscope. There are three major types of skin biopsies; punch biopsy, shave biopsy and excisional biopsy. All three types are highly invasive and can cause discomfort to patients. Figure 2.5 shows the procedure for each type of biopsy.

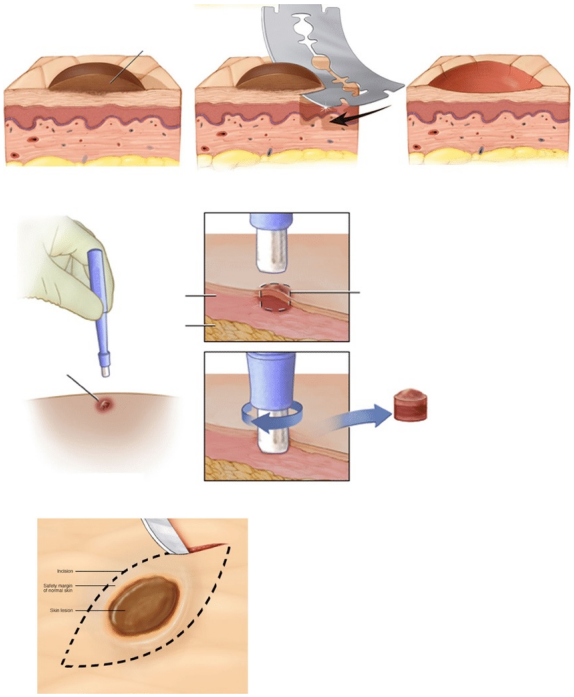


Figure 2.5: Major types of skin biopsies [12].

Can be observed that the doctor uses a sharp tool for cutting into the top layer of fat beneath the skin (epidermis, dermis and superficial fat) and removing the suspicious site for examination. These procedures require local anesthesia and the

removal of the entire suspicious spot, irrespective the size, a suture is often needed to close the wound, and all these techniques produce scarring [12].

In general, full-thickness skin wounds (wounds that extend beyond dermis and epidermis and go into the subcutaneous tissue or even all the way to the bone or tendons) that are relatively large heal by scarring; this healing process is the outcome of an immediate organ response to quickly fill and close large voids in skin tissue with a haphazard arrangement of connective tissue elements. Scar tissue is stiff and lacks normal function because the arrangement of extracellular matrix and specialized dermal structures that confer function to normal skin is missing.

In contrast to scarring, healing by remodeling is a process that replaces tissue while maintaining tissue architecture and dermal structures on the microscopic scale. Remodeling occurs in every organ as we age and grow, and almost every tissue has the capacity for local remodeling without scarring. While scarring is stimulated by large-scale tissue damage, remodeling is stimulated by microscopic tissue damage. This principle was recently used to develop a wound healing technique known as fractional skin grafting, which consists in harvesting hundreds to thousands of microscopic full-thickness skin grafts from a healthy donor site to graft them on the wounded site [13]. Figure 2.6 shows the concepts of scarring and remodeling; 2.6a shows a tissue section where microsamples of skin were taken, and 2.6b shows a tissue section after conventional skin removal for skin graft treatment. Figures 2.6c and 2.6d show the same tissue sections after 7 weeks, can be observed that section c is fully recovered without scarring while section d shows pigmentation and texture issues.

Considering all the advantages that the microscopic tissue damage concept offers, such as absence of scarring, healing by remodeling, less pain than conventional biopsy, among others, was decided to extract skin micro-samples for its chemiluminescence response analysis for the diagnosis of inflammation. By using this technique, the procedure can be considered as a minimally invasive test.

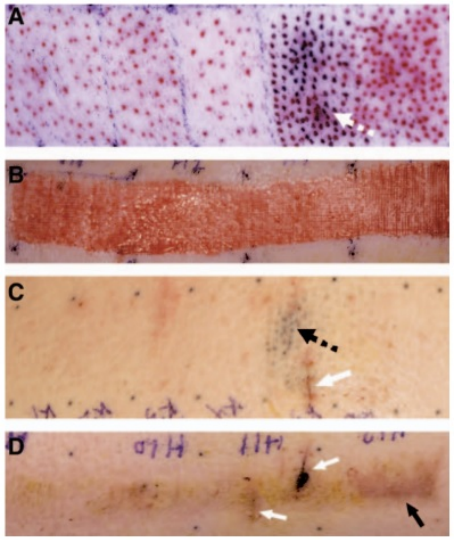


Figure 2.6: Healing process for fractional skin harvesting and conventional skin graft procedures [13].

Now that was decided to design a system for inflammation detection in skin samples based on chemiluminescence measurements, the development of the prototype is detailed in the following section.

2.6 Development and prototyping

The inflammation detection system designed in this work, is mainly divided in two parts; skin sample extraction and chemiluminescence analysis. Was decided to extract the tissue samples using the technique of fractional skin harvesting, in order to avoid scarring, and to keep the procedure minimally invasive. For this reason, a tool or device for the extraction of skin micro-columns is required. For the chemiluminescence analysis, a light isolated chamber for the measurement of light emission is needed. The design and prototype of both components are described in detail below.

2.6.1 Microbiopsy device

In order to collect microscopic skin samples in a minimally invasive way, the design of a microbiopsy device was needed. All the components, functioning principle, materials and details are explained below.

Collecting needle

The geometry of the needles used for the collection of microsamples is shown in figure 2.7. They are made out of 304 stainless steel, 22 gauge (0.72 mm outer diameter,

0.41 mm inner diameter),common hypodermic needles with two grinding cuts at 12 degrees [14, 13]. This specific shape allows the extraction of tissue in a easier way than the conventional biopsy punch, which requires to rotate the tool in order to cut the sample. In the case of the collecting needle, when the contact between needle and tissue starts, the contact area is really small (represented by the two red lines in figure 2.7), which reduces the needed force to penetrate the skin, and facilitates the tissue extraction. In comparison to the conventional biopsy punch, where the contact area is a circle and results in higher force applied to cut the skin and therefore more pain.

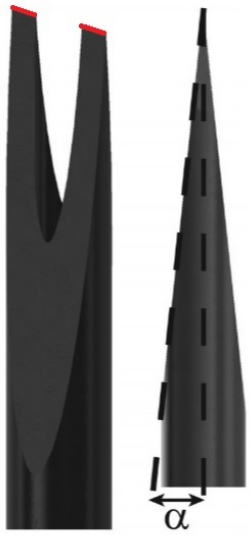


Figure 2.7: Collecting needle [14].

In order to reduce the force needed for sampling, and the remainings of metal particles on the edges of the needles after the cutting process, polishing and cleaning procedures are performed.

After the cutting process, the needles are placed in a container with alcohol for 3 minutes. Then they are left at room temperature to dry. Once the needles dried, an electropolishing process takes place.

Electropolishing is and electrochemical process that removes material from a metallic workpiece, reducing the surface roughness. Tipically, the work-piece is immersed in a temperature-controlled bath of electrolyte and serves as the anode; it is connected to the positive terminal of a DC power supply, the negative terminal being attached to the cathode. A current passes from the anode, where metal on the surface is oxidised and dissolved in the electrolyte, to the cathode [101]. In this particular case, the electrolyte solution used was SS Electropolish E972, the bath temperature was set to 110 F, the voltage used for 22G needles was 4 V, the current

3 A, and the polishing time of 5 minutes. The polishing time can be reduced by increasing the current, but in this case, the parameters were kept fix in order to apply the same treatment for all the needles used in this work.

Once the electropolishing routine is done, the changes in the surface roughness are clearly significant, but due to the contact between the electrolyte solution and the needles, a cleaning procedure is performed. It consists of 3 phases: immersion in citric solution, raise with hot water, and dry with hot air. The immersion in citric solution lasts 5 minutes, the citric solution used was Citri DeOX 964. Rinsing with hot water, and drying with hot air last 5 minutes too.

Sampling device and procedure

Due to the geometry of the needle, the tissue sample sticks to the walls of the needle once it penetrates the skin. In order to control ejection and placement of the sample a simple mechanism was designed, the operation principle and its components are explained below.

The tool has four basic components: body, pin, spring, and harvesting needle. The body and pin were 3D-printed in Accura® 60, a material resistant to autoclaving conditions, the spring and pin are made out of stainless steel, and the harvesting needle was machined using 22-gauge stainless steel (0.72 mm outer diameter, 0.41 mm inner diameter) and two grinding cuts at 12 degrees [14, 102]. The operation mechanics is shown in Figure 2.8. First, the tool is placed on top of the skin. Next, the harvesting needle is pushed to cut the skin from the epidermis to the subcutaneous tissue. The geometry of the cutting needle forces tissue into the needle, does not require a rotational motion, and demands less force for cutting the skin than the circular cutting geometry of a standard biopsy punch tool. The needle cutting geometry is shown in Figure 2.7. After reaching the subcutaneous tissue the tool, containing a full-thickness skin sample, is pulled back. The sample separates from the subcutaneous tissue and stays inside the needle because the subcutaneous tissue is fat and the skin tissue adheres to the inner walls of the needle. Finally, the skin sample is ejected into the cuvette by pushing it out with the pin.

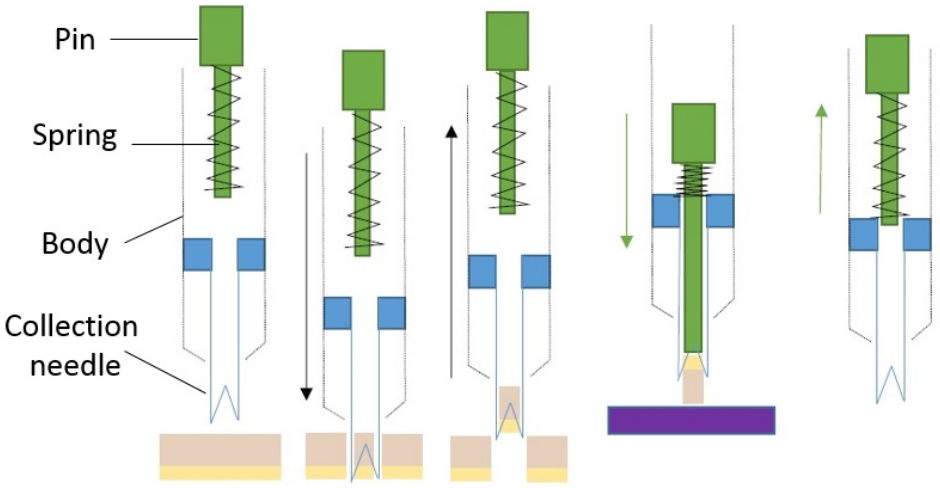


Figure 2.8: skin micro-sampling procedure.

All the materials involved in the sampling device are resistant to autoclaving conditions, since the tool is going to be in contact with the skin, sterilization is strongly required. As mentioned before, a cleaning and polishing processes are performed on the needles in order to reduce the force needed for tissue extraction, and to eliminate the undesirable metallic particles produced during the cutting process, this also allows the extraction of multiple samples with the same needle, without affecting its sharpness. The sampling device was designed to be disposable after the extraction of all the skin samples needed for the chemiluminescence test in each patient.

Initially because of the high resolution an material diversity that 3D printing offers, was decided to print all the components of the sampling device, but since the pin is too thin, the required resolution and cost increased, and the mechanic resistance of the element got reduced, which complicated the sample ejection. In order to avoid those problems, just a fraction of the pin was printed and a thin wire was used as an extension of it to eject the skin sample. The wire and collecting needle were attached to the pin and body by using Master Bond, a glue resistant to autoclaving conditions.

Two different versions of the microbiopsy tool were designed, 3D printed and tested for pig skin extraction. Figure 2.9 shows the first version. In this case, the body has a rectangular groove, and the pin has a squared wedge which has to be inserted into the groove in order to be locked and assembled, the length of the guide allows the pin to be displaced the needed distance to eject the tissue sample.

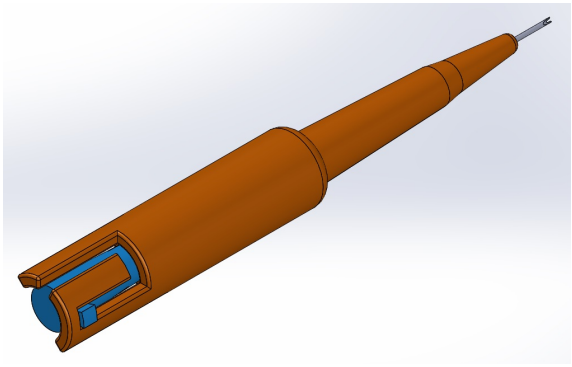
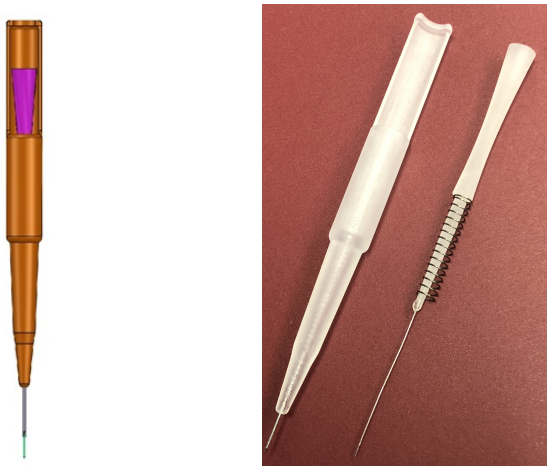


Figure 2.9: First version of the microbiopsy tool.

Figure 2.10 shows the second version of the microbiopsy tool. In this case, the body has a small stop on the top, which has the main function of keeping the pin inside the body. To assembly this version of the microbiopsy tool, the pin has to be inserted in a tilted way inside the main body avoiding the bending of the wire attached to the pin.



(a) (b)

Figure 2.10: Second version of the microbiopsy tool.

The main difference between the two versions of the microbiopsy tool, is the way to assembly the main body and the pin. The first version offers a more effective way to lock the tool, but the geometry is more complex than the second version, this reduces the tension resistance of the body, since the device is 3D printed (in several layers) the walls of the rectangular guide have a low resistance. Also the possibility to produce this tool by using plastic injection was considered, but with a complex geometry as the one used in the first version it would be impossible. Due to these reasons, the second version was chosen as a definitive version of the microbiopsy tool.

The designed sampling device is about the same size of a regular biopsy punch, which makes it easy to use and hold. In order to test functionality, experiments

were performed using pig and human skin. They showed that is possible to use the same sampling device for the extraction of more than 20 skin samples.

2.6.2 Chemiluminescence detection system

Lundqvist et al [103] report neutrophil chemiluminescence reactions longer than 30 minutes measured with a luminometer, but the concentrations used were of 10E6 cells. In these case lower concentrations of neutrophils and shorter chemiluminescence responses are expected. Also, it was already mentioned that the luminometers are expensive and bulky which is an impediment for portability. Due to these reasons, the design of a portable light isolated chamber for chemiluminescence measurements was needed.

Photocounting module

For real time chemiluminescence measurements, a photon counting module (PDM9107-USB, ET enterprises, Uxbridge, England) shown in figure 2.11. It is easy plug-and-play operation, no external power supply required, low noise, 25 mm active diameter and spectral range of 280 to 630 nm.

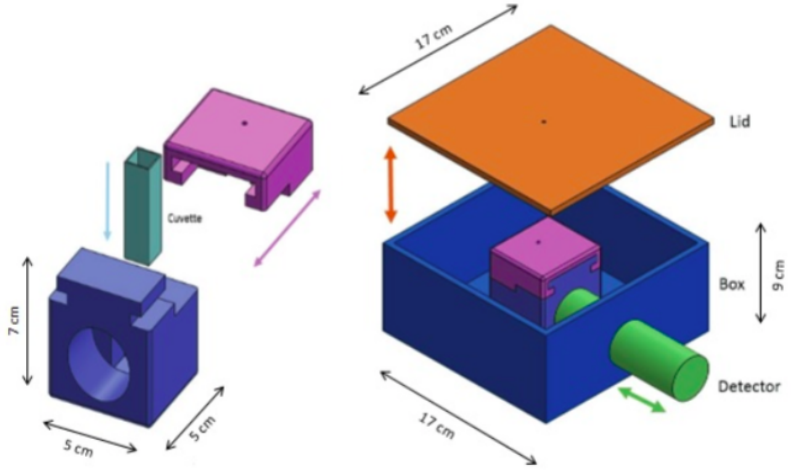
Figure 2.11: Photocounting module [15].

Chemiluminescence chamber

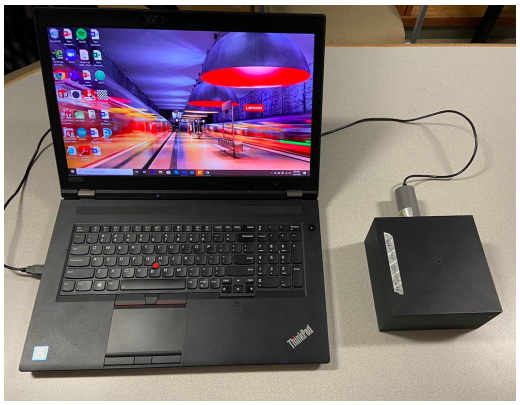
The light isolated chamber was designed using SolidWorks and 3D printed in black ABS material with a 3D printer Fortus 380 mc stratasys®, it is shown in figure 2.12. It consists of 2 boxes, the one on the right side is where the cuvette is placed with the skin samples. Once the cuvette is positioned, the small box is collocated inside the chamber, the photocounting module is slide trough the big aperture, and the chamber is closed with the lid as shown on the left of Figure 2.12 . Both chamber and the small box have a small orifice on top, where the reagents are deposited using a pipette. The main requirement for this chamber is the color, it should be black in order to isolate the sample from the light of the surroundings. If there is no black

material available for printing, the chamber could be built in a different color, and a layer of light reflecting paint should be applied on the inner walls. This component of the chemiluminescence measurement system could be expensive, since it requires long time for printing, but it could be replace with other materials, and it is not completely necessary to be produced using 3D printing technology.

When chemiluminescence reactions are measured with conventional luminometers, the reagents are usually mixed before placed inside the device, it works well when the reactions are slow (more than 20 minutes), but for faster reactions some of the light emitted can be missed at the beginning of the chemiluminescence phenomena. In this case, the orifice on the lid of the chamber was added to monitor the light emission before and after the deposition of the reagents.



(a) (b)



(c)

Figure 2.12: Light isolated chamber. a) Inner chamber. b) Full assembly of the chamber. c) Picture of the actual chemiluminescence detection system

2.7 Testing, experimentation

For development and validation purposes, experiments with chemical compounds, isolated neutrophils in vitro, isolated neutrophils combined with pig and human skin, and pig skin with inflammation were conducted. The experiments with chemical compounds were performed to test the sensitivity of the chemiluminescence system and its capability to measure the light emission from a well known chemical reaction. The experiments with isolated neutrophils were conducted in order to compare the performance on the proposed system and the data reported in the literature, and also, to determine the minimal amount of cells that can be detected with the proposed design. The experiments with human and pig skin were planned to mimic a real inflammation scenario, to test the capability of the system to detect light coming out of tissue samples. And finally, the experiments with inflamed skin were performed to test the main purpose of the system, the detection of inflammation based on the neutrophil respiratory burst activity. All these experiments are described in detail in the following sections.

2.7.1 Materials and methods

Isolation and preparation of human neutrophil samples

Neutrophils were isolated from 10 ml blood samples of healthy donors using the EasyStep direct human neutrophil isolation kit (Stemcell technologies, Vancouver, Canada). After the isolation procedure, neutrophils were centrifuged at 200 rcf (relative centrifugal force) for 5 minutes, and then re-suspended in Iscove's modified Dulbecco's (Fisher scientific, Hampton, NH) medium and 10% FBS. This solution was used to prepare additional solutions with different concentrations of neutrophils. Right before the experiments were performed, the neutrophil concentrations were centrifuged at 200 rcf for 5 minutes and resuspended in 100 μl of KRG solution (Alfa Aesar, Haverhill, MA).

Light emission measurements

The cuvette was positioned in front of the photon counting module to record light emissions in real time, as shown in figure 2.12. Once the samples were inside the isolation chamber cuvette, the data acquisition was started followed by the addition of 4 μl of HRP, 5 μl of PMA and 100 μl of luminol solution (Sigma Aldrich, St Louis, MO) through a small aperture on the isolation chamber lid. PMA activates neutrophils, HRP accelerates the reaction, and luminol amplifies the signal.

Histology analysis

To verify the presence of neutrophils for the experiments performed with inflamed skin, a 6 mm biopsy was taken from each inflamed and control skin section and placed in formalin immediately after performing experiments. Immunohistochemistry analysis with markers against porcine neutrophils (myeloperoxidase activity) was carried out in wax embedded sections of 5 μm thickness using rabbit polyclonal antibody against myeloperoxidase (Abcam, Cambridge, UK).

2.7.2 Statistical analysis

To determine the significance of the obtained results, statistical analysis was performed using the software Prism 8 (GraphPad Software, Inc, La Jolla, CA). Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used as the test of significance between groups for the data obtained from the experiments described in sections 3.4 and 3.5, $p < 0.05$ was considered significant. Data is presented as mean $\pm$ SD.

2.7.3 Experiments with chemical compounds

To test the capability of the designed system to detect light emissions from chemiluminescence reactions, experiments were performed using a well known chemiluminescence reaction: luminol in 0.5 M sodium hydroxide (solution 1) and a 5% solution of potassium hexacyanoferrate III (solution 2) in water. Two different groups of experiments were designed to evaluate the resolution of the system: varying volumes, and concentrations of each solution.

For the experiments varying the concentration of each solution, the total volume of the sample was fixed to 100 μl and the luminol/potassium hexacyanoferrate solution ratios were 10/90, 30/70, 50/50, 70/30, and 90/10. For the experiments varying the total volume of the sample, the concentration of each solution was fixed to 50% luminol and 50% potassium hexacyanoferrate and the total volume of the sample was changed to 40, 60, 80, 100 and 120 μl. In all experiments, the light emissions were measured 5 times during independent experiments.

The main aim of these sets of experiments was to determine the sensitivity of the chemiluminescence system by changing reagent concentrations and volumes, to test if the system was able to detect those changes in terms of light emission.

2.7.4 Experiments with neutrophils in vitro

Chemiluminescence is a well established procedure to measure activation of neutrophils as reported in the literature [104, 105, 106, 107, 103]. In these reports, light

emission is measured as a function of time in high concentrations of neutrophils (1E+6 cells) [106]. In the present work, samples with neutrophil concentrations varying from 5,000 to 50 cells were used to establish the minimum concentration of cells that the system can detect.

2.7.5 Experiments with live tissue

Neutrophils move from blood to tissue when activated by the presence of bacteria or other pathogens. A simple experiment to mimic the presence of neutrophils in tissue with inflammation was conducted. Different concentrations of neutrophils were injected into excised live skin tissue samples from discarded pig and human skin. The skin samples were divided in several 25 cm^2 areas. Different concentrations of neutrophils were injected in each section of the skin tissue using an insulin syringe. After one minute, 5 microsamples were extracted from random locations of each section using the microsample punch tool shown in Figure 2.10. The microsamples were placed in the detection chamber with HRP, PMA, and luminol. As control, 5 microsamples collected from skin sections that did not receive an injection were exposed to the same reagents. The same experiment was repeated using human skin. In this case, the possibility of measuring light emission coming from tissue samples was tested.

2.7.6 Experiments with inflamed tissue

The following in vivo studies were not designed to investigate the chemiluminescence response to variations in inflammation and infection levels, that is, the studies were not designed for the development of our device and protocol. However, they provided an opportunity for carrying out preliminary validation experiments using live pig skin samples with inflammation. The study protocol was approved by the MGH Institutional Animal Care and Use Committee. The protocol was a laser study that donated three samples. The first sample was skin at 72 hours after irradiation with a Fractional CO_2 laser (15 mJ at 5% density and 10600 nm wavelength) and a Pulsed Dye Laser (10 mm diameter, 1.5 ms exposure time, 8 mJ/cm^2 fluence, and 595 nm wavelength). The second sample was skin at 72 hours after irradiation with only the Fractional CO_2 laser (15 mJ and 5% density and 10600 nm wavelength). The third sample was non-irradiated normal skin. As before, five microsamples from treated and untreated areas were extracted using the biopsy punch tool shown in Figure 2.10. Also, 6 mm skin biopsies were collected for histology.

2.8 Results

2.8.1 Reagents chemiluminescence

The average chemiluminescence signal as a function of time for 5 different concentrations of luminol and potassium hexacyanoferrate solutions (luminol/potassium hexacyanoferrate %) at a constant 100 μl volume is shown in Figure 2.13a. The area under the curve representing the total average light emission for each concentration is shown in Figure 2.13b. Error bars correspond to the standard deviations of 5 independent experiments. Variations in concentration affect the length of the duration of the chemiluminescence signal. The concentration luminol/potassium hexacyanoferrate 10/90 showed the lowest emission intensity and the shortest time emission. The highest intensity and the longest emission occurred when the concentration of luminol solution increased to 30% and the potassium hexacyanoferrate decreased to 70%. Above 30% luminol, the duration of the emission decreased. Variations in concentration did not affect significantly the intensity of the chemiluminescence signal, regardless of concentration the maximum intensity value was about 9E+6 RLU. In these experiments, the 30/70 concentration of luminol/potassium hexacyanoferrate gave the highest emission intensity and the longest emission duration. the statistical analysis showed that the responses of all other concentrations were significantly different from controls.

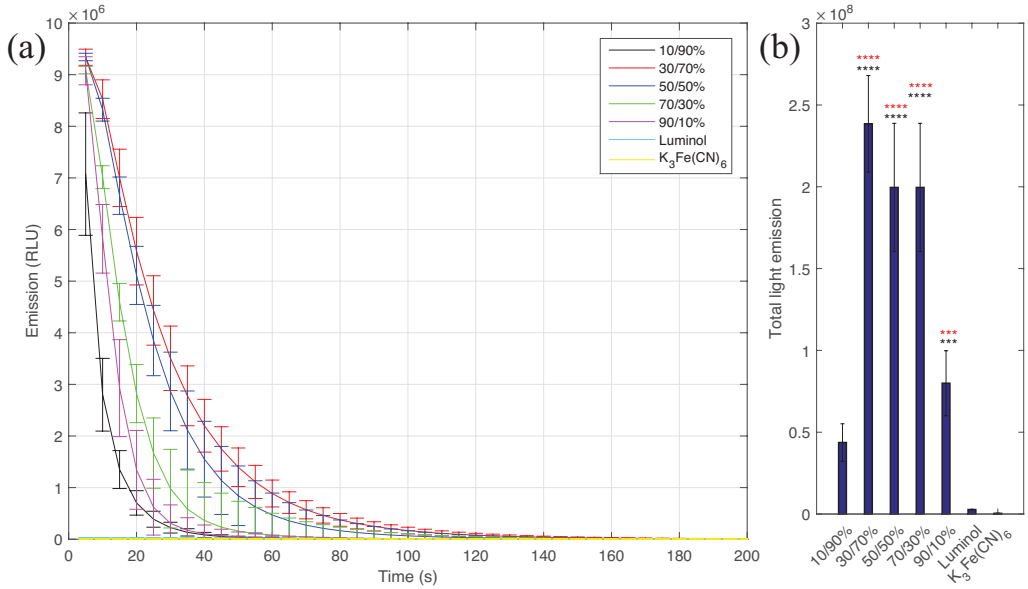


Figure 2.13: Chemiluminescence response of different concentrations of luminol and potassium hexacyanoferrate solutions at a constant volume.

Figure 2.14 shows average chemiluminescence measurements as a function of time and total light emissions for 5 different volumes (40, 60, 80, 100 and 120 μl) at a constant solution concentration of 50% luminol and 50% potassium hexacyanoferrate.

Error bars correspond to the standard deviations of 5 independent experiments. The differences in emission intensity and duration were small when the sample volume increased from 40 to 120 μl. For the volumes used in these experiments the intensity and duration of the chemiluminescence process do not depend on the volume of the sample. The statistical analysis showed that the response of each volume is significantly different from the control measurements, with p <0.0001.

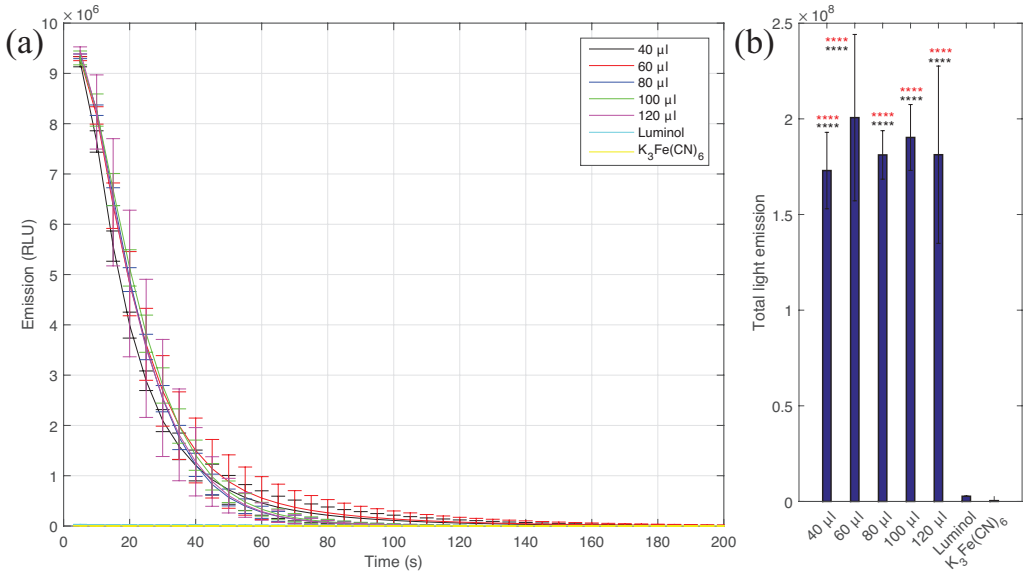


Figure 2.14: Chemiluminescence response of different volumes at a fixed concentration of 50% luminol and 50% potassium hexacyanoferrate.

2.8.2 Neutrophil chemiluminescence

Figure 2.15 shows average chemiluminescence emissions as a function of time and total light emissions for 3 different concentrations of cells activated with PMA: 5,000, 2,500, and 50. Error bars correspond to the standard deviations of 5 independent experiments. As expected, the intensity and duration of the light emissions decreased as the concentration of cells in solution decreased. The system is capable of detecting the light emission produced by the chemiluminescence of activating 50 neutrophil. The statistical analysis showed that responses from all the concentrations are significantly different to the corresponding controls.

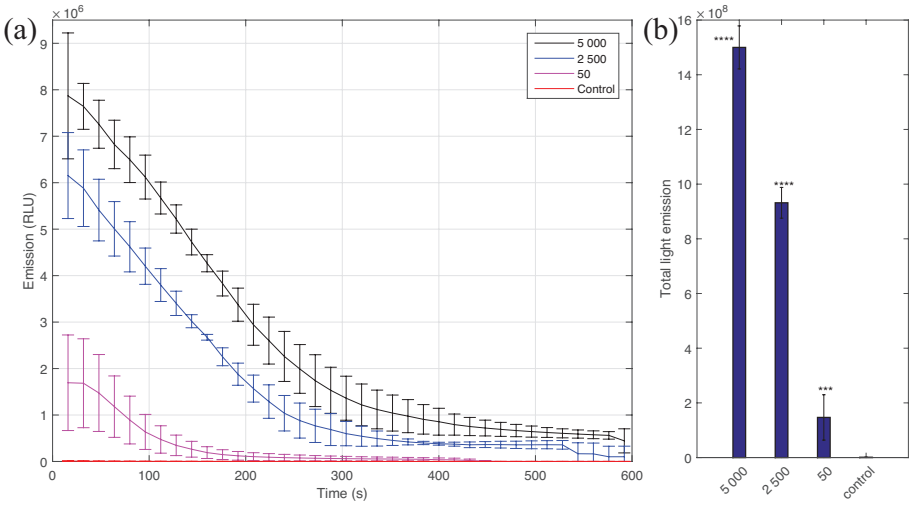


Figure 2.15: Chemiluminescence response of different concentrations of isolated neutrophils activated with PMA in vitro.

2.8.3 Neutrophil chemiluminescence in pig skin

Figure 2.16 shows average chemiluminescence emissions as a function of time and total light emissions of 5 skin microsamples collected from live pig skin injected with neutrophils. The Figure shows chemiluminescence emissions from a control site and two sites injected with different concentrations of cells. Error bars correspond to the standard deviations of 3 independent experiments. The duration of the light emission is similar but the intensity and total light emission decrease with the number of cells. Relative to cells in solution (Figure 2.15), the emission intensity is lower but the duration is longer. The statistical analysis showed that the chemiluminescence response of cells injected into tissue samples is significantly different to control measurements, $p < 0.05$.

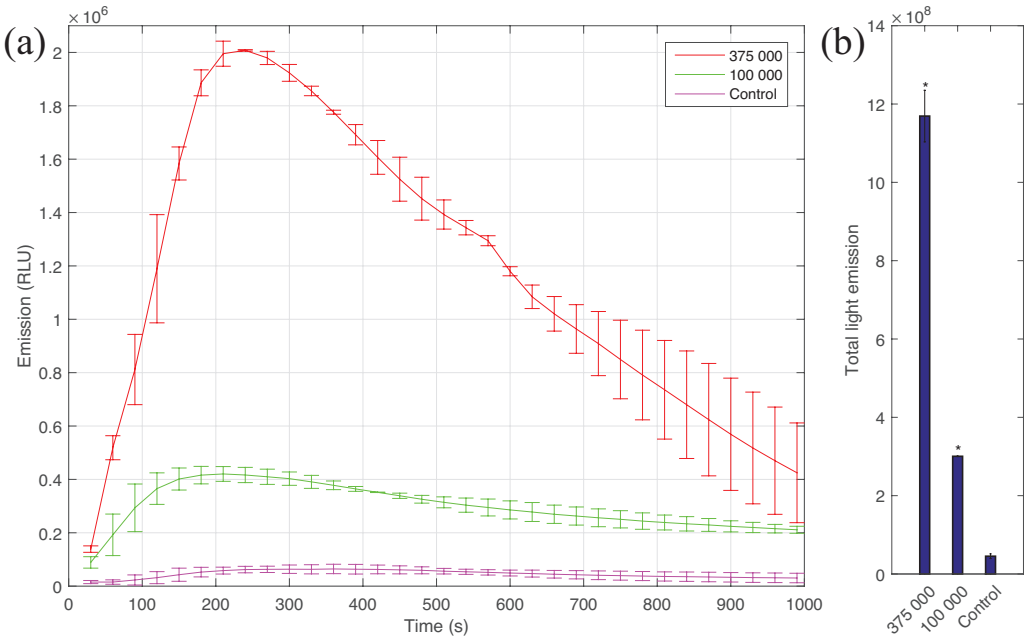


Figure 2.16: Chemiluminescence response of different concentrations of isolated neutrophils injected into pig skin and the corresponding control (no cells injected).

2.8.4 Neutrophil chemiluminescence in human skin

Figure 2.17 shows average chemiluminescence emissions as a function of time and total light emissions of 5 skin microsamples collected from live human skin injected with neutrophils. Total light emissions. Error bars correspond to the standard deviations of 3 independent experiments. As in the experiments with pig skin, the duration of the light emission is similar but the intensity and total light emission decreased with the number of cells. Relative to cells in solution and experiments with pig skin, the emission intensity is lower but the duration is longer. This set of experiments shows that it is feasible to measure chemiluminescence signals in small tissue samples, which suggests that it is possible to apply the proposed methodology to skin with inflammation in vivo and in situ. The statistical analysis showed that the results obtained by injecting 5000 cells into skin are significantly different to control measurements, $p < 0.001$.

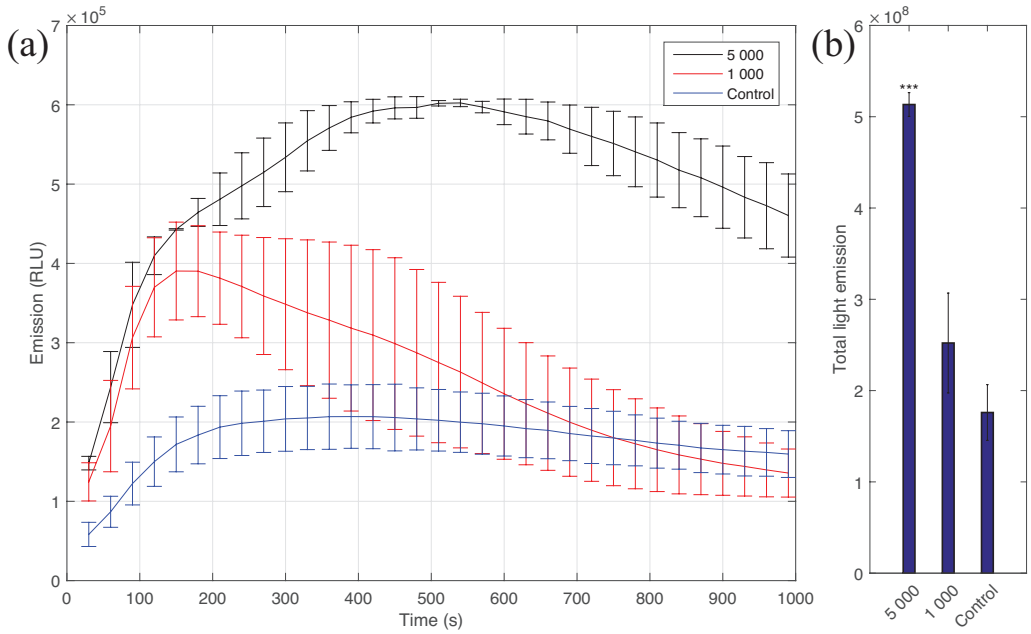


Figure 2.17: Chemiluminescence response of different concentrations of isolated neutrophils injected into human skin and the corresponding control (no cells injected).

2.8.5 Neutrophil chemiluminescence of inflamed skin

Figure 2.18 shows the average chemiluminescence response as a function of time and the total light emission of five pig skin microsamples. The samples were collected from skin exposed to laser irradiation, which elicited an inflammation response, and a control site that was not irradiated. Figure 2.18c corresponds to control tissue (no laser treatment applied). Treatment 1 (Figure 2.18d) corresponds to exposure to irradiation by a CO_2 and PDL lasers. Treatment 2 (Figure 2.18e) corresponds to exposure to irradiation by a CO_2 laser only. Error bars correspond to the standard deviations of 3 sets of samples. Accumulation of neutrophils is indicated by the circles. Scale bar represents 100 μm.

The intensity and total light emission by chemiluminescence of the skin samples with inflammation are higher than those of the samples from the control site. The duration of the light emission is also longer for skin with inflammation. There are considerable differences in the intensity of chemiluminscence between the skin exposed to laser irradiation and the unexposed skin. The results from the histology analysis show the presence of neutrophils at a higher concentration in the skin subjected to a radiation insult, see Figures 2.18d, 2.18e. The statistical analysis showed that the chemiluminescence response for both laser treatments is significantly different to the control, $p < 0.0025$ for T1 and $p < 0.0001$ for T2.

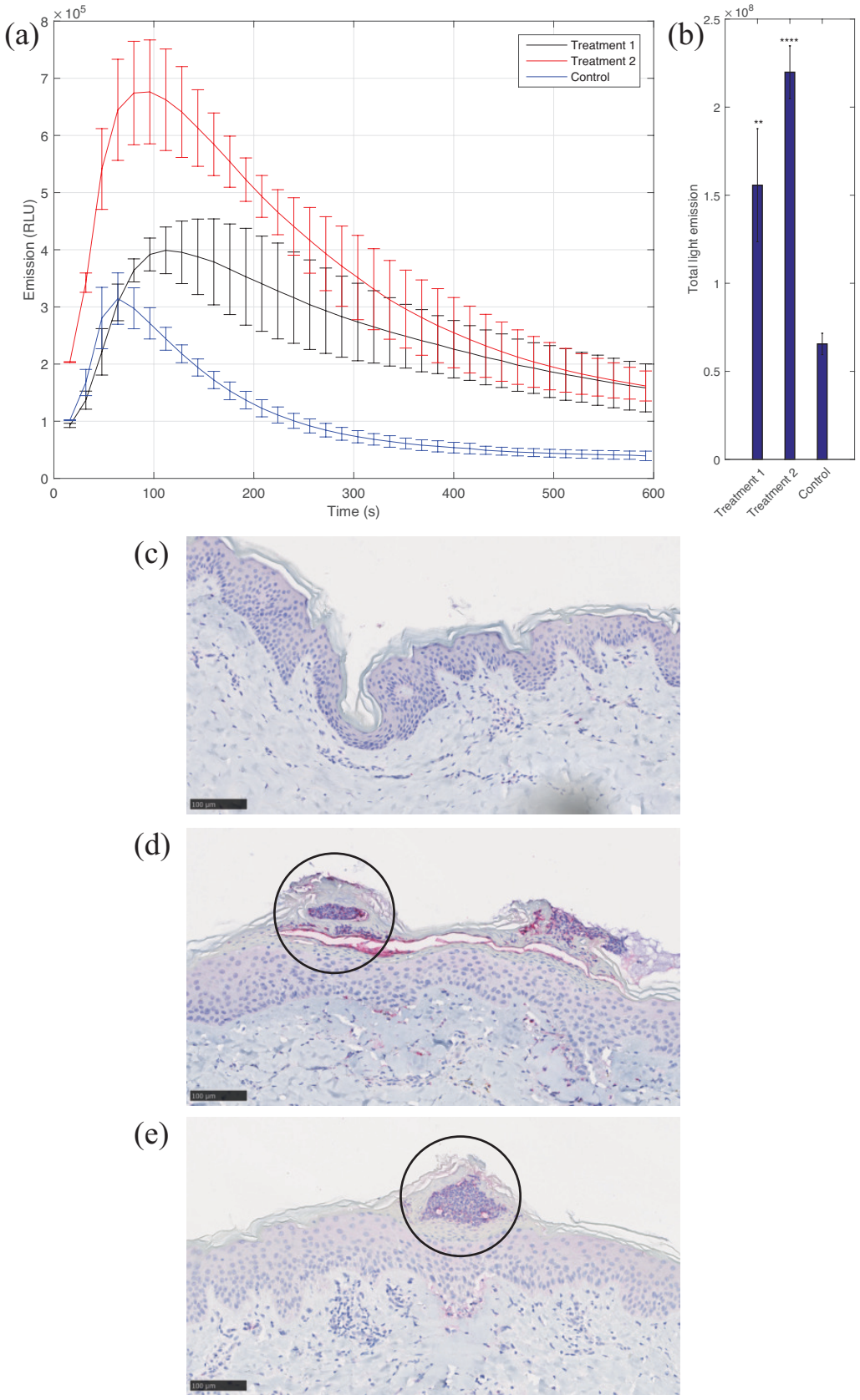


Figure 2.18: Chemiluminescence response in pig skin with and without inflammation.

Figure 2.19 shows a summary of all the design process involved in the development of the chemiluminescence detection system, for revealing the presence of neutrophils.

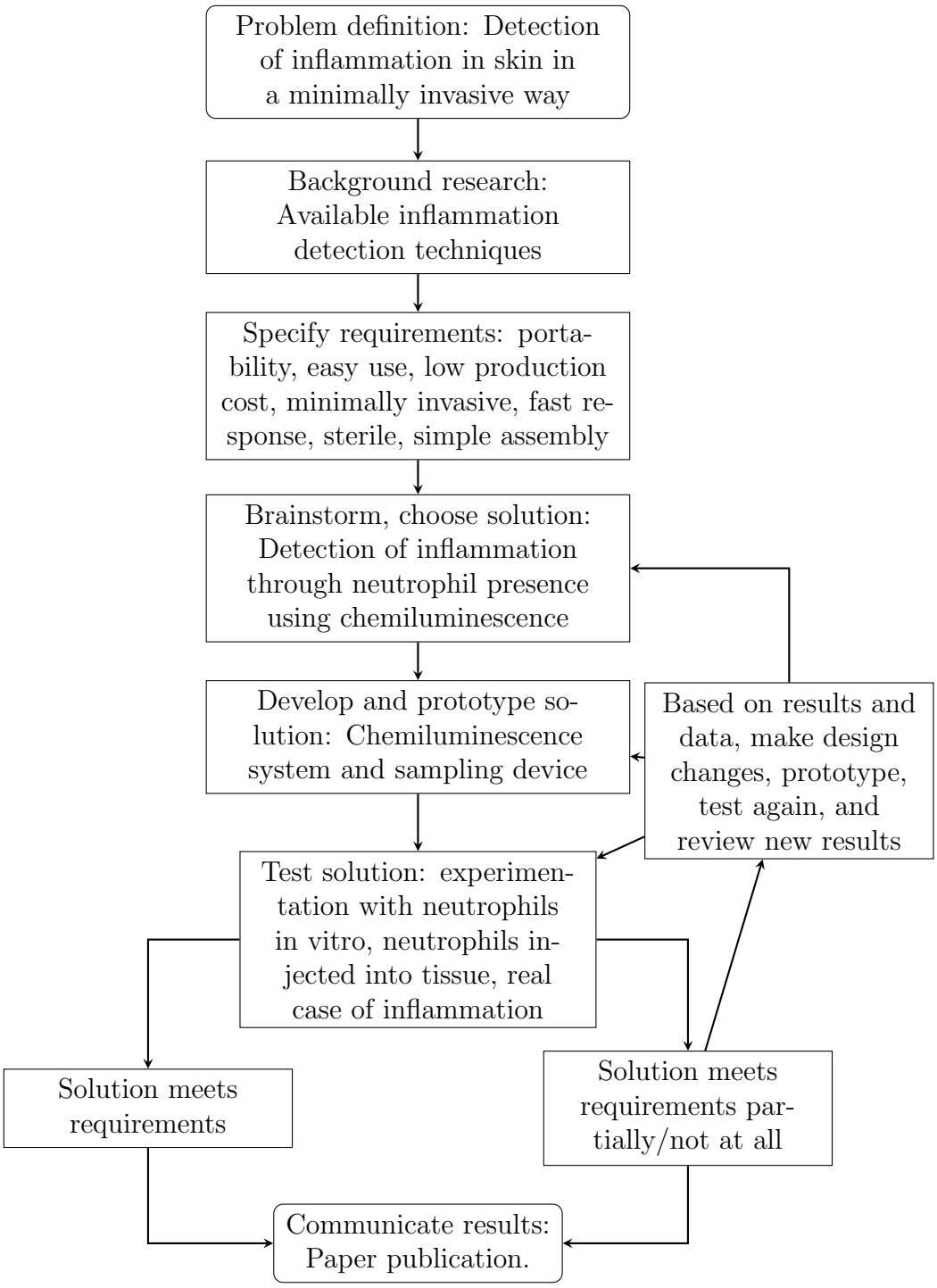


Figure 2.19: Design methodology followed for the design of the chemiluminescence system for detection of inflammation.

2.9 Conclusions

The results of the experimentation using known chemiluminescence reactions (Figures 2.13 and 2.14) show that is possible to measure the light emission of the mixture of chemical components, and the small changes produced by the variation of their concentrations and volumes. This points to the possibility of using the designed system for chemiluminescence reactions in general, in other words, this simple design could replace a luminometer, and offer the advantage of being portable and not bulky as commercial luminometers normally are.

The results obtained form isolated neutrophils in vitro (Figure 2.15) are comparable to what was reported in the literature, since the chemiluminescence response has the same behavior. With those experiments was determined that the designed system is able to measure the activation of at least 50 cells in vitro.

The experiments of isolated neutrophils injected to pig and human skin were used to try to mimic a real inflammation scenario. The results obtained showed what was expected, the chemiluminescence response increases when the neutrophil concentration increases. But still, the difference between neutrophil concentration looks inconsistent. This can be due to the way that the experiments were performed, since is hard to control the place where the cells were injected. Anyways the experiment helped to confirm that measuring light emission coming out of skin micro-samples is possible.

The preliminary results of inflamed skin, show the feasibility of measuring inflammation trough chemiluminescence response. It was also checked with histology analysis. But still, experiment repetition is needed.

With the results obtained, can be concluded that the initial hypothesis for inflammation detection was fulfilled. In other words, the absence or presence of neutrophil activation can be related to inflammation levels (proven with tissue histology). In this case, neutrophil activation was measured with chemiluminescence reactions.

The conventional techniques for inflammation detection, and the new procedures reported in the literature, require long time for sample preparation, tissue or cell culture, specific staining, sophisticated equipment for sample management, special storage components among others, and the analysis could take even days. Most of these facilities are available in clinical laboratories, but not in low resource settings. This is the main difference between the existing and developing techniques, and the proposed procedure for inflammation detection. Considering time consumption, the proposed technique takes no more than 20 minutes, including tissue extraction, reagent preparation, and chemiluminescence measurement which is significantly faster than conventional and developing procedures.

Chapter 3

Design of a smartphone-based fluorescence microscope for detection of pathogens

With the escalating use of smartphone devices in everyday life, there has been rapid growing trend for adapting them into sensing and diagnostic needs related to medical health care [108, 109]. The high level of seamless connectivity, portability and robust functionality integrated on these devices hold high promise in democratizing and decentralizing quality health care [110]. This constitutes a solution for bridging the existing gap between healthcare professionals and patients, especially in rural areas and developing regions that are distant from centralized laboratories [111]. Mobile health (m-Health) aims to implement smartphone-based or integrated wireless technologies to offer primary, video conferences, short and multimedia messaging services or other associated applications [112, 113]. One of the most enabling technologies integrated into smartphones has been the inclusion of portable digital cameras in the form of complementary metal-oxide-semiconductor (CMOS) sensors. These sensors transform electromagnetic waves within the visible spectrum into digital signals, thus enabling the capture and recording of images. Currently, the inclusion of a high definition (HD) camera is generally ubiquitous in the combination with the increasing processing and memory capacity integrated on these devices have allowed to capture digital images/photographs with high resolution.

In principle for m-Health applications, pictures of physical signs can be captured, shared and used remotely for an initial assessment by a physician. By this, the diagnosis can be delivered quickly, and the proper encryption data can not only be efficiently recorded but also instantly shared with healthcare professionals in different locations. Mere physical signs, on most of the cases, are however, insufficient to provide an accurate diagnosis or drive a medical decision. Therefore, additional

routine tests are performed at a centralized clinical laboratory to provide specific analysis that can lead to evidence-based decisions. This is critical for a successful treatment, management and the delivery of an integral and quality health care [114]. However, most of the tests available at the clinical laboratory including microscopy, biochemistry and molecular techniques have an inherent drawback because instruments such as microscopes are commonly delicate, bulky and expensive, thus confining precise clinical diagnostics to centralized and specialized laboratories. The development of portable and integrated solutions for delivering diagnostics at the point of care (POC) has become a cornerstone to decentralize medical care. Despite a number of systems developed and commercialized in the last decade, these systems are seldom used or too expensive to be implemented at the primary care level [111, 115]

Smartphones, albeit not being designed and developed for clinical applications, can be adapted for this purpose using compatible attachments that include the necessary hardware to perform microscopic imaging, and interface with diagnostic tests integrated in lab-on-a-chip devices, as shown in Figure [116, 117, 118]. Parallel advancements within fields of molecular analysis, biosensors, mathematical algorithms, microfabrications, 3D-printing and microfluidics have made possible to adapt smartphones as portable, versatile and highly connected read-out platforms with the capability of capturing the microscopic world ranging from cells and tissues to individual DNA molecules. Smartphone-based diagnostics is thus becoming a promising developing field that enables decentralization and democratization of clinical laboratory tests ad advanced molecular techniques, making the delivery of precise diagnostics in remote areas and limited resource settings practically possible [110].

Based on the advantages that smartphone-based diagnostic devices offer, in the present work, the design of smartphone-based fluorescence microscope for the detection of pathogens is proposed. The steps of the design methodology (described in section 2.1) were followed, and each step is described in detail in the following sections.

3.1 Problem definition

As mentioned in section 1.2, an important factor involved in skin diseases is the presence of pathogens. Fluorescence microscopy is a widely used imaging technique with several applications such as drug detection, cancer diagnosis, and pathogen detection. The drawbacks of fluorescence microscopy are the high cost of fluorescent microscopes and the training required to use them, limiting their use in low-resource settings. A cost-effective, easy to use and to reproduce, and portable fluorescence

system is needed in order to assist the diagnosis of skin diseases in low-resource settings.

In order to develop a low cost fluorescence system for pathogen detection, the main concepts of interaction between light and matter were revised in the literature. They are discussed in the following section, which corresponds to the second step in the design methodology (background research).

3.2 Background research: Light-matter interaction

Light can be described as either an electro-magnetic wave or a stream of photons. At its simplest, the wave theory of light allows to describe aspects of light related to the propagation of light as it us affected by mirrors, lenses, or other objects. On the other hand, the particle theory of light allows to understand the quantum nature of light as seen when the absorption of light increases the energy in a system by an amount which is wavelength dependent not radiance-exposure dependent [119].

The two theories are related in that the energy of a photon is proportional to the frequency of the associated wave, namely:

$$E = hv \tag{3.1}$$

where E is the photon energy, h is the Planck's constant ($6.62x10^{-34}Js = 4.14x10^{-15}eVs$), and v is the frequency of the wave (which is related to the wavelength, λ, by the speed of light, c; specifically, $c = v\lambda$). Beyond the simplest explanations, both (wave and particle) come with their own set of insights about the nature of light and the interactions of light with materials. Understanding basic light-matter interactions requires the use of both views [119].

Should be noted that the linkage between wave and particle theory of light leads to a simple observation: as the wavelength of light increases, the photon energy decreases. This decrease is important because high energy photons can drive chemical reactions than lower energy photons cannot.

The interaction of light with any material is dependent upon the properties of the incident light and the optical properties of the material. These properties control the propagation of light within, back from and through the material as well as any absorption of the light.

The interaction of light with biological tissues is a complex process because the constituent tissue materials are multilayered, multicomponent, and optically inhomogeneous [16]. As shown in figure 3.1, the basic effects of light-tissue interactions include reflection at a material interface, refraction when light enters a tissue struc-

ture that has a different refractive index, absorption of photo energy by the material, and multiple scattering of photons in the material.

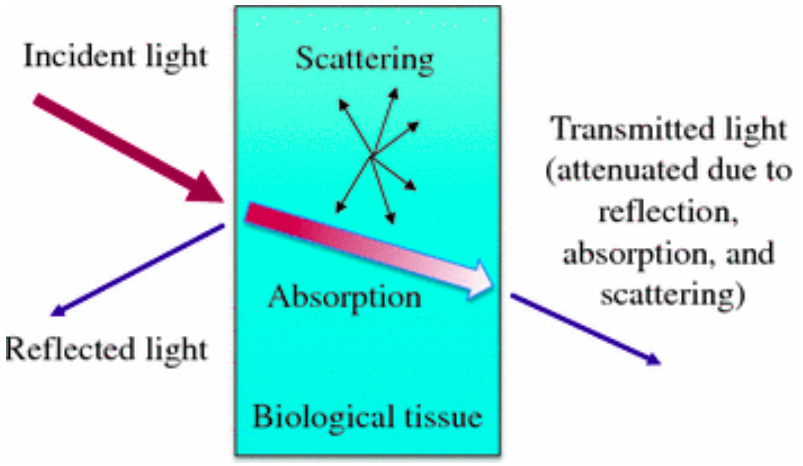


Figure 3.1: Basic effects of light-tissue interactions [16].

Atoms and molecules contain electrons with certain natural frequency, at which they tend to vibrate. When light with the same natural frequency impinges upon an atom, then the electrons of that atom will be set into vibrational motion, in other words, the electrons will absorb the energy of the light and transform it into vibrational motion. During its vibration, the electrons interact with neighboring atoms in such a manner as to convert its vibrational energy into thermal energy. Some light is scatter in all directions when it hits very small particles as gas molecules or much larger particles, the amount of scattering depends on how big the particle is compared to the wavelength of light that is hitting it.

Reflection and transmission of light occur because the frequencies of the light do not match the natural frequencies of vibration of the objects. Then, instead of vibrating at large amplitude, the electrons vibrate for brief periods of time with small amplitudes of vibration; then the energy is reemitted as a light wave. If the surface is smooth, the light will reflect at the same angle as it hits the surface, this is called specular reflection. For a rough surface, reflected light rays scatter in all directions, this is called diffuse reflection. Both cases are shown in figure 3.2. If the object is transparent, then the vibrations of the electrons are passed on to neighboring atoms through the bulk of the material and reemitted on the opposite side of the object.

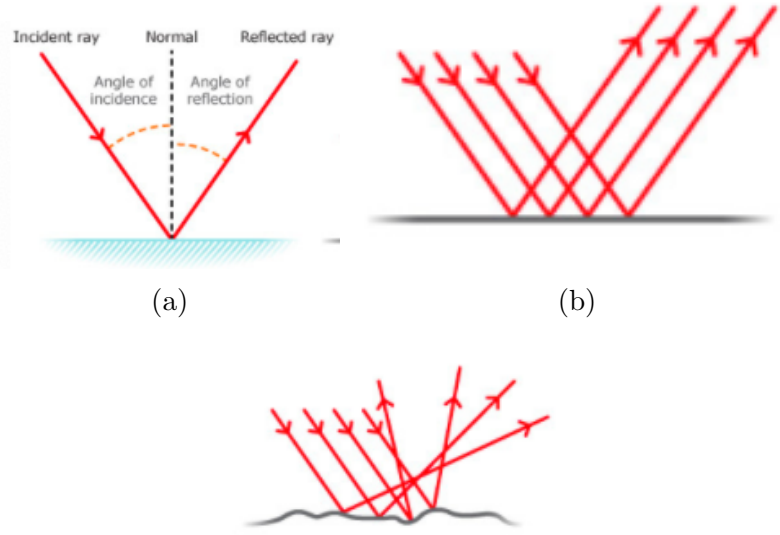


Figure 3.2: Light reflection. a) Reflection of a light ray. b) Specular reflection. c) Diffuse reflection [16].

The major optical parameters of the incident light include the following:

- wavelength of the incident light, equivalent to photon energy.
- power or energy of the incident light.
- spot size, which can either describe the area irradiated or the diameter of the irradiated area.
- irradiance or radiant exposure, where the former is power per unit area and the latter the energy per unit area.
- duration of the irradiation.
- spatial profile, which is related to the spot size; it describes how the irradiance varies across the beam.
- temporal profile, describes how the irradiance varies with time.
- spectral profile, describes the variation of the irradiance as a function of wavelength.

There are several parameters that are used to describe how a material affects the propagation of light within that material:

- the absorption coefficient, describes the absorption of light within the material.
- the scattering coefficient, describes the scatter of light within the material.
- the anisotropy describes the variation in direction in which the light propagates following a scattering event.

Should be noted that each of these material optical properties is generally wavelength dependent. Some of the parameters can be dependent on the irradiance or change over time. Some of the parameters are temperature or pressure dependent. Now that these concepts were introduced, the process of fluorescence is described in the following section.

3.2.1 Fluorescence, and fluorescence microscopy

Fluorescence microscopy is a major tool with which to monitor cell physiology. The underlying process of fluorescence involves the absorption of light energy (a photon) by an indicator followed by the emission of some of this light energy (as another photon) a few nanoseconds later. Because some energy is lost in this process, the emitted photon has less energy than the absorbed photon. Light with a short wavelength (toward the blue) has higher energy that light with a long wavelength (toward the red). Therefore, light emitted from an indicator usually has a longer wavelength than that of the absorbed (excitation) light. This change is called Stokes shift [17]. The molecular transitions explaining these processes can be depicted in terms of Jablonski energy diagrams (Shown in figure 3.3). Excitation (from S_0 to S_1) induced by 488 nm laser light (blue) requires one photon or by two-photon 800 nm light (red) requires two photons. After relaxation to the lowest energy levels, the reverse transition (from S_1 to S_0) releases a photon of longer wavelength (green). The incidence of photons at 592 nm (STED wavelength) induces the transition from S_1 to high levels of S_0 and suppression of fluorescence.

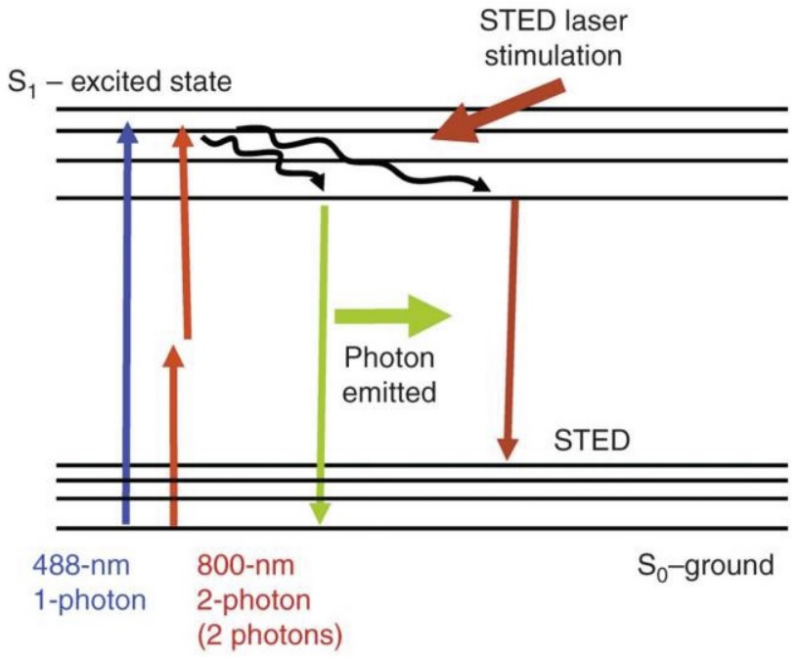


Figure 3.3: Changes in electron state of fluorescence indicators during photons excitation and emission (Jablonski profiles) [17].

One- or two-photon fluorescence

The above scenario applies to the absorption of single photons; each photon absorbed results in the release of a lower energy photon. However, it is also possible for an indicator to simultaneously absorb the energy from multiple photons but only emit one photon. Importantly, the energy of each of the two absorbed photons is less than energy of the emitted photon. Thus, red light can be used to generate green light. This principle underlies "two-photon" or "multiphoton" microscopy. This process can only be achieved by having a very high spatial and temporal density of photons, because the two absorbed photons must arrive simultaneously, which requires a relatively specialized, high-powered, pulsed laser. The pulses serve to package the photons.

Fluorescence light separation

The goal of the fluorescence microscope is to separate emitted light (dim) from excitation light (bright); fluorescence indicators with large Stokes shifts are advantageous for this. The separation of the light is generally achieved with optical filters and the key to successful imaging is their selection with respect to the indicators being used. Figure 3.4 shows the operation diagram of most fluorescence microscopes. An excitation filter cube containing a dichroic mirror (DM 1) directs excitation light (from a bulb or laser; filters with an excitation filter) to the specimen and passes emitted fluorescence to the emission cube for further separation (multiple indicators). The barrier filter B prevents excitation light from reaching the detectors. Emitted fluorescence is separated by DM 2 into two beams. The emission filter (Em 1 and Em 2) block unwanted light. Fluorescence is detected by cameras (wide-field) or PMTs (laser-scanning). For transmitted illumination of a bright-field image, long wavelength light is selected.

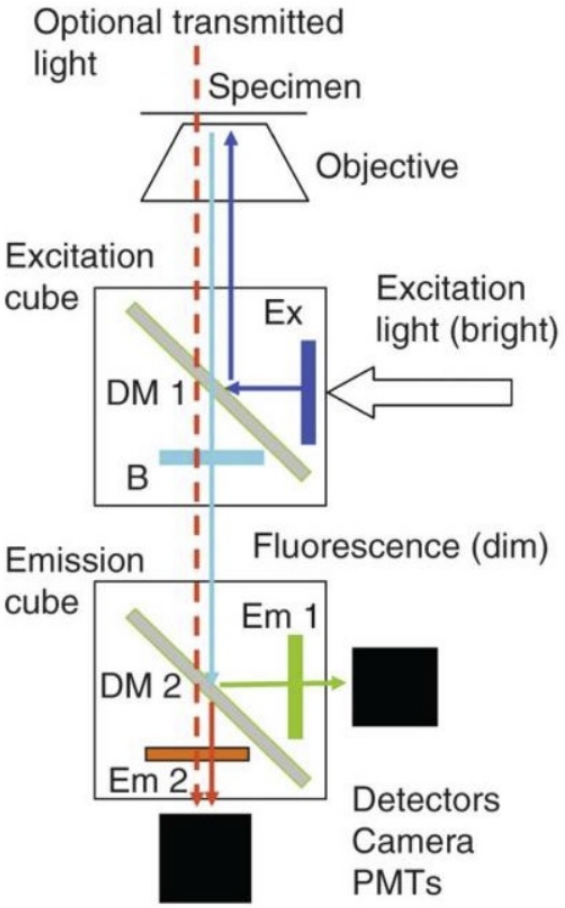


Figure 3.4: The basic light paths of a fluorescence microscope [17].

Although it is often reported that a particular indicator has a single absorption and emission wavelength (a maximum peak value is often cited in the indicator specifications), in reality indicators, especially genetically encoded fluorescent proteins, have broad spectrum of excitation and emission wavelength. Moreover, the excitation and emission spectra of different indicators frequently overlap, thereby increasing the importance of considering the appropriate excitation and emission when multiplexing indicators. Filter choice is generally a best compromise between passing emitted light and blocking excitation light.

Filter cube In many fluorescence microscopes, the filter cube is the conventional element used for aligning filters in the excitation and emission light path (figure 3.4). The traditional filter cube typically carries an excitation filter, a dichroic mirror at 45°, and a barrier/emission filter; the cube directs light from the excitation source to the specimen and from the specimen to the detector. an important issue when loading filters into the cube is their orientation. Because many filters work by reflected interference, it is important to ensure the filter is correctly orientated in the direction of the light path. In many modern fluorescence imaging systems, excitation and emission filters are not located in a filter cube, but rather in an

automated filter changer where they can be rapidly switched in and out of the light path. In addition, excitation light can be dynamically selected by devices such as monochromator or an acoustooptic modulator, potentially avoiding the need for an excitation filter.

Dichroic mirror Initially, fluorescence microscopy was performed with transmitted light, but because the emitted fluorescence is weak in comparison to the excitation light, it was necessary to view the fluorescence with filters capable of blocking very bright transmitted excitation light. Overall, this scheme was not very practical and a reflected optical design was adopted. With this epi-illumination, the dichroic mirror serves as the primary optical element separating the excitation and emission light. The common mode of operation is that the shorter excitation wavelength is reflected toward the specimen and the longer emitted wavelength is transmitted by the dichroic mirror. However, dichroic mirrors can also be used to reflect longer wavelengths and to transmit shorter wavelengths.

Blocking filters To enhance the selection of appropriate excitation and emission wavelengths in addition to the wavelength discrimination by the dichroic mirror, it is common to add excitation emission filters (shown in figure 3.4) The excitation filter ensures only the required wavelengths are transmitted. Usually a band-pass filter with good blocking of light outside the specified band-pass wavelengths is used. However, a key consideration of fluorescence microscopy is obtaining sufficient light to create a useful image; passing light through filters always results in a loss of intensity. Modern light sources have a high light intensity, minimizing this concern for the excitation filters, but it is an important criterion when assessing the suitability of emission filters. The bandwidth (spectral range that passes through a filter) and percentage of the light transmitted by the filter will determine image brightness. When using a single indicator, there is only one source of fluorescence. Thus, the emission filter needs only to block any spurious excitation light. Here, the choice of a long-pass filter has the advantage that all the light from emission spectrum can be collected. Blocking filters must be especially good for total internal reflection (TIRF) microscopy, because a large fraction of the excitation light is reflected directly back into the objective.

Orientation of dichroic mirrors and filters Often manufacturers indicate the orientation of a filter by either an arrow that should point in the direction of the light propagation direction or some form of marking to indicate the front surface that should face the light source. This minimizes spatially translated reflection from the uncoated side. Orientation of interference filters (filters that operate by reflecting or

transmitting light rather than absorbing light) is not important but in older filters that also incorporated absorptive elements, the reflective elements are best oriented facing high-intensity light sources.

3.2.2 Mobile phone-based biosensing

Early detection and accurate diagnosis are critical for the effective treatment and prevention of diseases. In many cases, diagnosis unfortunately requires costly and time-consuming procedures and instruments, which limit their application and use to centralized settings with relatively advanced infrastructures and well-trained healthcare professionals. Although such resources are readily available in developed countries, the cost of healthcare can still be prohibitive for patients to seek early diagnosis. In developing countries and low-resource environments, however, access to the adequate medical equipment may not even be available. Unfortunately, most infectious diseases are still endemic in developing countries, significantly elevating the need for cost-effective and easy-to-use medical technologies. To address this issues, much research has been devoted to develop diagnostic tools for use at the point of care (POC). While the specific requirements may vary, POC devices should ideally be portable and cost-effective, while also providing reliable and rapid results to enable immediate clinical decisions to be made. The availability of such systems would not only reduce the costs and turn-around times associated with medical testing, but also help provide rapid and reliable healthcare to remote and resource limited settings [120].

Optical imaging techniques, in general, offer a number of important advantages for developing medical devices for diagnosis at the point of care. Optical imaging can provide real-time and high-resolution microscopic and macroscopic information toward rapid and accurate diagnosis. The recent advances in a wide range of optical technologies such as optoelectronics, optical fibers, micro-optics and optical micro-electro-mechanical systems (MEMS) further enable the miniaturization of optical imaging platforms as well as reduction in their costs. Moreover, with the rapid growth of the consumer electronics market in recent years, high-performance imaging devices such as camera phones have become ubiquitous. In addition to being portable, compact and cost-effective, camera phones are also becoming a platform of choice for the development of various POC diagnostic tools.

Light microscopy has been a powerful tool for biomedical research and clinical applications for several countries. Microscopic inspection of specimens (e.g. tissue, sputum, or blood film) is still regarded as the gold standard for diagnosis of many diseases, especially for infectious diseases, such as malaria and tuberculosis (TB). Among various optical microscopy platforms, fluorescence microscopy has particular

importance due to its high sensitivity and specificity. Conventional bright-field and fluorescence microscopes, however, are relatively bulky and costly, impeding their use beyond well-established and advanced laboratory facilities.

Among the major concerns of mobile phone users is health, an area that can greatly benefit from biosensors [121, 122]. A mobile phone contains all the components required for a common analytical reader: the screen, which can act as display and controller; an input to capture a signal, which could work via the camera [123, 124, 125, 126, 127]; ambient light sensors [128] and headphone jacks [129]; memory to store the data; and several wired and wireless (Wi-Fi, Bluetooth, NFC, etc.) connectivity modes. Therefore, considering the billions of mobile-phone users in the world, these phones are an invaluable resource for biosensing. This premise leads us to the emerging "diagnostic communication" technology (DCT).

The data transmission capabilities of mobile phones are important for health applications: for example, through an internet connection, users can access data libraries (e.g. their medical records) or send biometric measurements to health specialists in real time. In addition, connectivity through GPS could enable studies on global health or even environmental monitoring.

Regarding the possibilities for signal measurement, most of the mobile phones currently on the market feature an HD camera than can detect visual stimuli at high resolution and sensitivity, either in solution or on a substrate [123, 124, 125, 126].

Sometimes adapters or other devices need to be connected to the mobile phone, in order to maintain the distance between the camera and the sample constant; to make a dark chamber for fluorescence; or simple to integrate the biosensing process without compromising the phone's portability. However, creating a universal design is challenging, such as mobile phone has a different design and size. One possible solution for this problem is 3D-printing at home. Three-dimensional printers and related materials are becoming more affordable and offering increasingly higher resolution and material strength, thereby enabling ready fabrication of personalized adapters for any mobile phone [130].

Optical-based biosensors are advantageous for their simplicity and low cost. Using these devices, a qualitative response (e.g. Yes/No) can often be gauged by the naked eye, although quantitative measurement requires an optical detector. In fact, the area of quantification is one in which mobile phones are poised to play a decisive role [131, 132, 133, 134, 135, 136].

Light microscopy provides a simple, cost-effective, and vital method for the diagnosis and screening of hematologic and infectious diseases. In may regions in the world, however, the required equipment is either unavailable or insufficiently portable, and operators may not possess adequate training to make full use of the images obtained. Counterintuitively, these same regions are often well served by

mobile phone networks, suggesting the possibility of leveraging portable, camera-enabled mobile phones for diagnostic imaging and telemedicine.

Luminescence and fluorescence detection

Regarding fluorescence detectors, common choices for the source of the excitation light include lasers [137, 125, 138] and LEDs [139, 140]. Lasers have the advantage that their light is monodirectional and powerful, and can penetrate the sample without losing too much signal on the way. However, the laser must remain motionless during the assay and the beam has to be placed perpendicular to the detector (the camera) to avoid reading errors or damaging the sensor. This in turn requires a wider and more complex adapter, one which is often equipped with mirrors to redirect the beam. A representative example of the resolution that lasers can provide for fluorescent techniques can be found in the work of Wei et al [137], who used a mobile phone camera to take images of isolated fluorescent nanoparticles 100 nm in diameter. Alternatively, LEDs, which are less powerful than lasers, can be used without the risk of damaging the camera or the user's eyes. Moreover, LED adapters are simpler, amenable to miniaturization and portable.

According to the reviewed literature, was decided to design a potable, low cost, reproducible, and easy to use phone-based fluorescence microscope. The system requirements are mentioned in the following section:

3.3 Requirements specification

As mentioned before, is expected to be able to use the fluorescence system in low-resource settings, based on this fact, the following requirements have to be satisfied.

- Low cost. Since the prototype is expected to be used in low-resource settings. The difference in price, compared to the commercial fluorescence microscopes should be significant,
- Easy to use. Since the proposed device is not a sophisticated system, the operation of it should be intuitive.
- Resolution of at least 10 μm. Some applications, as cancer detection require this resolution.
- Versatility. Multiple analysis, with different excitation/emission pairs should be able to be performed easily.
- Minimal training required.
- Low maintenance. Since the system is expected to be used in low-resource settings, the components should not need to be replaced frequently.

3.4 Choosing a solution: Design of a phone-based fluorescence microscope for biological applications

Fluorescence microscopy is a widely used imaging technique with several applications from drug detection to cancer diagnosis, depending on the targeted fluorescing entity. The drawbacks of fluorescence microscopy are the high cost of fluorescent microscopes and the training required to use them, limiting their use in low-resource settings.

In this work, a simple monolithic design for a portable, easily printed, low cost attachment to convert a smartphone to a fluorescence detector was developed. Compact and readily available off-the-shelf optics and power source to simplify the reproduction of the device were used. The use of this instrument does not require any modification or tuning, making it easy to use in resource poor settings that may lack trained personnel. The imaging performance of this device was evaluated using a USAF resolution target and fluorescence microparticles. The feasibility of using this device for detection of weak fluorescence intensity has also been assessed. The proposed configuration is shown in Figure 3.5.

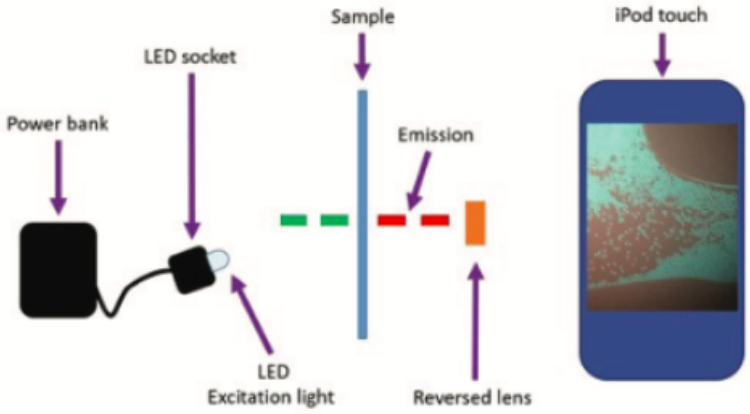


Figure 3.5: Basic operational scheme of the phone-based fluorescence microscope and integrated components.

3.5 Development and prototyping

The main components of the device and the operation principle are illustrated in the figure 3.5. It consists of an iPod® touch 6th generation for image acquisition, a reversed lens (exactly the same lens than the iPod® 6th generation has, but placed in opposite position) which reduces aberrations that ball lens could produce, a USB-powered LED mount that provides power and a mounting socket for LEDs within a

single compact housing, a power bank that serves as power supply for the iPod® and the LED mounting socket, and a channel that connects the surface of the sample and the LED.

The USB-powered LED mount has a 5V input voltage and is compatible with any two-pin through hole LED, which facilitates the replacement of excitation light for different assays. The mount is powered via a micro-B USB port on the back of the mount. The power bank has a capacity of 5000 mAh, and two-port output, one to power the LED mount and the other for the iPod® in case it is out of charge. This power bank also has a display that shows the remaining charge capacity.

The concept of using the exact same lens of the smartphone in a reversed position for the elimination of aberrations was introduced by Switz et al [141]. This option provides good resolution and magnification (20x) with simple and accessible components. An important fact in this case, is that the reversed lens has to be positioned exactly at its focal distance (3.3 mm) away from the iPod® camera, in order to ensure the elimination of aberrations and to provide sharp pictures. Due to this reason, the prototype has to be produced with a method capable of provide fraction of millimeter of resolution as 3D printing.

In order to fix the position of each component a structure was designed using SolidWorks (Figure 3.6) and printed in F123 ASA plastic, which along with ABS, is one of the most common materials used for 3D printing. In some cases depending on the sample, the light intensity produced by the USB-powered LED mount is high enough to saturate the camera. There are several ways to accomplish the light intensity reduction, as the addition of neutral density filters, iris diaphragms, or an electrical circuit with a variable resistance to reduce the current delivered to the socket. But all these options increase the final cost of the prototype. As shown in figure 3.6b, the structure has a channel that connects the top of the LED with the surface of the sample, the light absorption along the channel reduces the light intensity delivered to the sample in order to avoid the camera saturation in a simpler and cheaper way. The structure has a rectangular compartment for the placement of the power bank, and one support in each corner of the prototype, one of them is hollow and is where the LED and LED socket mount are located inside of it.

For the placement of the reversed lens, the printing of a small ring is needed (Figure 3.6c). The lens is located inside the ring to facilitate its manipulation, and placed in the cylindrical compartment in the main structure. This ring is positioned right at 3.3 mm from iPod® camera.

The prototype was built using a F170 stratasys 3D printer, the volume of support material needed is 3.108 in^3 and 14.662 in^3 of model material, using a slice height of 0.01 in the printing time is 6 hours and 38 minutes. Once the prototype is printed, it has to be placed into a bath with sodium solution at high temperature in order

to remove the support material and to provide a functional prototype, this process takes around 5 hours.

In this design all the components are fixed, the only movable element is the sample under analysis. The section where the sample is inserted is big enough to observe the entire microscope slide. The slide is moved manually.

The most important parameter of this structure is the distance between reversed lens and sample, it should correspond to the focal distance of the iPod® camera lens, if the sample is not positioned at this focal distance, aberrations will be present. Due to this reason a 3D printer with the capability of printing layer of 0.254 mm or less is required.

The material used for 3D printing should be opaque to reduce the light from the surroundings.

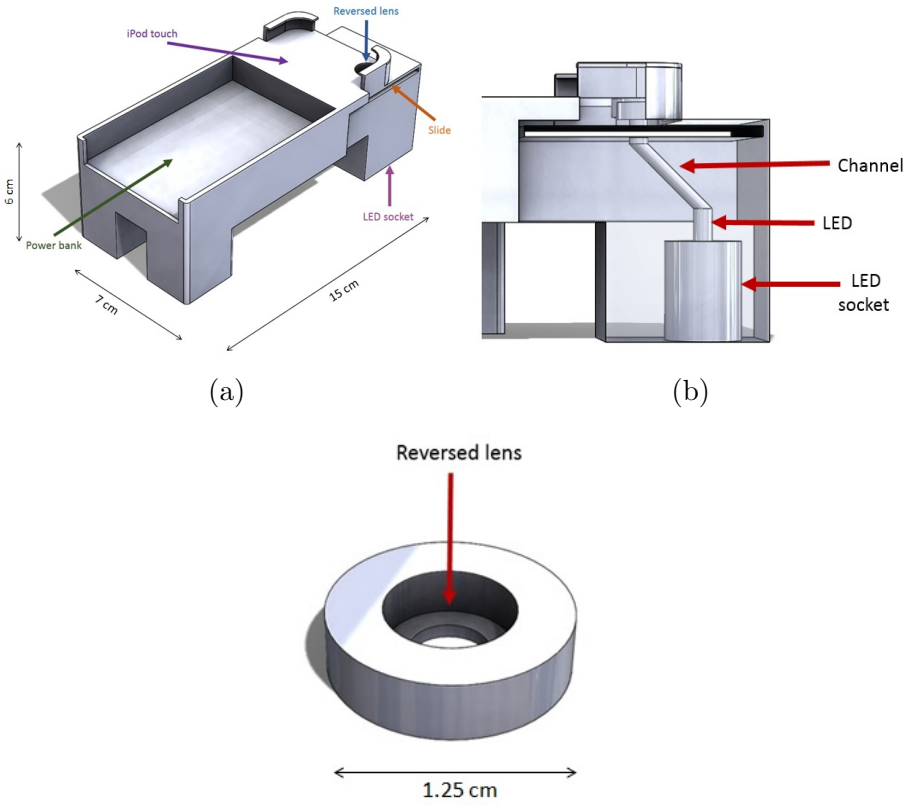
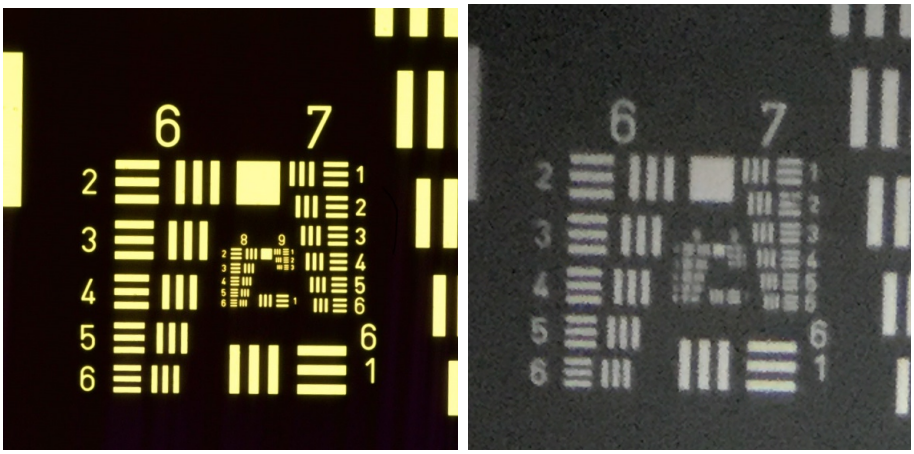


Figure 3.6: 3D Printed prototype and components.

3.5.1 Optical characterization of the system

To determine the resolution of the prototype, a 2" x 2" USAF target (1951 1x Contrast Resolution Target, Edmund Optics, Barrington, NJ) was used. For comparison, pictures were taken with a commercial fluorescence microscope with a 20x

eyepiece (Nikon Eclipse TE2000-S, Nikon Instruments, Mellville, NY), and the prototype, as shown in Figure 3.7.



(a) (b)

Figure 3.7: Resolution test, using USAF target. a) Using a commercial microscope with 20x eyepiece. b) Using the designed prototype.

The resolution target has horizontal and vertical bars arranged in "elements" within "groups", with each group comprising six elements. The resolution of an optical system in line-pairs/millimeter (lp/mm) is calculated using Eq. 2.2, and in μm using Eq. 3.2 as follows:

$$\text{Resolution(lp/mm)} = 2^{\left(\text{Group Number}+\frac{\text{Element Number}-1}{6}\right)}, \tag{3.2}$$

$$\text{Resolution}(\mu m) = \frac{1000}{\text{Resolution in lp/mm}}. \tag{3.3}$$

In Eq. 2.2, the group and element numbers represent the location on the target when the alternating black and white bars are just starting to blur together. Figure 3.7(a) shows the image of the USAF target taken with the prototype under brightfield illumination. The white lines start to blur only after group 7, element 2. Using these values, Eq. 2.2 gives us a resolution of 143.7 lp/mm, which when used in Eq. 3.2 gives us a resolution of 6.9 μm. For comparison, the image taken with a standard microscope Figure 3.7(b) gives us a resolution of 512 lp/mm or 1.9 μm.

3.6 Testing and experimentation

In order to test the designed prototype for the detection of fluorescence and resolution, two main types of experiments were performed; with fluorescence microparticles, and with biological samples. Both sets of experiments are described in detail in the following sections.

3.6.1 Fluorescence of microparticles

To test the resolution and capability of the designed system to detect fluorescence, experiments were performed using fluorescent microparticles of 3 different sizes and excitation/emission wavelengths (Table 3.1). For comparison, images were taken using a commercial fluorescence microscope with a 20x eyepiece (Nikon Eclise TE2000-S, Nikon Instruments, Mellville, NY). The fluorescent particles are microsphere suspensions that are internally dyed and feature bright, high-contrast colors. All the microparticle samples were prepared following the next steps:

Sample preparation

1. With a 10 μl pipette, take 5 μl of the fluorescent microparticles to image.
2. Place the particles on a microscope slide.
3. Place a glass cover slide on top of the sample.

The sample was collocated in the slide compartent of the prototype and images were taken. Different LEDs were used as excitation light source, the spectra intensity distribution of each of them is shown in figure 3.8.

Particles	Diameter	Excitation wavelength	Emission wavelength
A	2 μm	365 nm	445 nm
B	3 μm	542 nm	612 nm
C	10 μm	490 nm	525 nm

Table 3.1: Microparticles

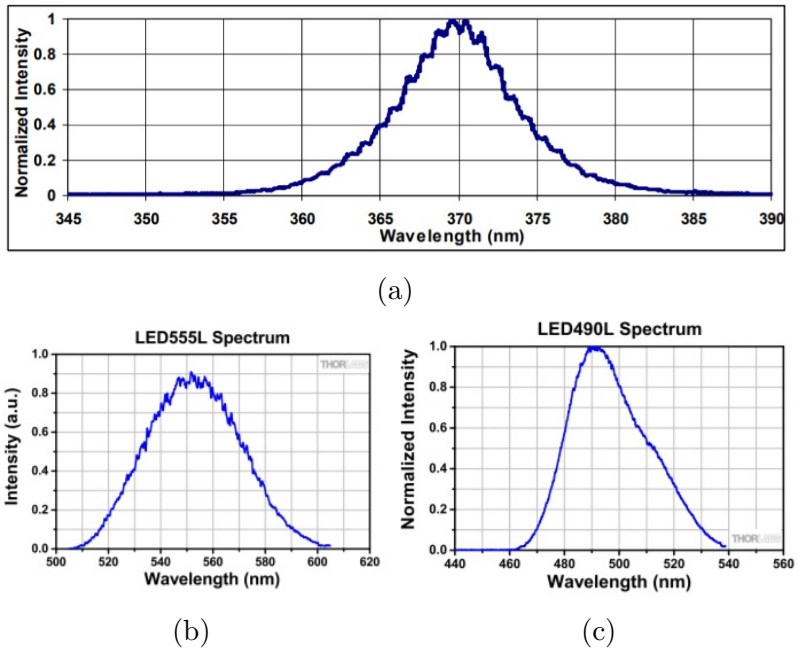


Figure 3.8: Spectral intensity distributions. a) LED used for particles A. b) LED used for particles B. c) LED for particles C [18].

3.6.2 Test with biological samples

The designed system is mainly oriented to be used in biological applications or as a point-of-care tool where the resources are limited. To test the prototype in a more realistic scenario, three experiments were performed using biological samples: blood, tissue and methyl cellulose.

Blood sample

This experiment was performed to test if the resolution of the system was enough to image cells from a blood sample. The Sample was prepared by following the next steps:

Sample preparation

1. Collect a 3 μl sample of blood.
2. Place the sample on a microscope slide.
3. Place a cover slide on top of the sample.

In this case, an excitation light was not needed to image the sample. For comparison, a bright field picture was taken with a commercial digital inverted microscope with a 20x eyepiece.

Tissue sample

a 8 μm thick cryosection of fatty liver from a rat was stained for alpha smooth muscle actin with a standard immunofluorescence protocol. For comparison, pictures were taken using a commercial fluorescence microscope with a 20x eyepiece.

Methyl cellulose sample

One typical medical application of fluorescence assays is the detection of fungal infections in the skin, to demonstrate the feasibility of using this prototype to detect dermatophytes experiments with methyl cellulose were performed. The sample was prepared by following the next steps.

Sample preparation

1. Add 10 gr of methyl cellulose to 1 liter of distilled water.
2. Using a heating magnetic stirrer, get the mixture to boil for 5 - 10 minutes until small amorphous aggregates are formed.
3. Sterilize the solution for 16 minutes at 121 C and 15 psi steam pressure.
4. Allow the solution to stand overnight at room temperature for complete dispersion (the solution will be cloudy but uniform, can be stored at room temperature and used for one year).
5. With a 10 μl pipette, take 5 μl of the methyl cellulose solution.
6. Place the methyl cellulose volume on a microscope slide.
7. With a 10 μl pipette, take 5 μl of calcofluor white stain, and place it on top of the methyl cellulose sample.
8. With a 10 μl pipette, take 5 μl of potassium hydroxide, and place it on top of the sample.
9. Place a cover slide on top of the sample.

The cellulose in methyl cellulose and dermatophytes binds to staining substances such as calcofluor white (CFW) and fluoresces at 430 nm for a 365 nm excitation wavelength. Images were taken using the designed prototype and the commercial fluorescence microscope with a 20x eyepiece.

3.7 Results

3.7.1 Fluorescence of microparticles

The fluorescence images obtained with the designed prototype and commercial fluorescence microscope for microparticles of 3 different sizes and excitation/emission

wavelengths are shown in figure 3.9. Figures 3.9a and 3.9b correspond to particles of 10 μm diameter, 365/445 nm excitation/emission wavelengths. Figures 3.9c and 3.9d correspond to particles of 3 μm diameter, and 542/612 nm excitation/emission wavelengths. Figures 3.9e and 3.9f correspond to particles of 2μm diameter, and 490/525 nm excitation/emission wavelengths. The pictures in the right column were taken using the commercial fluorescence microscope with a 20x eyepiece. Pictures in the left column were obtained with the designed prototype, and making use of all the digital zoom provided by the iPod camera.

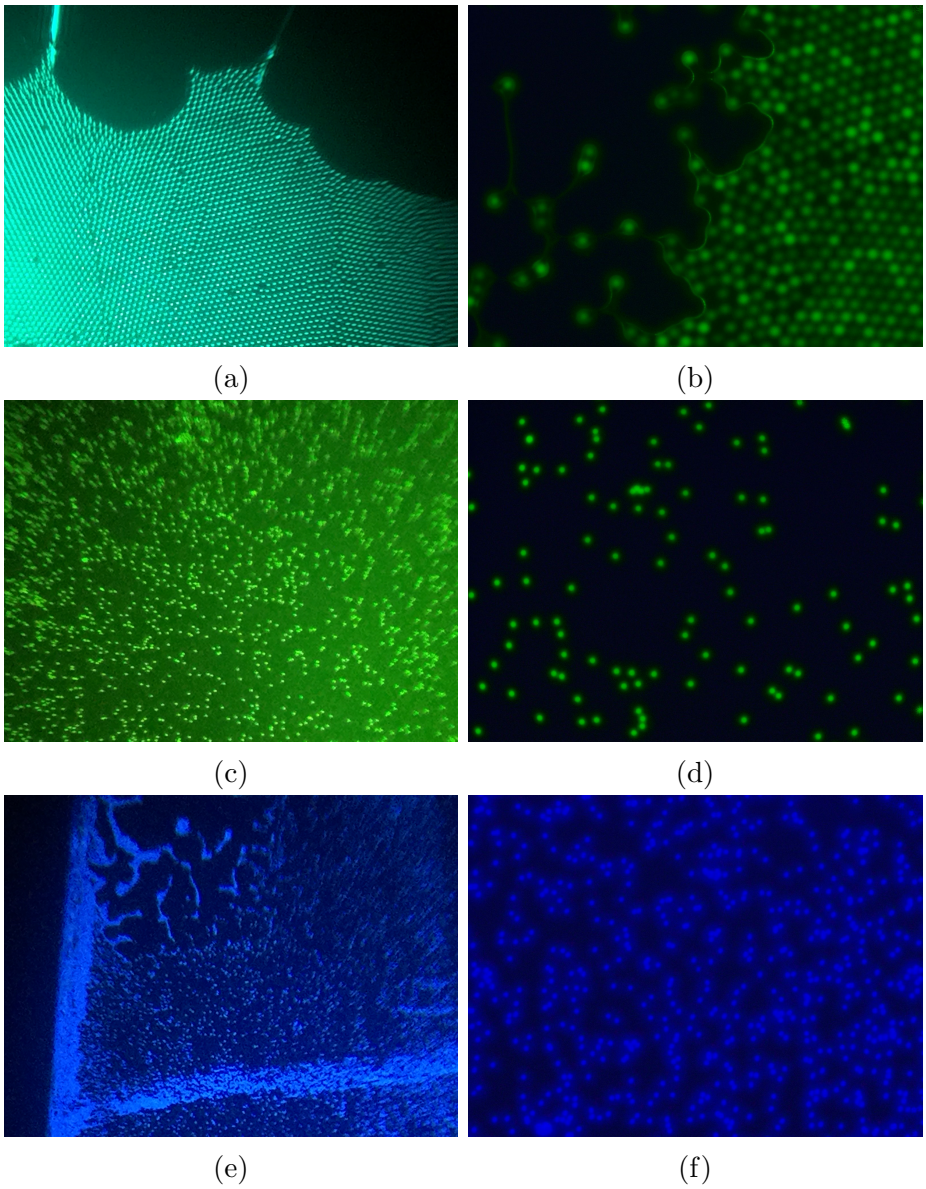


Figure 3.9: Fluorescence test for microparticles with different sizes and excitation/emission wavelengths.

3.7.2 Test with biological samples

The images of biological samples obtained with the designed prototype and commercial microscopes are shown in figure 3.10. Figures 3.10a and 3.10b correspond to a 10 μl blood sample. Figures 3.10c and 3.10d correspond to a methyl cellulose sample stained with calcofluor white. Figures 3.10e and 3.10f correspond to a fatty liver cryosection with standard immunofluorescence staining. The pictures in the left column were taken with the prototype, making use of all the digital zoom provided by the iPod camera. The images in the right column were acquired with a commercial fluorescence microscope and a commercial digital inverted microscope with 20x eyepieces.

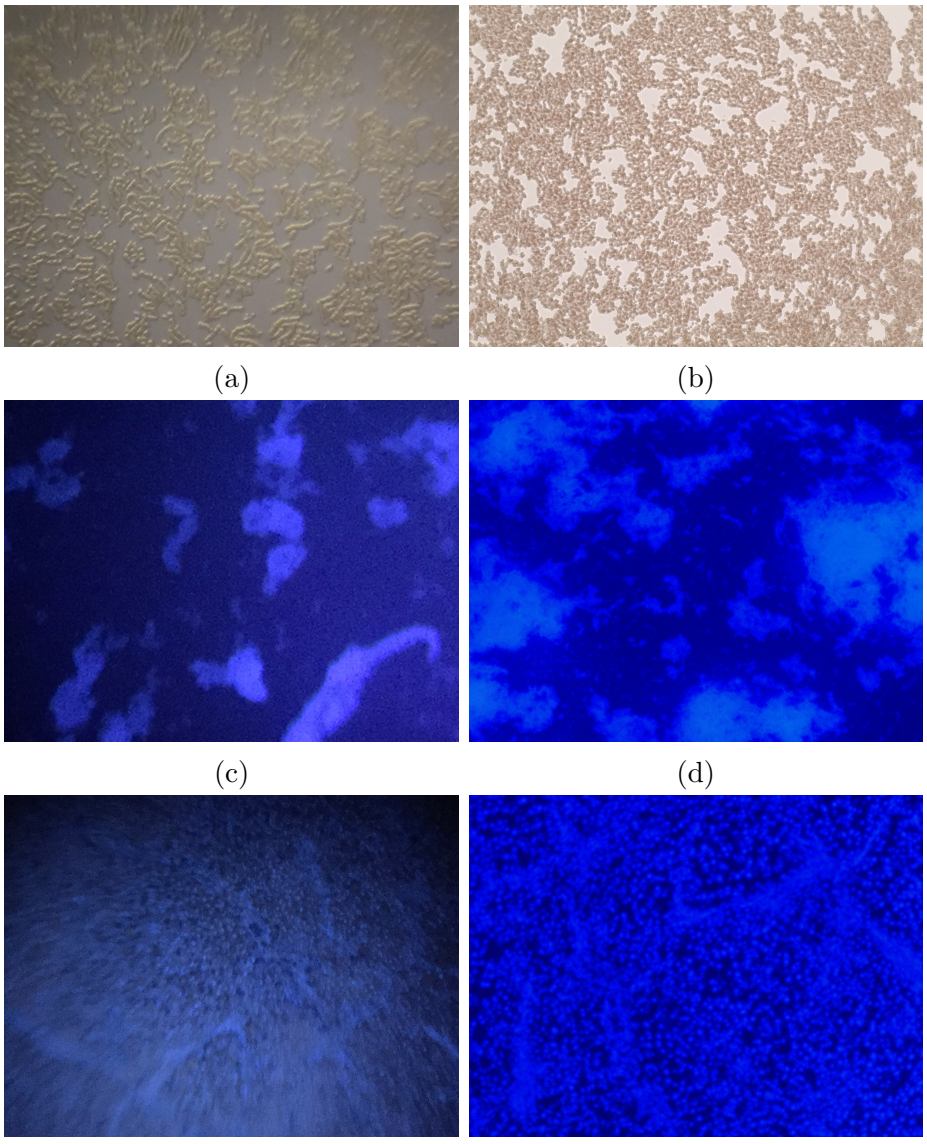


Figure 3.10: Comparison between images acquired with the designed prototype and commercial microscopes for biological samples.

3.8 Limitations

In some cases, depending on the sample, the application, and the excitation/emission peaks, fluorescence can be hard to differentiate from light absorption. In other words, in some applications, the interpretation of the results would depend on the experience of the user. An example of these situations is presented below.

A set of experiments were performed in the "Basic Science Laboratory" at the Stomatology school of Universidad Autónoma de San Luis Potosí. The experiments consisted of the extraction of epithelial cells from a volunteer's mouth. Two samples of cells were taken using disposable and sterile dental brushes, by swabbing the inside of the cheeks. After the sampling, the brushes were washed in PBS solution in an Bio Plas tube and left for 5 minutes for letting the cells to sediment. 2 drops of acridine were added to one of the Bio Plas tube. Acridine attaches to the nuclei of the epithelial cells, and fluoresces at 525 nm under 505 nm light excitation. As the other sample was not stained with acridine, it was kept as a negative control (without fluorescence).

The excitation/emission spectra of acridine is shown in figure 3.11.

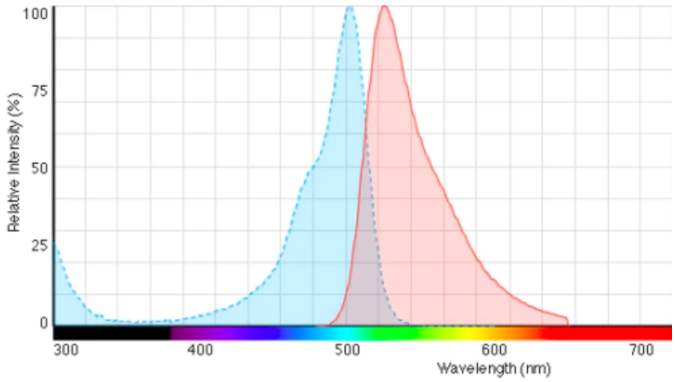


Figure 3.11: Excitation/Emission spectra of Acridine [19].

A sample was taken from each Bio Plas tubes to be analized under microscope. In this case, a commercial microscope was used to acquire images. To add the fluorescence response, the samples were irradiated with a LED, its emission spectra is shown in Figure 3.12.

From figures 3.11 and 3.12 can be noticed that the emission peak of the LED used as excitation wavelength does not match with the excitation peak of acridine. Due to this reason, low fluorescence should be expected.

Figure 3.13 shows the pictures obtained from samples with/without the addition of acridine. In figures 3.13a and 3.13c the nuclei of the epithelial cells can be observed as dark dots. After LED irradiation on the stained sample, those nuclei should fluoresce, but instead, they look even darker, and some other areas look bright.

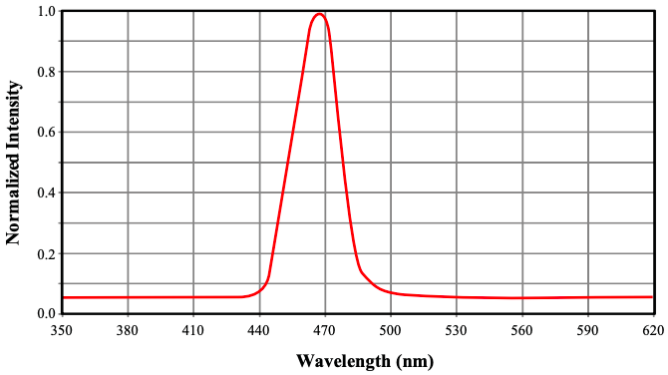


Figure 3.12: Emission spectra of the LED used as excitation source for epithelial cells analysis [18].

Due to this reason, was determined that the blue dots can not be a fluorescence response, which actually is confirmed with in the figure 3.13d. As the sample used for figures 3.13c and 3.13d was not stained with acridine, the sample should look completely dark, but instead, some areas look bright, which could be confused with fluorescence.

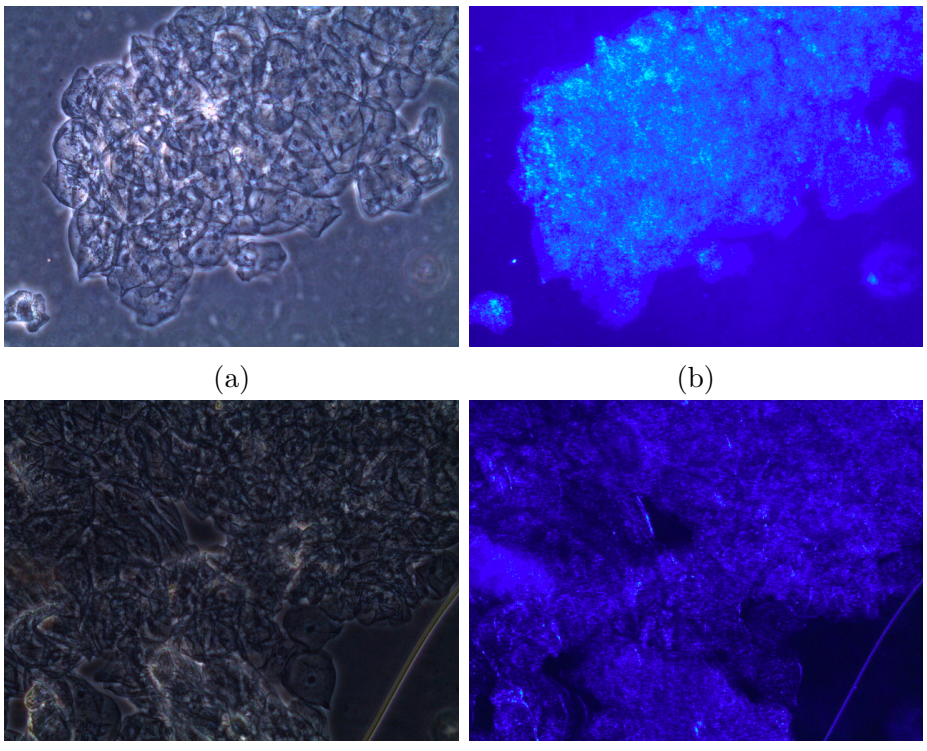


Figure 3.13: Fluorescence response from epithelial cells. a) Bright field picture of epithelial cells (acridine added). b) Picture of epithelial cells irradiated with LED (acridine added). c) Bright field picture of epithelial cells (no acridine added). d) Picture of epithelial cells irradiated with LED (no acridine added).

With the results obtained from the experiments described previously, can be concluded that the addition of optical filters should be considered depending on the application and the excitation/emission peaks. By using a filter to exclude all wavelengths different from the emission spectra, the fluorescence response could be easily confirmed.

3.9 Redesign

As shown in the previous section, the results obtained when the excitation light is different than UV, optical filters are needed, in order to visualize just fluorescence emission. As mentioned in section 3.5, with a reversed lens configuration, the lens must be placed 3.3 mm away from the iPod camera and the surface of the sample. Which eliminates the possibility of filter addition (space limitation). Due to this reason, the replacement of reversed lens configuration with a commercial microscope objective is proposed. This modification would increase the distance between lens and sample, or camera and lens, but also the resolution of the system, and it would reduce the presence of optical aberrations.

During the experimentation, was noticed that, depending on the magnification and size of the sample, small and controlled slide displacements are needed. To include that function in the design, the addition of a manual linear stage, and a xy microscope stage (shown inf Figure 3.14) is proposed.

(a) (b)

Figure 3.14: Commercial components considered for slide displacement [18].

3.10 Testing and experimentation

Considering the addition of all the components mentioned in the previous section, fluorescence assays with excitation different than UV could be performed. In order to test the feasibility of the concept experiments were performed.

3.10.1 Fluorescence of epithelial cells

In order to determine if is possible to detect fluorescence in an assay where the excitation wavelength is different than UV, experiments were performed with epithelial cells stained with ethidium bromide. An emission filter was acquired in order to eliminate the wavelengths that do not correspond to fluorescence, and was placed in front of objective of a conventional microscope. For sample excitation, an LED was placed in front of the sample.

Sample preparation

Epithelial cells were extracted from a volunteer's mouth. A sample of cells was taken using a disposable and sterile dental brush, by swabbing the inside of the cheeks. After the sampling, the brush was washed in PBS solution in a Bio Plas tube and left for 5 minutes for letting the cells to sediment. Ethidium bromide was added to the sample, it attaches to the nuclei of the epithelial cells, and produces fluorescence according to the spectra shown in Figure 3.15.

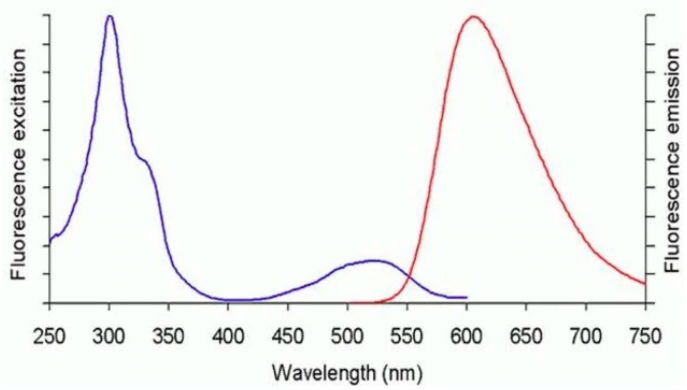


Figure 3.15: Fluorescence spectra of ethidium bromide [19].

After the addition of ethidium bromide a sample of cells was collected on a slide, and a cover glass placed on top of the sample. The sample was excited by using a 525 nm LED, its emission spectra is shown in Figure 3.16. A 600 nm filter was used, in order to eliminate all the wavelengths below 600 nm (Fluorescence emission peak).

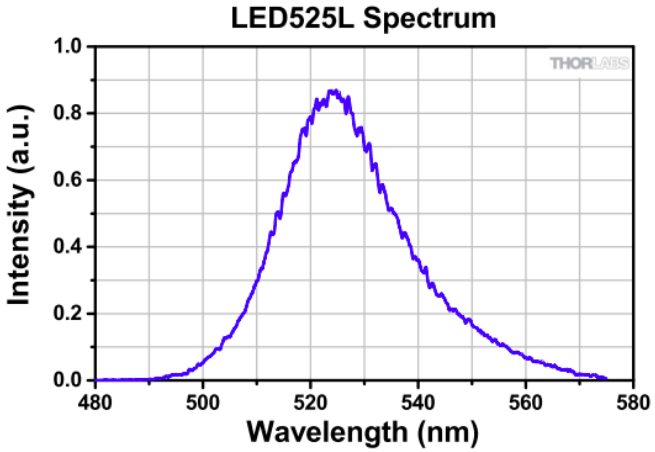
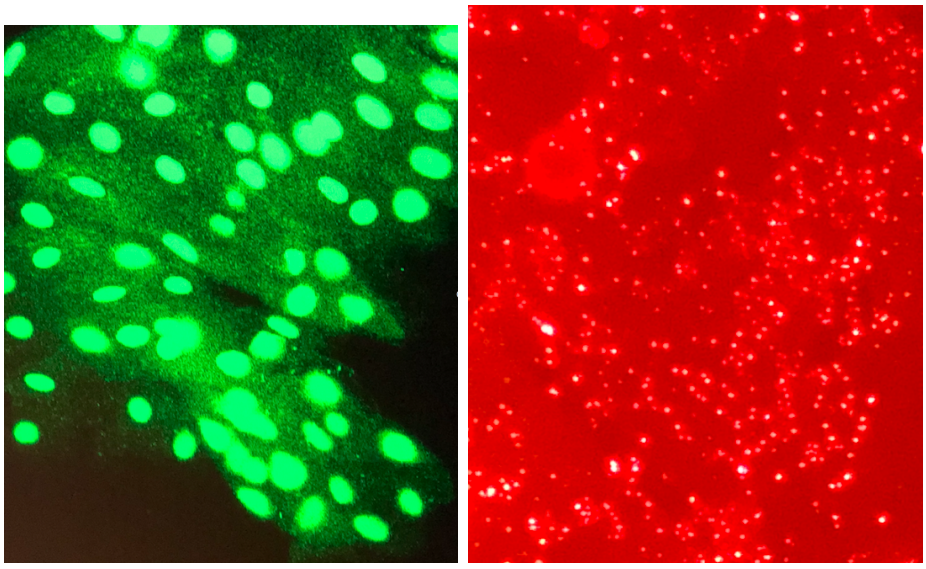


Figure 3.16: Emission spectra of the LED used for epithelial cells stained with ethidium bromide [18].

3.10.2 Results

In order to validate the concept, the obtained images were compared with the performance of a commercial fluorescence microscope. The obtained images are shown in Figure 3.17. Figure 3.17a shows the fluorescence response using a commercial fluorescence microscope, the cell nuclei appear as shiny green dots, than can easily be distinguished from the cell structure. Figure 3.17b shows the fluorescence response using the proposed set up (conventional microscope with adapted filter), even though the magnification is different, shiny red dots can be distinguished, and also cell structures in red color with lower intensity.



(a) (b)

Figure 3.17: Images obtain from epithelial cells stained with ethidium bromide. a) Picture obtained with commercial fluorescence microscope. b) Picture obtained with a conventional microscope with adapted filters.

The results showed that is possible to detect fluorescence in excitation wavelengths different than UV with the implementation of optical filters. Also, is clearly observable that optical aberrations are not presented due to the presence of a microscope objective.

Figure 3.18 shows a summary of all the steps of the design methodology followed for the development of the system for detection of fluorescence.

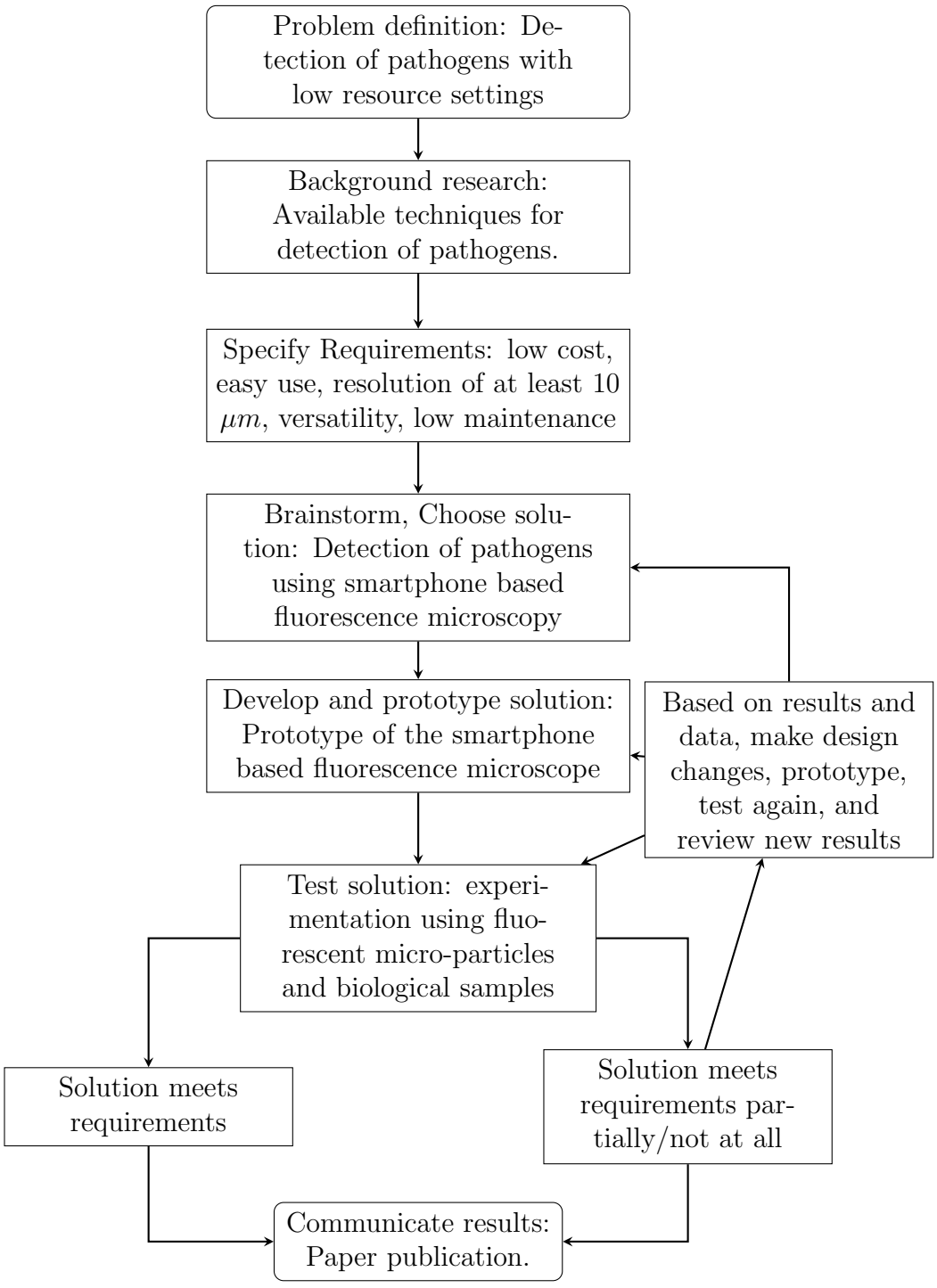


Figure 3.18: Design methodology followed for the design of the system for detection of pathogens through fluorescence microscopy.

3.11 Conclusions

The calculated resolution of the developed system was 6.9 μm, which was enough to image fluorescent particles of 3 μm diameter. The calculations, and the experimentation with particles, suggest the possibility to use the prototype for skin diseases diagnosis.

In the case of the images obtained from epithelial cells, is hard to distinguish the nuclei of the cells, this could be due to the disorganization of the sample, since it was not cultured properly in one layer. Can be observed in Figure 3.13 that the cells are accumulated and disorganized, which complicates the visibility of nuclei.

Is well known that phone-based fluorescence microscopes are not new, nowadays phone-based technology is very common in several applications. The difference between the design presented in this work and the ones found in the literature is the simplicity. Most of the prototypes reported, have complex optical systems, which increases the cost, and complicates the operation and assembly. In this case, the microscope is an attachment that requires minimal assembly and tuning. Several fluorescence assays can be performed with the prototype, just by replacing the LED with the required excitation wavelength.

Another important feature is that this prototype does not include any filter in its optical design. Which works well when the excitation/emission peaks of the samples are far away from each other, but not when they are close, in those cases, the presence of optical filters is going to be needed. All the samples used for the validation of this system have excitation/emission peaks that are not close to each other, so as it can be seen in Figures 3.9 and 3.10, fluorescence is easily distinguished.

Even though the experimentation was not completely successful for applications with excitation different than UV, preliminary results suggest that the hypothesis can be fulfilled with some modifications in the optical design of the prototype (addition of commercial components). Those modifications would increase the final cost of the device, but still that cost would be ten times less than a commercial fluorescence microscope cost.

As mention before, there are several specific techniques for pathogen identification and detection, but they require long time for sample preparation, or tissue/cell culture, which reduces its accessibility in low resource settings. Fluorescence microscopy is the gold method for pathogen detection, but it requires the use of expensive, complex, and sophisticated equipment, that also limits their use in developing regions. In this case, the design of a portable fluorescence detector bring the possibility of implementation of preliminary fluorescence assays in low resource settings. The main aim of these assays is to trigger an alarm for the performance of specialized studies (as the conventional techniques).

Conclusions

The present work reports the development of a device and procedure to identify the presence of neutrophils in skin. At this stage of development, this is an enabling technology for investigating its utility in different clinical applications — it could also find value as an investigational tool in preclinical models. A concrete example of a clinical application is the diagnosis of cellulitis, which cannot be replicated in animal models. As mentioned in the introduction, cellulitis is a common infection of the skin difficult to diagnose because many inflammatory conditions, termed pseudo-cellulitis, present similar symptoms. A feature of cellulitis is heavy neutrophil infiltration around blood vessels, hence, there exists the potential to improve the diagnosis of cellulitis with a differential chemiluminescence test.

The method of sampling microscopic skin tissue for evaluating the presence of neutrophils using luminol-enhanced chemiluminescence exhibits several advantages. The procedure is simple and fast and does not require user specialization to extract a certain number of microbiopsies, place them in a cuvette, add reagents, and record the light emissions. The complete procedure takes about 15 minutes or less and does not require cell isolation or tissue preparation. Isolation of neutrophils from blood can take up to one hour. Herein, chemilumniescence signals were acquired for 5 minutes, Figure 2.18, but a shorter acquisition would also reveal the presence of neutrophils and differences in concentration as the signals peaked and started decaying before 200 s, see Figure 2.18. The method is also relatively inexpensive to implement since the microsample punch tool and the detection chamber were fabricated using additive manufacturing and hypodermic tube grinding processes. The photon counting module is the most expensive component, but the cost is reasonable ($<$ \$1,075.00 dollars) and could potentially decrease by using inexpensive detectors as the signal is strong. The reagents have a long shelf life and are inexpensive and consumed in small volumes. The system is compact in size with a small foootprint, which makes it portable and suitable for point of care diagnostics.

The proposed method is minimally invasive and does not produce scarring. A full-thickness skin biopsy of less than 0.7 mm in diameter stimulates rapid replacement of the lost tissue by remodeling, which results in new skin tissue that is both functionally and aesthetically normal. Stimulating healing by remodeling using mi-

croscopic wounds has been extensively demonstrated in fractional laser procedures for improving photoaged skin and various lesions, like wound scars [142, 143]. As opposed to procedures with standard punch biopsies, stitches are not required to close the small wounds created by the microsample punch tool. In normal skin, the wounds epithelialize or create a barrier closing the wound within a day and the scab covering the wounds falls off by day three. The extraction procedure is less painful because of the cutting geometry and motion of the needle: a quick forward motion as opposed to a rotational one [144]. The extraction and healing characteristics of this microsampling technique could lower the time and number of biopsies thresholds for sampling the skin. Furthermore, our preliminary results show that we can detect chemiluminescence in 5 microsamples, but this number could be reduced by determining the minimum number of samples needed to gather physiological information.

In the experiments with tissue, the time duration and intensity of the chemiluminescence signal were longer and higher, respectively, than those obtained from experiments with neutrophils in vitro. These differences may be related to diffusion and cell concetration. In tissue, the reagents have to diffuse through the skin to reach neutrophils to jump-start the chemical reaction, and the number of cells are presumably less. For the experiments where different concentrations of neutrophils were injected, it is hard to know the cell concentration for each skin microsample. The microsamples were taken randomly from the 25 cm^2 skin area where they were injected.

The experiments with inflamed skin suggest that the device and method are able to detect different levels of inflammation with the extraction of 5 skin microsamples. The light intensity and total light emission were lower in the skin treated with CO_2 and PDL lasers, Figure 2.18. The CO_2 laser causes non-selective partial damage to the skin, operating at 5% irradiation surface implies that 95% of the skin surface was not irradiated. The PDL causes selective but uniform damage within the circular area of the 10 mm irradiation diameter. The PDL operates at 585 nm wavelength, which selectively targets blood vessels. Thus, measurements suggest that less neutrophils were present in the skin treated with both lasers. We are not certain about why less neutrophils are present, however, one hypothesis is that the PDL collapsed the blood vessels affecting cellular transport to the injured site. These results suggest that a simple, fast and minimally invasive chemiluminescence assay could aid in the quantitation of different levels and types of inflammation.

The method has limited specificity and sensitivity. Experiments with cells and tissue required PMA to activate neutrophils and luminol to amplify the signal, without these reagents the system was not able to detect chemiluminescence in control experiments. However, in the presence of bacteria the neutrophils may not need PMA

for activation. The luminol-enhanced chemiluminescence is mainly attributed to neutrophils because these are the most abundant cells that produce chemilluminescence: they represent about 60% of the circulating leukocytes while the eosinophils and basophils, which also produce chemiluminescence, represent about 1%. The production, circulation, and leukocytes ratios change in response to biological threats. These changes are a function of the type of hazard; for example, the production and circulation of lymphocytes increase with viral infections while neutrophils increase with bacterial infections. As evidenced by the chemiluminescence from controls, there is a baseline signal which is probably a combination of the presence of neutrophils and other chemical species able to oxidize luminol. Therefore a reference measurement in skin without inflammation may be necessary to define changes. The results presented herein are limited by the number and type of in vivo experiments with skin with inflammation. However, preliminary results suggest feasibility and warrant further experimentation.

Observations and comments from people with high experience in healthcare environments and tissue biology, the chemiluminescence detection system shows potential to be used in several applications. One of them is NETosis, which is a regulated form of neutrophil cell death that contributes to the host defense against pathogens and its linked to several diseases, such as autoimmune diseases, diabetes, atherosclerosis, and vasculitis [145]. In other words, the designed system could be used with different chemical components to produce a chemiluminescence signal that could be related to the presence of NETosis.

The present work also reports the development of a phone-based fluorescence microscope for the detection of pathogens. It has the advantage of being a simple attachment for a smartphone. It does not need to be adjusted or tuned, which makes it easy to use and requires minimal training. The system is easy to reproduce since the structure is one fixed component, and it does not require major assemblies. It is low cost too, all the components needed are available for a reasonable price, the most expensive component is the structure, this is due to the material needed for 3D printing, which can be reduced by optimizing the current design.

The designed phone-based fluorescence microscope does not require filters, at least for the applications mentioned in this work. Which is a huge advantage in comparison to the similar systems reported in the literature. For applications where the excitation and emission wavelengths are too close to each other, the implementation of filters will be needed. For the applications where the excitation wavelength corresponds to UV, the presence of filters can be discarded, since the iPod camera has already an UV filter.

Although the reversed lens configuration offers a system without the presence of aberrations, the pictures in Figures 3.9 and 3.10 show some, this could be due to

the positioning of the reversed lens, which means that is possible that the lens are not perfectly parallel to the sample or to the iPod camera. This can be corrected by changing the position of the reversed lens.

According to the calculations made, the designed prototype has resolution of 6.9 μm, the commercial fluorescence microscope used for the image comparison has resolution of 1.9 μm (3.6 times higher). But the cost of a commercial fluorescence microscope of that resolution is more than 6 times the cost of production of the presented prototype.

Another advantage of this phone-based fluorescence microscope over others reported in the literature is that, it can be used in low resource settings, since it does not require a fix voltage source. The battery included in the design is enough to feed the iPod and the LED for more than one hour.

The prototype presented in this work can be used for a big range of excitation/emission wavelengths, since the LEDs can be replaced easily, the only restriction in this case, is that the LED has to be two-pin through hole.

The pictures in Figures 3.9 and 3.10 show some background light coming from the LED (noise), this can be due to the intensity of the LED, which can be modified by the inclusion of neutral density filters, or by changing the shape or the size of the channel that conducts the light from the LED to the sample. The images acquired have good resolution and are comparable to the ones obtained with the commercial microscope, but they can be improved in order to implement this system in clinics.

For applications where the location of pathogens is not required, but just the presence or absence of them, the system for chemiluminescence detection could be modified to detect those pathogens by using fluorescence. The changes required to integrate both systems (chemiluminescence and fluorescence) would be minimal, as the addition of an excitation light source, and optical filters to discriminate the responses that do not correspond to fluorescence. With those adjustments, the development of a low cost, portable and integral system for detection of specific cell reactions and presence/absence of pathogens would be achieved.

At this stage of development, the obtained results suggest that is feasible to assist the diagnosis of skin diseases by using the systems presented in this work.

In the following section, the contributions generated by the author of this work are listed.

As mentioned before, most of the techniques for inflammation and pathogen detection require sophisticated equipment, sample preparation, specific staining, special sample storage and management, and special training among others. These features limit their application in low resource settings, and are the main difference compared with the proposed techniques. With the results obtained until now, the designed systems require modifications and more experimentation for validation,

but they show potential to be used in low resource settings for the performance of preliminary assays, that could trigger an alarm for certain diseases, or special clinical analysis. The aim of this work is not to replace a physician, but to provide fast assays in order to facilitate the physician's work.

Contributions

- Design and development of a low cost system for the measurement of chemiluminescence reactions.
- A technique for identification of neutrophil presence in skin samples based on chemiluminescence.
- Design and development of a microbiopsy device for tissue sampling in a minimally invasive way.
- A minimally invasive procedure for assisting the diagnosis of inflammation in skin microsamples through chemiluminescence.
- Design of a low cost phone-based fluorescence microscope with 6.9μm resolution.
- Design and development of two tools for assisting the diagnosis of skin diseases in low-resource settings.
- The publication "Point-of-care detection of neutrophils in live skin microsamples using chemiluminescence" in the Journal of Biophotonics.
- Participation in the Photonics West BIOS 2019 SPIE (Society of Photographic Instrumentation Engineers) conference (February 2019, San Francisco, CA, USA), with the talk "Chemiluminescence detection system for revealing the presence of neutrophils in skin microsamples"
- Participation in the 2019 Annual Meeting of the American Society for Laser Medicine and Surgery (ASLMS) (March 2019, Denver, Colorado, USA) with the talk "Chemiluminescence detection system for revealing the presence of neutrophils in skin microsamples"

All the obtained results were satisfactory, but still some improvements are considered as future work, in order to increase the efficiency of both systems, and to validate the proposed methodology for inflammation diagnosis. Those features are mentioned below.

Future work

- Experimentation with the chemiluminescence system in a inflammation study to validate its capability of minimally invasive detection of different levels of inflammation in skin samples.
- Experimentation or numerical modeling to determine the optical number of tissue samples needed to get a precise measurement.
- Possible integration of chemiluminescence-fluorescence system.
- Numerical modeling to optimize the chemiluminescence reaction.
- Numerical modeling to optimize the light distribution inside the chambers.
- Improve the lens position in the phone-based fluorescence microscope to reduce aberrations.
- Experimentation with the phone-based fluorescence microscope using different biological samples.
- Implementation of filters in order to reduce the light lacking from the excitation light.
- Implementation of changes in the structure for the reduction of material, cost, and printing time.
- Determination of technical specifications for proper performance, as LED power, irradiance, work distances, etc.

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