
Single Fiber Optical Probe for In Vivo Raman Spectroscopy

*Adaptations of a Raman Spectrometer for In Vivo Use Aiming at
Diagnosis of Skin Cancer*

BACHELOR THESIS FOR APPLIED PHYSICS
THE HAGUE UNIVERSITY OF APPLIED SCIENCES
DEPARTMENT OF DERMATOLOGY
CENTER FOR OPTICAL DIAGNOSTICS AND THERAPY
ERASMUS MEDICAL CENTER, ROTTERDAM
M. JANSEN – 12120073 – JUNE 2016
COMPANY COACH: I. P. SANTOS



Preface

This thesis was written as a part of the final project for the degree of Bachelor in Applied Physics, for the Hague University of Applied Sciences.

The project was performed in the Raman spectroscopy group of the Center of Optical Diagnostics and Therapy (CODT) at the Erasmus University Medical Center.

I would like to thank Inês and Elisa for guiding me through the internship, creating a fun and motivating environment and for working with me to finalize the thesis.

Abstract

Raman spectroscopy is an optical technique that is based on the inelastic scattering of light. It is non-invasive, fast, objective and easy to use. These traits make it an ideal tool for use in medical diagnosis.

Melanoma is the deadliest form of skin cancer. Currently, melanomas are visually diagnosed by general practitioners or dermatologists. All skin lesions that are suspected of being melanoma are excised. However, only a small percentage of those excisions is actually a melanoma. This indicates that some excisions could be prevented. It is also reported that some melanomas are missed during clinical diagnosis, further emphasizing the need for an easy-to-use and objective tool to aid doctors in their diagnosis. Raman spectroscopy could be such a tool.

Santos et al. have developed a focused-beam Raman spectroscopy setup that measures Raman spectra of excised skin lesions. It uses a novel InGaAs detector to measure in the High Wavenumber spectral region using a Near Infrared (976 nm) laser to decrease laser-induced tissue fluorescence that might otherwise overwhelm the typically very weak Raman signal. However, in order to adapt the setup for *in vivo* use and eventually clinical use, the optical layout with a focused-beam geometry had to be expanded with a fiber-optic probe, thus enabling a flexible solution to perform Raman measurements directly on the skin of the patient.

This thesis focuses on the adaptation, feasibility and verification of a fiber-optic adapter using a single fiber probe for measurements. To do this, a convenient adapter that could be removed and replaced easily to be able to compare the existing to the adapted setup had to be designed first. Calculations were done to find the requirements and limitations of such an adapter and the construction was then outsourced to the engineering department.

Once the removable adapter was available, the setup was re-aligned and optimized for use with the fiber-optic probe. Tests were done to approximate the sampling depth of the fiber probe in skin tissue, which was up to about 300 μm . This depth is sufficient for early melanoma detection. The adapter was found to produce results that were repeatable to within less than one percent.

Finally, spectra obtained using the single-fiber probe were compared to spectra obtained with the existing focused-beam setup. The results showed that the signal-to-noise ratio was consistently significantly higher using the focused-beam setup. However, the fiber adapter consistently showed a slightly lower average level of noise. It is still possible that spectra taken with the fiber adapter are of sufficient quality to be able to distinguish melanomas from non-melanomas, but more tests are needed to determine this.

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List of Abbreviations

a.u.	Arbitrary Units
CCD	Charge Coupled Device
FWHM	Full Width at Half Maximum
GRIN	Graded Index
HWVN	High Wavenumber
InGaAs	Indium Gallium Arsenide
NA	Numerical Aperture
NIR	Near Infrared
NIST	National Institute of Standards and Technology
PVC	Polyvinyl Chloride
RMS	Root Mean Square
SMA	Sub Miniature A
SNR	Signal-to-Noise Ratio
SWIR	Short Wave Infrared
TIR	Total Internal Reflection
VPH	Volume Phase Holographic

1.Introduction

Melanomas are pigmented skin tumors and they are the deadliest form of skin cancer. They are derived from pigment-producing cells in skin, the melanocytes. Melanomas are caused by mutations. These mutations cause a rapid multiplication of the melanocytes and could be triggered by damaged DNA, which is most often caused by ultraviolet radiation (e.g. sunburn). Melanomas can develop from existing pigmented benign lesions or appear *de novo*.^{[1][2]}

Melanomas are clinically diagnosed through visual inspection of morphological aspects of the lesion by a general practitioner or dermatologist, involving recognition of patterns and structures. Melanomas sometimes closely resemble benign pigmented skin moles, making them hard to distinguish and diagnose even for specialized dermatologists. This results in a far-from-perfect diagnosing accuracy.^[1] If the lesion is considered clinically suspicious of melanoma, it is surgically excised and sent for histopathological diagnosis. The histopathological diagnosis is currently used as the gold standard and requires a pathologist. The histopathological processing of samples is laborious and, most importantly, requires all suspicious lesions to be excised and processed. Over the past twenty years, a tool called dermoscope, which is a type of magnifier, has been used to aid the doctor in his clinical diagnosis.^[3] Dermoscopes have helped to improve the clinical diagnosis accuracy, but it is still far from ideal. Out of all excised skin lesions suspected of melanoma, only 23% (or less for less experienced examiners) turns out to be a melanoma after histopathological diagnosis. Furthermore, it is reported that general practitioners miss up to 30% of melanomas when performing examinations.^[4] Therefore, there is a clinical need for a diagnostic tool that could aid the general practitioners and dermatologists to increase their melanoma recognition accuracy.

Raman spectroscopy is an optical technique that is widely used to characterize biological tissues and to detect molecular changes associated with pathological processes (e.g. distinguishing malignant from non-malignant tissue).^[5] It is an attractive technique for clinical diagnostics because it is, i.e., non-invasive, non-destructive, requires no sample preparation and it only needs seconds to measure a spectrum.^[6]

However, up to now, Raman spectroscopy has almost been limited to non-pigmented tissue. When exciting pigmented skin with visible light, it is absorbed by melanin in the pigment which generates high laser-induced tissue autofluorescence. This laser induced tissue fluorescence spectrally appears as an intense and broad background, which may completely overwhelm the weak Raman signal to the extent that the Raman signal is no longer detectable.

Shifting to near-infrared (NIR) wavelengths of around 1000 nm and measuring in the High Wavenumber (HWVN) spectral region can reduce sample and laser fluorescence considerably. However, the HWVN spectral region of the Raman signal will shift above the cut-off wavelength of Charge Coupled Device (CCD) detectors due to the wavelength shift. CCDs are the preferred method of detecting Raman signal due to their very low readout noise and dark noise in combination with a multichannel detection scheme.^{[5][7]}

As a part of the RASKIN project, Santos et al. have developed an NIR ex-vivo focused-beam Raman spectroscopy setup, which is dedicated to perform measurements on excised pigmented lesions suspicious of being melanoma in the HWVN area using a novel Indium-Gallium-Arsenide (InGaAs) detector.^[5] This equipment is currently being used to do measurements on excised pigmented skin lesions shortly after being taken from patients in the Leiden University Medical Center.

A next step in this research project is the translation from *in vitro* measurements on excised skin samples to *in vivo* measurements directly on the patient. In order to adapt this setup for *in vivo* measurements, a practical fiber-optic probe needs to be implemented and verified.

Raman signal that is acquired using a fiber-optic probe has often had a significant problem associated with it. This is that the laser light that is used generates a Raman signal in the material (usually fused silica) of the fiber it travels through for certain excitation wavelengths, which is often also more intense than the Raman signal of interest. This unwanted signal must be filtered out using a filter, but since the Raman signal needs to reach the spectrometer, a separate collection fiber is also required. The intense Rayleigh scattered light must also be filtered out of the Raman signal, requiring another filter for the collection fiber. This would make the setup complicated and expensive.

However, using near-infrared (NIR) laser light and measuring in the HWVN Raman spectral region have proved to circumvent or reduce these issues. This is because fused silica optical fibers show little to no Raman signal in the HWVN area. Furthermore, if *in vivo* measurements can be performed on pigmented skin lesions by a single optical fiber, the use of a simple and cheap fiber-optical probe for assessment of pigmented skin lesions suspected of being melanoma can be made possible.^[7]

In order to show that a single fiber optical probe can be used *in vivo* in a clinical environment, the existing setup had to be adapted to be used with a single fiber. Additionally, due to the novelty of the setup and technique, a proof of concept was required. In order to achieve this, comparison measurements were done to compare the existing focused-beam setup and the adapted fiber-probe setup. The goal of this thesis was to adapt the existing focused-beam Raman setup to use a single-fiber probe for *in vivo* measurements, and to compare the quality of the resulting setup to the existing one.

2. Raman Spectroscopy and Optical Fibers

This chapter will cover the theory required to understand the used methods and materials. First, Raman Spectroscopy will be covered, since this is the base of the research. Next, the theory behind optics and optical fibers will be discussed. Considering both of these are broad subjects, only the necessities for this project will be covered here.

2.1. Raman Spectroscopy

Raman Spectroscopy is widely used for many different applications in many different fields of work. It is based on the inelastic scattering of incident light which results in shifts in the wavelength of the scattered light. This shift is called “Raman Scattering” or “Raman Shift”, and it can serve as a means to obtain information about the chemical composition of the material being illuminated. This chapter will explain Raman Scattering and the principle of Raman Spectroscopy.

2.1.1. Raman Scattering

Raman Scattering, or Raman Shift, is the base of all Raman Spectroscopy. This effect describes the inelastic scattering of incident light on a surface, which holds information about the surface that was illuminated. This is different from the more common (by a factor of about 10^6) Rayleigh Scattering, where incident light is scattered with the same wavelength.^[8]

When photons interact with molecules, they polarize the molecule’s electron cloud, raising electrons to a so called ‘virtual energy state’. After an extremely short time, commonly about 10^{-14} seconds, the electrons drop back down to their ground state, emitting a photon in a random direction with the same energy as the incident photon. This is due to the fact that the electron must gain and lose the same energy if it travels between the same energy states. The light is now Rayleigh scattered and the wavelength remains the same.^[9]

It is also possible that an electron that was raised to a virtual state falls back to a different energy than it started with. This also emits a photon from the molecule, but it will have a slightly higher or lower energy. Falling back to a different energy happens because the ground state of a molecule has several so-called ‘vibrational states’ or ‘vibrational modes’ that are slightly higher in energy than the ground state. When electrons fall back to one of these vibrational modes, the resulting shift in energy (and thus wavelength) of the emitted photon is called ‘Stokes Scattering’. Similarly, electrons can be raised from a vibrational state instead of from the ground state and then fall back to the ground state. This results in a photon being emitted of slightly higher energy than the incident light, which is called ‘anti-Stokes Scattering’. These terms combined are what is known as Raman Scattering (or Raman Shift). Rayleigh scattering is about 10^6 times more common than Raman Scattering, meaning Raman signal is relatively weak. All three forms of Scattering (Rayleigh, Stokes and anti-Stokes) are illustrated in Figure 1.^{[9][10]}

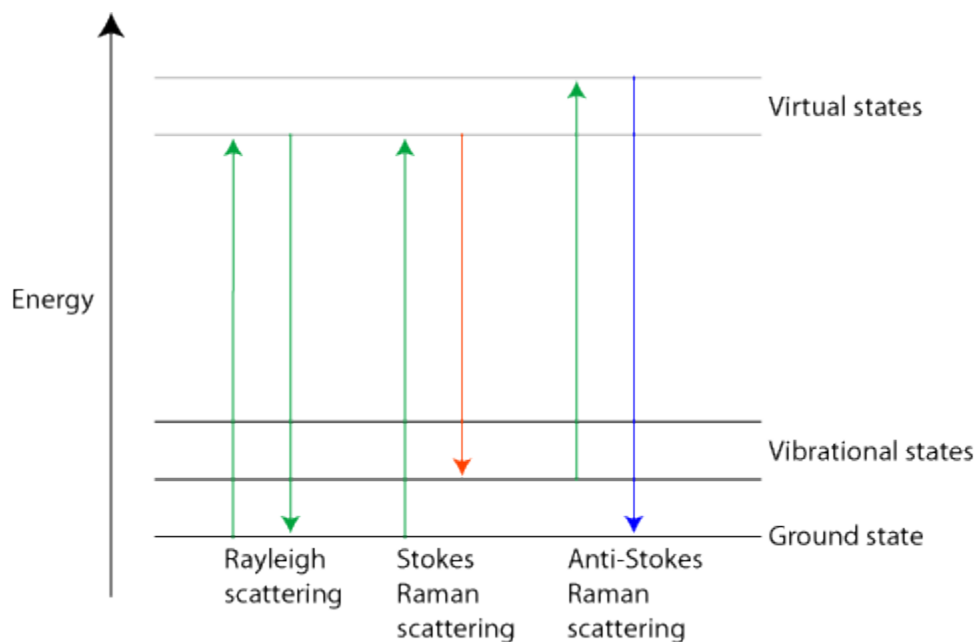


Figure 1, Electron energy transitions caused by incident photons for three types of scattering. Electrons are excited to a virtual state by incident photons and subsequently fall back to a lower energy state. The state they fall back to determines the energy of the emitted photon.^[9]

The shift in wavelength due to Raman Shift is determined by the energy levels of the vibrational states of the molecule in which electrons are being excited. The energy level of the vibrational states is dependent on the reduced mass μ of a molecule, as well as the bond strength k . Since molecular vibrations are similar to simple harmonic motion, the bond strength k resembles the spring constant of a spring.^[11]

The reduced mass μ for a molecule of two atoms is given by equation 2.1:

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \quad (2.1)$$

Where m_1 and m_2 are the masses of the individual atoms present in the molecule and μ , m_1 and m_2 are in kilograms (kg). Using this, the wavelength λ (nm) of the vibrations can be calculated using equation 2.2:

$$\lambda = 2\pi c \sqrt{\frac{k}{\mu}} \quad (2.2)$$

Where c is the speed of light in $\text{m}\cdot\text{s}^{-1}$, k is a positive constant in $\text{kg}\cdot\text{s}^{-2}$ and μ is the reduced mass. Anti-Stokes shift is not commonly used in Raman spectroscopy because it has an even lower intensity than regular Stokes shift. This is because it only occurs when electrons are already in a vibrational state as a result of Stokes shift, which is already uncommon (by a factor 10^{-6}).^[9]

Raman spectra are commonly given in wavenumbers, as these directly relate to energy. Conversion between wavelength and wavenumbers is done using equation 2.3 below.

$$\Delta w = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda_1} \right) \quad (2.3)$$

Where both wavelengths are given in centimeters (cm), Δw is the Raman shift expressed in wavenumbers (cm^{-1}), λ_0 is the initial wavelength and λ_1 is the shifted wavelength.

2.1.2. Wavenumber Regions

A typical Raman Spectrum, as shown in Figure 2, consists of two regions: the fingerprint region and the High-Wavenumber (HWVN) region.

The fingerprint region contains more detailed information than the HWVN region, as it has more spectral nuances. It is typically defined as the range $400\text{-}1800\text{ cm}^{-1}$ and is used to identify molecules by their vibrational states, as explained in section 2.1.1. The peaks in the fingerprint region are caused by vibrations within the molecules that are present.

The HWVN region, with wavenumbers ranging from $2500\text{-}4000\text{ cm}^{-1}$, does not contain as much detailed information as the fingerprint region. However, research has shown that this region does still contain sufficient information for medical research and diagnostics.^{[7][12]} Moreover, laser induced fluorescence is strongest in the fingerprint region, although it is present in the HWVN region as well. The HWVN region Raman spectrum of tissue is dominated by stretching of O-H bonds and C-H bonds in molecules that are present in the sample. The HWVN region has the benefit of being outside of the range of background Raman signal generated by laserlight guided through silica fibers. However, some materials still show strong background peaks in this region, meaning a careful choice of fiber materials is important.^[7]

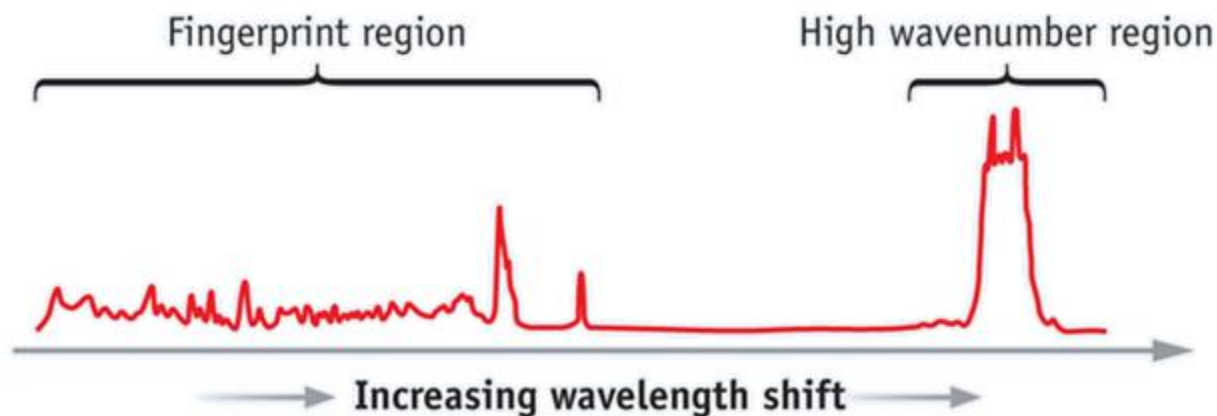


Figure 2, typical Raman spectrum showing the fingerprint region ($400\text{-}1800\text{ cm}^{-1}$) and the high wavenumber region ($2500\text{-}4000\text{ cm}^{-1}$).^[13]

2.1.3. Wavelength Dependence

Raman Scattering, like Rayleigh Scattering, is wavelength dependent. The scattering occurs because particles much smaller in size than the wavelength used scatter the light evenly across their entire surface. Due to the random nature of the particles, the collected light has random phases and is therefore incoherent. The intensity at a certain point can be approximated by the sum of the contributions of each scattering particle. When this intensity is averaged over all angles, the result is what is known as the 'scattering cross-section' σ_s :

$$\sigma_s = \frac{2\pi^5 d^6}{3\lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right)^2 \quad (2.4)$$

Where the scattering cross-section σ_s (m^2) multiplied by the amount of particles per unit volume N (m^{-3}) gives the fraction of light that is scattered. The approximated radius of the scattering particles is d (m) and n (-) is their refractive index.^[14]

The equation indicates that light scattering is inversely proportional to the fourth power of the wavelength. In other words, light of shorter wavelengths is scattered more strongly than light of longer wavelengths. This means that shorter wavelengths show stronger Raman scattering. However shorter wavelengths also produce more laser-induced fluorescence. Because this fluorescence is orders of magnitude stronger than the weak Raman scattering, using longer excitation wavelengths instead could be preferable regardless of the loss in Raman intensity.^[9]

2.2. Optics and Fibers

Since their appearance in the 1970s, optical fibers have started to dominate many fields in optics. They are fast and efficient in transporting data. They are also relatively cheap, flexible and small. When combined with several optical components, fiber optics could enable an otherwise very bulky setup to be used in a practical way. Here, essential theory behind fiber optics and important optical components will be covered.

2.2.1. Optical Fibers

Optical fibers are cylindrical waveguides. Fibers consist of 2 to 4 parts, numbered in Figure 3, which will be explained below. The first two layers are essential for the principle of light transmission while the third and fourth are optional layers that each add protection to the fiber.

1) Fiber Core

The fiber core is the part through which light propagates. Common core materials are (transparent) glass and plastics. Light stays in the core because of Total Internal Refraction (TIR), sizes range from several micrometers to several hundreds of micrometers or even millimeters.^[15]

2) Cladding

The cladding of a fiber is the layer that directly surrounds the core. This layer will always have a lower refractive index than the core, to allow for TIR to take place, making the cladding essential in light propagation through the fiber. The cladding is often made of a transparent material like silica.^[15]

3) Coating

The coating of a fiber, sometimes referred to as buffer, is a protective layer around the cladding. It serves to protect the fiber from moisture and from breaking or bending too much, and is usually made with an acrylate or polyimide material.^[15]

4) Jacket

The jacket is the final layer of protection of an optical fiber. Jackets range from simple plastic tubes to Kevlar-reinforced stainless steel tubes. The jacket is the first line of protection against stresses from handling and storage. The jacket is usually not attached directly to the coating itself to allow for the fiber to freely move inside it.^[15]

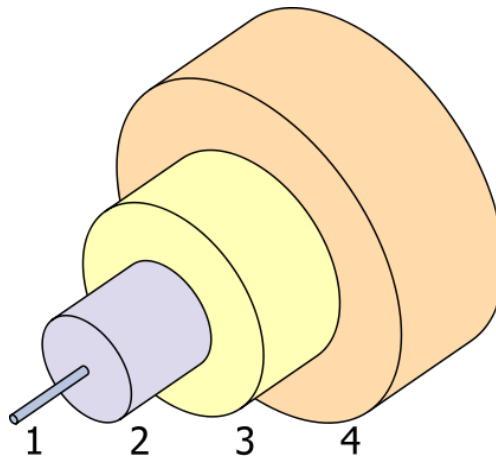


Figure 3, Typical (single mode) optical fiber configuration with, from 1 to 4: core, cladding, coating and jacket.^[15]

Total Internal Reflection

When light hits a surface of lower index of refraction, it bends away from the so called 'normal', which is an imaginary line that is perpendicular to the surface that is being illuminated. For a certain angle, the angle of refraction would be 90 degrees. That would mean that the light would travel along the surface of the object being illuminated, such as glass or water. Any angle larger than this would cause the light to be internally reflected instead of penetrating the surface. This phenomenon is called Total Internal Reflection, or TIR for short.

The angle at which this happens is called the critical angle θ_c (°) and it follows from Snell's law:

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \quad (2.5)$$

Where n_1 and n_2 are the indices of refraction of the source medium and target medium respectively. A graphic representation of the critical angle is shown in Figure 4.

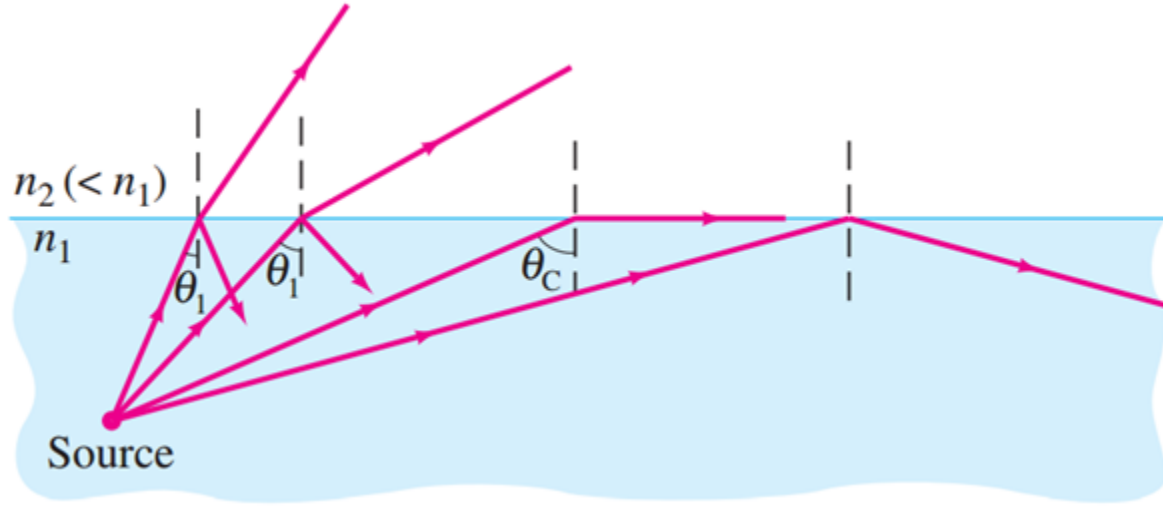


Figure 4, simple graphical representation of the critical angle of light traveling from water to air.^[16]

TIR is important in fiber optics, since all fibers rely on it to transport light from one place to another. However, different fibers rely on it in different ways. Because a fiber is a waveguide, light only remains trapped under certain specific conditions; that is, the angle of incidence θ_i (°) and phase ϕ (rad) of the incident light have to satisfy the waveguide condition as described in equation 6.^[8]

$$\left[\frac{2\pi n_1 d}{\lambda}\right] \cos(\theta_m - \phi_m) = m\pi \quad (2.6)$$

Where d represents the diameter of the waveguide (in this case, the fiber core) and $m = 0, 1, 2, \dots$ is an integer known as the mode number of a waveguide. Essentially, the waveguide only works for specific modes where θ_m and ϕ_m correspond with a mode number m . Since both phase and mode numbers are not relevant in this thesis (because the only requirement is that TIR happens), they will not be covered further.

Single Mode Fibers

Single Mode fibers only allow for one mode to propagate through them, e.g. only $m = 0$ light can propagate through them. This happens due to very specific characteristics of the used light and fiber. A typical core diameter is 5 μm and a typical cladding is around 125 μm . One benefit of allowing only one mode to propagate is that it allows the fiber to better maintain the fidelity of light transmitted through it, in turn allowing for a higher bandwidth through the fiber.^[15] However, since bandwidth is not relevant

to this project and since single mode fibers are more difficult to optically couple, these will not be considered further in this thesis.

Multimode Fibers

Multimode fibers allow for more than one mode of light to propagate through them. These fibers usually have a much bigger core diameter than single mode fibers, ranging from 50 up to 1000 μm . This gives them the ability to 'accept' light more easily and it also makes connections between fibers and optical elements simpler. For those reasons, this kind of fiber is commonly used in communication and research.^[15]

As mentioned before, TIR depends on the index of refraction of the target medium being lower than that of the source medium. Fibers achieve TIR in two different ways when looking at index of refraction, that is 'Graded-index' and 'Step-index'. Both of these fibers will be briefly explained below.

Step-index Fibers

Used for most single mode fibers and some multimode fibers, step-index simply means that the core and cladding have uniform but different refractive indices. Light travels through the core of refractive index n_1 and reflects against the surface of the cladding with refractive index n_2 . This causes light to travel in straight lines from each point of contact with the cladding as shown in Figure 5A.

Graded-index Fibers

As opposed to step-index fibers, graded-index (GRIN) fibers have a refractive index in the core that decreases over its radius. That is, the further from the center of the core, the lower the refractive index. This causes light to be gradually bent away from the cladding and back to the center of the core, effectively making it travel through the core in a sinusoidal path. Graded-index is common in multimode fibers since it reduces the modal dispersion (signal spread due to different velocities of different modes) which can be a problem in multimode fibers compared to single mode fibers. This is also shown in Figure 5B.

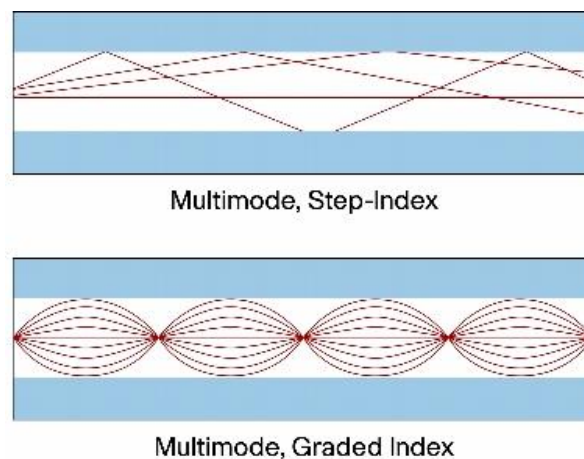


Figure 5, light paths in multimode step-index fiber, A (top).
Light paths in graded-index fiber, B (bottom).^[17]

2.2.2. Optics

Besides glass fibers, several other optical elements and principles are required in this research and spectrometry in general. They will be covered here.

Numerical Aperture

The Numerical Aperture (NA) is a dimensionless number that relates to the volumetric “cone” of light that an optical element can accept or emit. It is a single number that indicates the maximum acceptance angle. This angle is the maximum angle α_{max} (°) at which light can still enter an optical element, and relates to the NA (-) with the index of refraction of the source medium n_o (-):

$$NA = n_o \sin(\alpha_{max}) \quad (2.7)$$

The NA is important in fiber optic connections because, for example, if the NA of incoming light is bigger than the NA of a fiber, not all of the incident light will go into (or stay in) the fiber and signal will be lost as a result.^[8]

Lenses

Lenses are optical instruments that refract light in order to produce a real or virtual image. Lenses can be convex, concave or both. Convex means that a lens makes light converge, while concave means that it makes light diverge. The point where the light is focused is called the ‘focal point’.

The focused spot of a lens is, theoretically, infinitesimally small. However, in reality it always has a width. This width is called the ‘spot size’ ($2w_0$), which is an important number when using optical fibers because the spot has to fall within the fiber core so as not to lose light. See Figure 6. The spot size is given by the following equation:

$$2w_0 = \frac{4\lambda}{\pi} \cdot \frac{f}{d} \quad (2.8)$$

Where λ is the wavelength used in nanometers (nm), f is the focal distance of the lens in meters (m) and d is the width of the incoming beam of light in meters (m). Spot size is important when coupling fibers and lenses, because the core of a fiber is in the same order of magnitude. Even when the optics have been chosen in such a way that the spot size is less than the core of the fiber, it still requires very precise alignment to successfully couple the light.^[18]

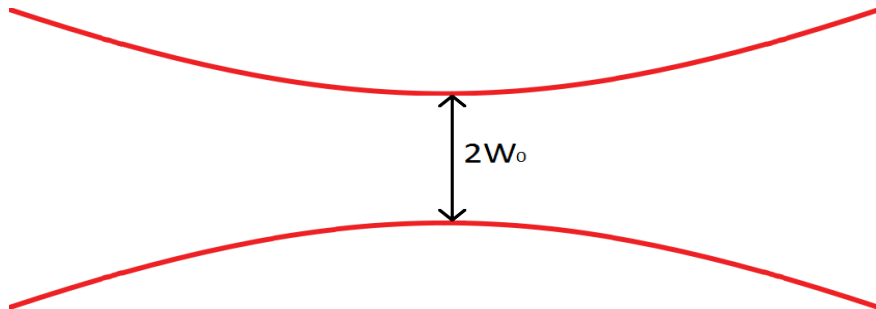


Figure 6, graphical representation of a focused beam. W_0 is the radius of the focused spot, meaning $2W_0$ is the size (or diameter) of the spot.

Mirrors

Mirrors are completely reflective surfaces. The basic rule for light reflecting off a mirror is that the angle of incidence θ_i (°) is equal to the angle of reflection θ_r (°), or:

$$\theta_i = \theta_r \quad (2.9)$$

Flat surfaced mirrors will always create an image that is virtual and the same size as the object, at the same distance as the object. However, some mirrors are spherical and will either diverge or converge the light that falls onto them. These mirrors, like lenses, have focal points. The location of this focal point f in meters (m) is directly proportional to the radius of curvature of the mirror r in meters (m):

$$f = \frac{r}{2} \quad (2.10)$$

In converging mirrors, this focal point is considered to be negative as it is located before the mirror itself, instead of behind it like with lenses. In divergent mirrors, however, the focal point is virtual because it is located in the virtual space behind the mirror.^[16]

Noise

Due to the naturally low intensity of Raman signal, noise and losses are of significant impact on its quality. The signal-to-noise ratio (SNR) is often used as a method to describe signal quality in (Raman) spectroscopy. It is defined as the ratio between collected signal and collected noise. Raman spectroscopy knows three types of noise, i.e. shot noise, dark current noise and readout noise.^[19]

Shot Noise originates from the quantized (particle) nature of light, and the statistical probability of emitted 'light particles' hitting a surface known as the Poisson Distribution of photon counting. The statistical probability in a laser beam is negligible, considering many billions of photons hit a surface to create the spot of the laser, and the brightness of that spot will not vary significantly over time.^[19] In a Raman signal that is detected, the amount of photons that hit the detector is small enough that the distribution does vary over time. In other words, if the intensity of the measured signal increases, the relative contribution of shot noise goes down. Shot noise is commonly approximated as the square root of the collected signal S . Equation 2.11 shows the shot noise N_{shot} in electrons per second ($e \cdot s^{-1}$):

$$N_{shot} = \sqrt{S} \quad (2.11)$$

Dark current is unwanted signal from the detector, which represents the signal measured if no photons are being collected. This is because electrons are being thermally generated and registered by the detector. This happens at all times and is unavoidable. Dark current noise N_{dark} is defined as the square root of the dark current signal S_{dark} , given as a flat amount of electrons per second ($e \cdot s^{-1}$).

Finally there is the readout noise that is inherent to the detector used. Readout noise is generated by the detector as it reads its pixels, and is always present. It is caused by the detector imperfectly reading the electron count on the chip.^[20] It is usually expressed in an amount of electrons per second Root Mean Square (RMS) to indicate that it is an averaged value over several measurements.

The total readout noise $N_{readout}$ is equal to the sum of the square root of the individual noises r_n (from each time a pixel value is read) squared, given in electrons per second ($e \cdot s^{-1}$):

$$N_{readout} = \sum \sqrt{r_n^2} \quad (2.12)$$

Combining these types of noise with the collected Raman signal S in electrons per second ($e \cdot s^{-1}$) results in the equation for the SNR ratio:

$$SNR = \frac{S}{\sqrt{N_{shot} + N_{dark} + N_{readout}}} \quad (2.13)$$

When the contributions of the dark current noise and the readout noise are negligible compared to the shot noise, a detector is called 'shot noise limited'. The signal-to-noise ratio is then approximated as follows:

$$SNR = \frac{S}{\sqrt{S}} = \sqrt{S} \quad (2.14)$$

Miscellaneous Components

The major components and principles have been covered, but a few smaller components with very specific functions remain. They will be covered here.

Filters

An optical filter is an element that transmits only a select part of wavelengths depending on its properties. Common types of filters are the absorptive filter, which absorbs some wavelengths while transmitting others, and the reflective filter, which reflects some wavelengths and allows others to pass through it. Filters can be longpass, shortpass and bandpass, meaning they transmit long, short or bands of wavelengths respectively.^[8]

Filters are important components in Raman spectroscopy. They are used, among other things, to block the very intense laser light from reaching the detector. To do this, they need a high optical density (higher density for a wavelength means less light passes through) for that specific laser wavelength. They also need to transmit Raman signal evenly, meaning the wavelength range of the signal needs to have the same (low) attenuation all across.^[5]

Grating

A grating is a simple optical element that disperses incident light wavelengths onto the detector. It can have a series of thin grooves (diffraction grating), or a thin holographic film (transmission grating). Incident light is diffracted by the grooves or the film in a pattern that is well-defined and that depends on wavelength and grating properties such as the distance between the grooves or the thickness of the film. Gratings are used to split rays of light into the respective wavelengths of its components, as light diffracts differently for each wavelength. The grating is responsible for the wavelength range of a spectrometer.^[8]

Slit

A slit is a thin, long gap in a surface through which light can pass. It usually has a width in the order of magnitude of the wavelength of the light used. Light that passes through the slit makes an interference pattern dependent on its wavelength and the distance (from the image) and size of the slit. A single slit creates one strong peak in its interference pattern, and determines how much light reaches the spectrometer. Furthermore, it is responsible for the spectral resolution of the spectrometer. In other words, the slit decides the smallest distinguishable difference in wavelength.^[21]

2.3. Human Skin

Because the goal of this thesis involves Raman measurements on human skin, it is important to know something about its composition and features.

2.3.1. Epidermis and Melanoma Detection

Human skin consists of multiple layers, each with its own function and origin. However, since most of these layers are irrelevant to this thesis, they will not be mentioned or explained further. For Raman spectroscopy used to identify melanoma, only one layer is of interest: the epidermis. The epidermis is the collective name for the few most superficial layers of the human skin and it is typically between 50 and 150 μm thick, depending on anatomy.^{[22][23]} The reason this layer is of specific interest is that, at its bottom, it contains the pigment-producing cells called melanocytes. As mentioned above, melanomas are derived from these cells.^[24] Melanocytes lay along the epidermis-dermis interface, which is a curved interface located about 50 to 150 μm deep. See Figure 7 for a graphical representation of the epidermis-dermis interface and one melanocyte. Note that there are usually more spread along the interface. The thickness and the composition of this layer are important because, in order to get relevant measurement data to detect melanoma, this layer needs to be penetrated (twice, back and forth) to reach the epidermis-dermis interface and collect Raman signal from this region.

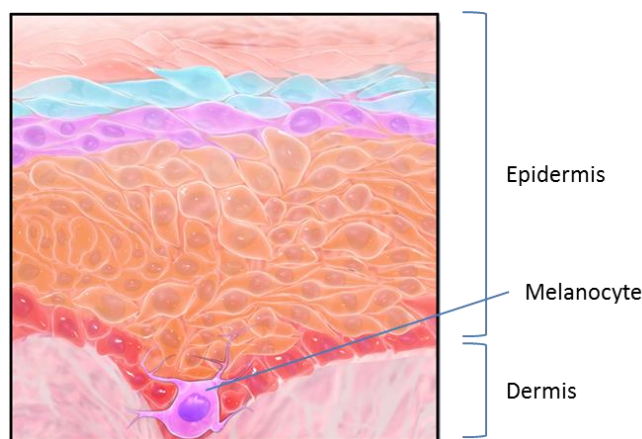


Figure 7, graphical representation of the epidermis. Also shown are a melanocyte and the dermis, which extends beyond the image. The dermis is a connective layer of tissue that protects against stresses.^[25] The top of the figure represents the surface of the skin.

In the earliest stage, melanoma is confined to the epidermis and will later extend into the dermis. Once it extends too far down and melanoma cells start to spread, treatment becomes difficult to impossible.^[12] Early detection, meaning when it is still closer to or inside the epidermis, is therefore critical. This makes the epidermis up to the epidermis-dermis interface the most interesting region for detecting early melanoma.

2.3.2. Raman Spectrum

Raman spectroscopy can be applied in medical field to detect and quantify biomolecular changes which could cause, or be caused by, diseases.^{[26][27][28]} The most prevalent application of medical Raman Spectroscopy, however, is in cancer diagnostics. Cancer is a disease which alters the molecular composition of cells. Therefore, Raman Spectroscopy is an ideal tool to detect these changes. In biological tissue, the composing (bio)molecules are influential in the defining shape of the spectrum. Some of the most influential molecules in human skin are water, proteins and lipids. Water content influences the OH stretching vibrations, while proteins and lipids influence the CH stretching vibrations.^{[22][29]} A typical HWVN Raman spectrum of skin with highlighted OH and CH regions is shown in Figure 8.

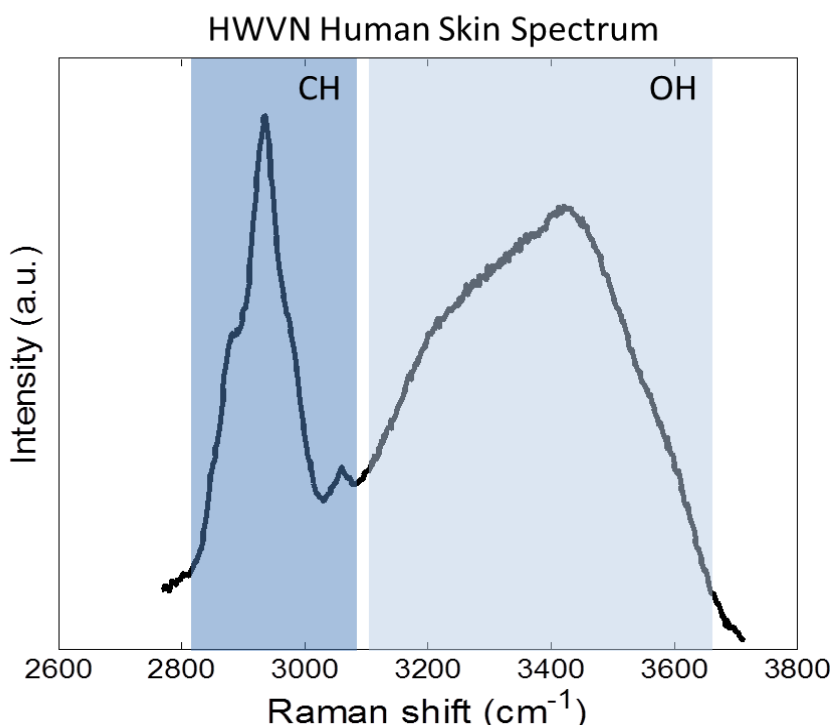


Figure 8, typical HWVN Raman spectrum of human skin. The CH and OH bands are highlighted. See appendix E for an explanation of arbitrary units (a.u.).

The OH stretching region specifically is depth sensitive, as the water content in the epidermis increases with depth.^[22] The lipid and protein contributions do not vary much with depth; however, recent research done by Santos et al. has shown that the lipid-specific peak in the CH stretching region might be indicative of melanoma.^[30] This makes the CH stretching region the primary focus of the measurements carried out in this project and this thesis.

3. Instrumentation & Goals

As mentioned in the introduction, the current setup uses a focused-beam ex-vivo approach to measure spectra of pigmented skin lesions suspected of melanoma. This chapter will cover the current setup and status of the project, as well as its aim for the future.

3.1. Instrumentation

Current measurements are done using a ‘focused-beam’ (also referred to as ‘open-air’) approach, meaning that excitation of a sample and collection of the signal happens in open air using nothing but a lens to focus and collect the light. Because of the unique nature of the research and the extraordinarily low level of signal that needs to be collected with sufficient quality, the setup, nicknamed ‘RASKIN’ after the project name, contains many custom built or customized components. Figure 9 shows the initial setup and its components.

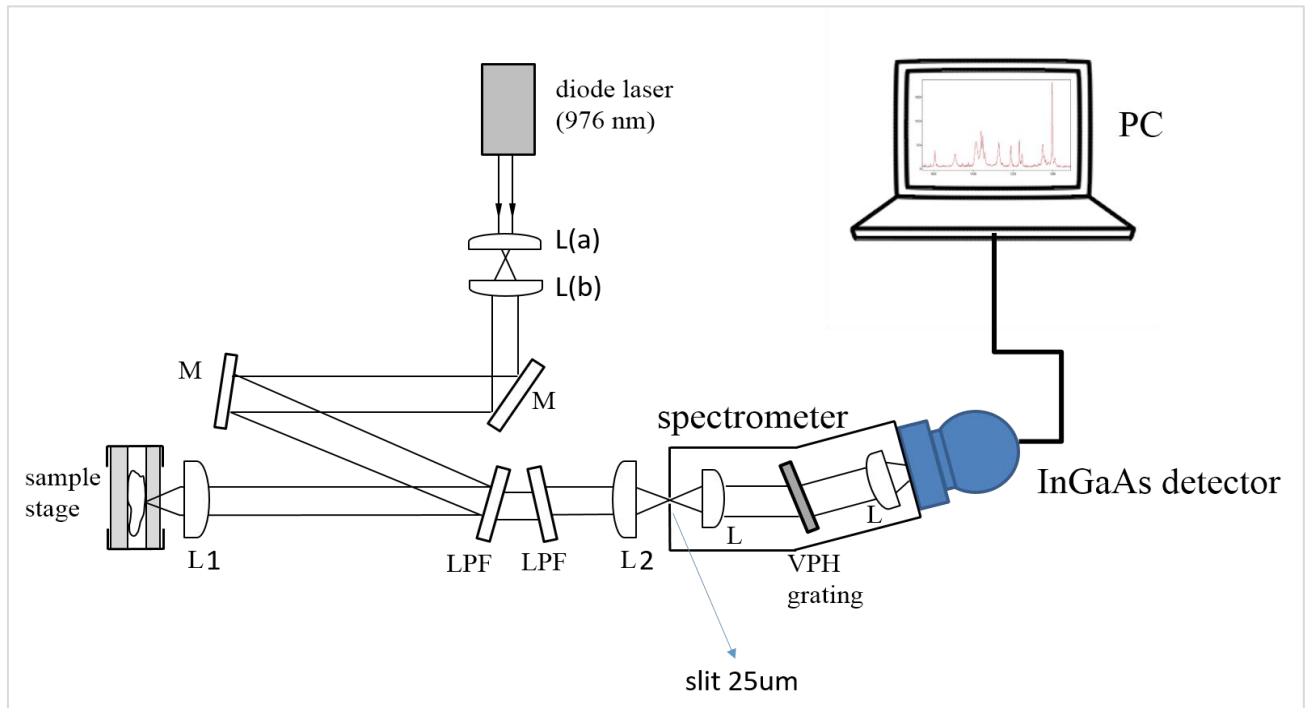


Figure 9, simplified representation of the RASKIN setup. The components and their separate functions are further explained in chapter 4.1.

General operation of the setup is as follows. A beam of near-infrared laser light is expanded by a set of two lenses (L(a) and L(b)) and then passes a set of mirrors that redirect the beam in a way that it is nearly parallel to the (in Figure 9) horizontal plane. This is necessary because the filter (LPF) only works for light that arrives under very small angles or no angle at all. The light is then focused by lens L1 on the sample, where excitation happens. The generated Raman signal is collected by L1 and travels back through both LPF filters to be focused on a slit in the spectrometer by lens L2. After passing the slit, the beam is collimated by lens L before passing through a transmission grating (VPH) and finally being focused on the InGaAs detector by another lens L. Detailed explanation and justification for each component will follow here.

3.1.1. Diode Laser

One of the main components of the spectrometer setup is the near-infrared, single-mode continuous wave diode laser (Class 3B, Model R-type, Innovative Photonic Solutions, Monmouth Junction, NJ, USA), which produces light with a wavelength of 976 nm with an output power of about 165 mW. As mentioned before, a wavelength in the near-infrared range helps to circumvent laser-induced tissue fluorescence. This type of fluorescence is strong and can completely overwhelm the Raman signal of interest. Moreover, this wavelength region helps to eliminate the Raman signal that is generated along the length of the whole fiber due to the fused silica of the fiber core. The main drawback of 976nm laser light is that it is invisible light. This makes visual alignment and tracing the beam of light more difficult and time consuming. Further, the invisible light is dangerous to the eyes because there is no warning or indication that the eyes are being exposed to it.

3.1.2. Filters

In order to ensure that laser light gets through to the camera, two long pass dichroic edge filters (Model Raman Edge Filter, Edmund Optics, Barrington, NJ, USA) are used. These filters cut off at 1064nm, causing any stray visible light or laser light to be filtered out before it gets to the spectrometer. If any of this light got through to the spectrometer, the Raman signal of interest would be overwhelmed by it.

Dichroic filters are angle dependent, meaning both the angle and amount of reflectance depends on the angle of incidence of light. They are commonly made for and placed at an angle of 0°. Initially, the concept was to use a dichroic mirror at 45° for the setup, however all the tested 45° mirrors introduced artefacts in the transmission curve. Because the percentage of transmitted Raman signal was not stable with wavelength, the (spectrally wide) Raman signal would vary with wavelength as well. Instead, two 0° filters were placed under an angle of about 7,5°. As visible in Figure 9, the beam of light remains parallel due to the second filter and the slight angle means the filtered light is reflected away from the light path.^[5]

3.1.3. Spectrometer

Spectrometer, in this case, is a collective name for a series of components that work together to focus only a specific part of the incoming signal on the camera chip. The first of these components is a slit that is 25µm wide and 3mm high. The slit determines the fraction of incident light that passes through to the detector. Smaller slits allow less light to pass through, but could increase the spectral resolution significantly.

After the slit, light passes through a lens that collimates the beam. Then it hits a Volume Phase Holographic (VPH) Transmission Grating (BaySpec). The VPH differs from reflective gratings in the fact that it uses a transparent layer that is placed in between layers of e.g. fused silica instead of being a series of slits or a relief pattern. The thin transparent layer usually contains a gelatin substance that has a periodically varying index of refraction. Incident light is phase-shifted by this difference, and is refracted in different angles for different wavelengths.^[31] The grating is, as a result, responsible for what is imaged in the spectral direction of the camera chip. Finally, the light is focused on the chip of the camera by a second lens, which is identical to the one used to collimate the light in the spectrometer.

3.1.4. InGaAs Detector

As mentioned before, measurements take place in the HWVN region to reduce tissue-induced fluorescence. Due to the high signal wavelengths associated with this region when using NIR excitation (between 1300 nm and 1550 nm), Charge Coupled Devices (CCDs) cannot be used as the camera. CCDs are limited by the bandgap of silicon, which does not allow them to detect signal over about 1100 nm.^[5]

Instead, a novel deep-cooled Indium-Gallium-Arsenide (InGaAs) detector for SWIR imaging, the “Cougar-640” (Xenics, Leuven, Belgium), is being used. The Cougar-640 uses a fundamentally new technique to read the chip, which is called ‘non-destructive readout’ or ‘Read While Integrate’. As the chip counts photons, a capacitor charges up and upon readout the charge is left intact. This means that, unlike other InGaAs detectors, the Cougar-640 does not have problems with high inherent readout noise when deep-cooled (to 77K). Each read out reduces the uncertainty in the slope of the fitted line through all the read outs, causing the total read out noise to go down by the square root of the amount of measurements.^[5] This, together with its ability to measure in the HWVN range, makes it an ideal camera to use for *in vivo* Raman measurements on tissue in this range.

Figure 10 shows a graphical representation of the block that contains the mirrors, filters and lenses L1 and L2. The corridor that leads from the first mirror to the filters and lenses is only about 13mm wide. This will prove to be a limiting factor later on in this project (4.1).

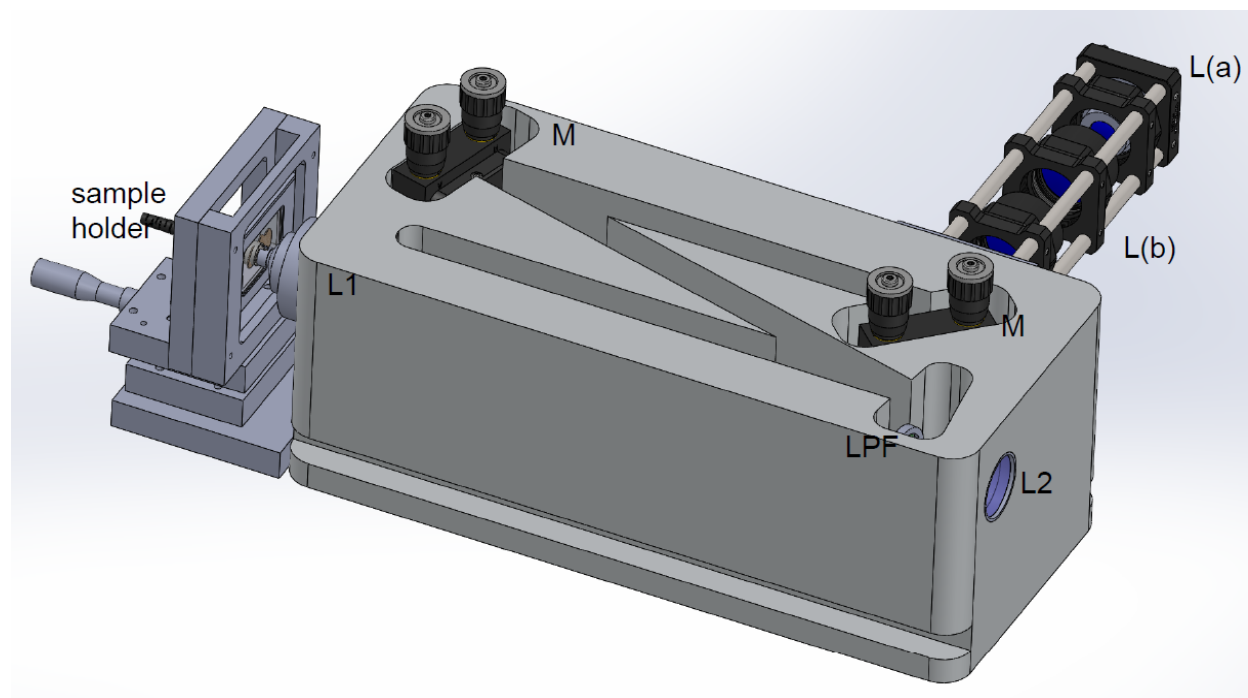


Figure 10, computer model of the block that contains the mirrors, filters and lenses L1 and L2. The laser light enters the block after L(b), L1 focuses light on the sample and L2 focuses the signal on the spectrometer.

3.1.5. Optical Fibers and Probe

Optical fibers are an important part of instrumentation for this project. The goal is to perform *in vivo* measurements on human skin, which is impossible with the open beam setup. Optical fibers offer flexibility and more ease of use, allowing *in vivo* measurements to happen easily and quickly.

Because of the delicate nature of fibers, they require regular cleaning and are prone to breaking. Measurements cannot be done with broken fibers, meaning several backup fibers have to be present at all times.

Due to previous research into optical fiber probes for Raman spectroscopy of human tissue^[7], an optical fiber with silica core and silica cladding was selected to measure with. The fiber (customized FG050LGA, Thorlabs Inc, Germany) has polypropylene inner tubing, threaded Kevlar reinforcement and black PVC outer tubing. The fiber has an NA of 0.22. It has a core size of 50 μm , which is the smallest one that is commercially available. Imaging bigger sizes on the slit would cause magnification problems, because the slit is only 25 μm wide. The fibers are SMA connectorized on one side to connect it to the setup, and are modified with a ferrule on the other side to measure samples with or to fit in a probe. Figure 11 shows an example of the fiber that was used.



Figure 11, image of an SMA-Ferrule connected fiber with 50 μm core of fused silica. Image courtesy of Thorlabs Inc.

The fiber was modified in-house to be able to fit in a stainless steel fiber probe. This probe offers increased stability and safety to the fiber tip, as most of it is secure inside the stainless steel tube. Figure 12 shows the probe holding the fiber.



Figure 12, picture of the finalized fiber-optic probe.

The tip of the probe can be unscrewed to replace the fiber that is inside with another one. This allows the rapid switching of fibers in case of one breaking, meaning measurements can still be done and patients will not be bothered.

3.2. Calibration

In order to convert the raw detector output to an accurate Raman spectrum of intensity versus wavenumber shift, extensive calibration has to be done. This paragraph will go through the standard calibration steps and explains why each is done.

- 3.2.1. Laser Power: First and foremost, the laser power output should be checked to ensure that maximum power (and thus signal intensity) is generated and achieved. The laser power is measured using an S130C power meter (ThorLabs, Germany).
- 3.2.2. Background Fiber/Optics: The laser light interacting with the fiber and optics in the setup generates a background signal that is present in every measurement, even without samples in place. To correct for this background signal, a measurement without sample is done to be able to subtract it. A typical background spectrum is shown in figure 1 of Appendix A.
- 3.2.3. Dark Current: Dark Current (DC) is an unwanted signal that is always present when the detector is measuring. It is caused by the thermal generation of electrons and is measured in a so-called 'dark frame'. In this dark frame, there is no sample and no (laser)light. A typical DC spectrum for this detector is shown in figure 2 of Appendix A.
- 3.2.4. Cyclohexane: To determine the exact excitation wavelength used by the laser, the Raman standard spectrum of Cyclohexane is used. The peaks in this spectrum are well defined, and can be used to calculate the wavelength which is used in the calculation of the Raman shift, as shown in equation 3. A typical Cyclohexane spectrum is shown in figure 3 of Appendix A.
- 3.2.5. Neon-Argon Lamp: A Neon-Argon lamp is used to calibrate the wavenumber axis of the spectrometer. This specific type of lamp is used because it has thin, well defined spectral peaks that correspond accurately with specific wavelengths. A list of known pixel positions is cross referenced with a list of the known wavenumber peaks of Neon-Argon to calibrate the wavenumber axis. A typical Neon-Argon spectrum is shown in figure 4 of Appendix A.
- 3.2.6. NIST Glass: Spectrometers have a wavelength-dependent detection efficiency. This means that measurement intensity may vary depending on which wavelength is being detected. NIST glass (SRM2246) is used to correct for this wavelength-dependent response. A typical NIST spectrum is shown in figure 5 of Appendix A.

3.3. Research Goal

The setup's focused-beam measurements are not the ideal methodological approach for assessment of skin lesions. This is because the ideal instrument needs to be able to measure *in vivo* and not in excised lesions. The ultimate goal of the RASKIN project is to create a compact Raman spectrometer with a handheld fiber-optic probe that can be moved around and operated easily. However, because this technology is very new and realizing such a compact setup is a very expensive task, a proof of concept is required first.

Thus, the aim of this thesis is to prove that measurements can be done *in vivo* with a single fiber probe connected to an adaptation of the current setup, and that the results can be used to assist general practitioners and dermatologists in identifying melanoma.

3.3.1. Sub Goals

To be able to properly reach the main goal of this thesis, a hierarchy of smaller goals needs to be achieved first. Figure 13 will show this hierarchy, starting with the main goal on the left side and fanning out to sub goals that are required to reach it on the right.

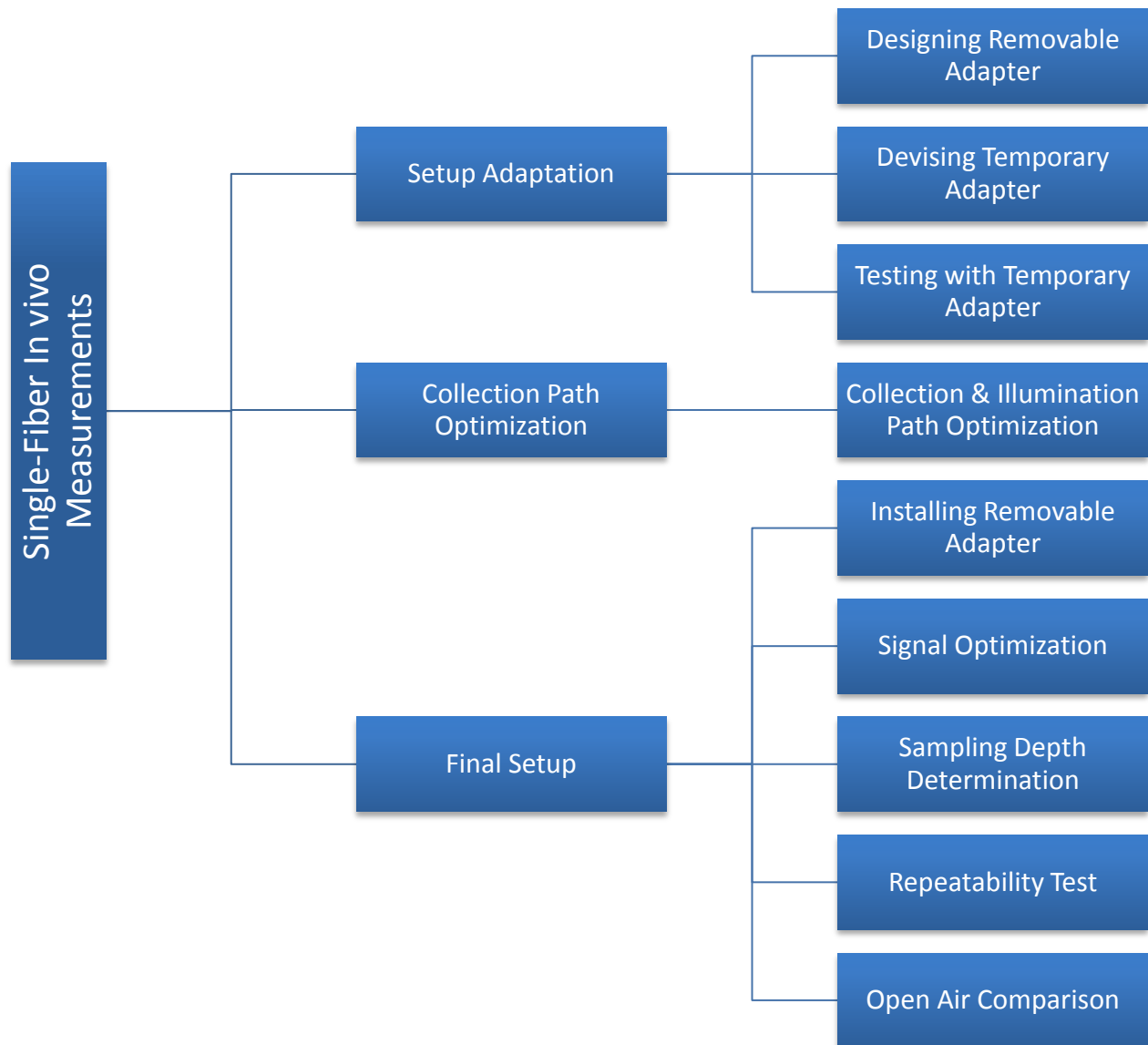


Figure 13, hierarchical representation of the main goal with its required sub goals.

4. Methods & Materials

As shown in the hierarchy before, the main objectives were (i) to adapt the setup for use with a fiber probe, (ii) to optimize the signal collection quantity and quality for effective and fast measurements and (iii) to prove that this method actually shows improvement over the current methods. To accomplish these objectives, several experiments or tasks were done for each one. Those experiments will be separately explained in this chapter.

4.1. Setup Adaptation

4.1.1. Removable Adapter

A removable adapter that allows to quickly transform the setup to be used with a focused-beam or with a fiber is required to effectively verify the method. This ability would serve to more easily be able to do measurements to compare both measurement methods.

However, due to the very small sizes and distances involved in working with fibers, the required accuracy of such an adapter is very high. This means that tolerances are low, and a very precise system had to be designed. First, a concept was theorized and sketched, as seen in Figure 14. This concept was brought to the engineering department which was then responsible for the finalized version of the adapter. Appendix D shows some results of calculations of limitations and requirements.

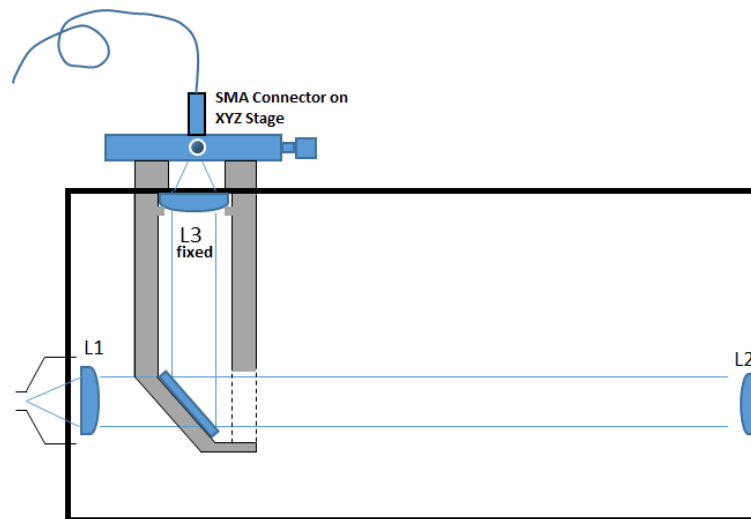


Figure 14, graphical representation of the removable fiber adapter concept.

The function of the adapter is simple; it can be inserted into the corridor between L1 and L2 (see Figure 10), which should be a near-perfect fit so that there is no room for horizontal offset.

A right-angle mirror (12.5mm, Silver, Thorlabs Inc, Germany) was used to fold the beam upward (where there is more room) towards an achromatic lens, L3 in Figure 14, (25mm FL, NIR II coated, EdmundOptics, Barrington NJ, USA) that was used to focus the light on the fiber connector. The connector itself was mounted in a 3-axis translatable stage to be able to place the fiber tip exactly in the

focus of the lens. A small metal plate was glued to the block containing the optics, which held dowel pins that were used to make sure the adapter was in the exact same location every time it was removed and placed back.

4.1.2. Temporary Adapter

Because of the complexity of the above design, and the need to wait for components to be delivered and built, a temporary adapter was required to be able to work and do verification measurements while waiting.

This adapter had to work in the same way, but did not need to have the same ease of use due to it only being temporary. This means the only requirements of the temporary adapter were that it could hold a fiber connector (SMA) and that it could translate the fiber precisely into the focus of L1, which is the lens in front of which the adapter would be placed. In Figure 10, the adapter would be placed in the location of the sample holder.

In order to meet the above requirements, a 3-stage translatable fiber adapter was used (Melles Griot) which could be controlled using micrometer screws. An SMA fiber connector was added to this using a groove in the center of the block. See Figure 15 for the block in position in front of L1.

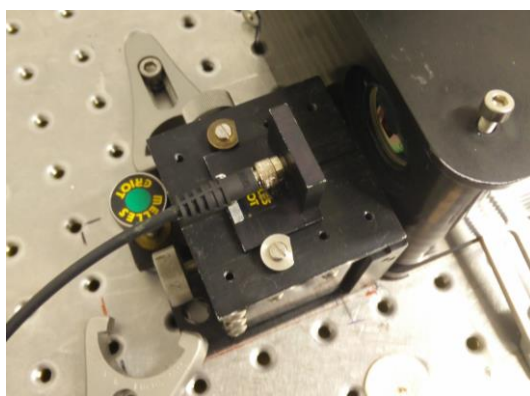


Figure 15, 3-stage translatable temporary fiber adapter for RASKIN setup.

While the block is in place in front of L1, the setup works in the same way as with the permanent fiber adapter. However, the temporary 3-axis stage was less accurate than the final one, and still required much manual alignment to be placed exactly in focus of the lens.

4.1.3. Testing the Temporary Adapter

As mentioned earlier, the temporary adapter was used to perform some tests and verifications during the development of the final adapter solution.

To verify whether the fiber could properly measure Raman signal measurements that are normally performed using the focused-beam setup were done. Calibration protocol according to Appendix B was followed to prepare the setup. After calibration measurements, signal was measured from different materials: Cyclohexane and skin, simply by placing the collecting end of the fiber in or on it.

4.2. Signal Optimization

One of the subgoals is to improve the Raman signal that is being collected, both in intensity and quality. This can be done by precisely (re)aligning the optical elements that are present in the setup.

4.2.1. Collection and Illumination Path Optimization

Important factors in improving the alignment of the illumination and collection paths are the optimization of laser power on the sample as well as maximization of the Raman signal on the detector. Increasing the amount of signal that is produced and thus collected can help reduce the amount of time that is needed to collect a quality spectrum (i.e. 'Integration time'). Moreover, this helps to reduce the influence of noise on the spectrum due to achieving a higher SNR. This is important because shorter measurement times mean less discomfort for patients when measuring *in vivo*.

Improving the alignment of the setup started by achieving the highest amount of power at the collecting end of the fiber as is possible.

Further optimization was done by improving the focus of L2 on the slit (Figure 16). To do this, lens L2 could be translated along its Z-axis (b in the figure).

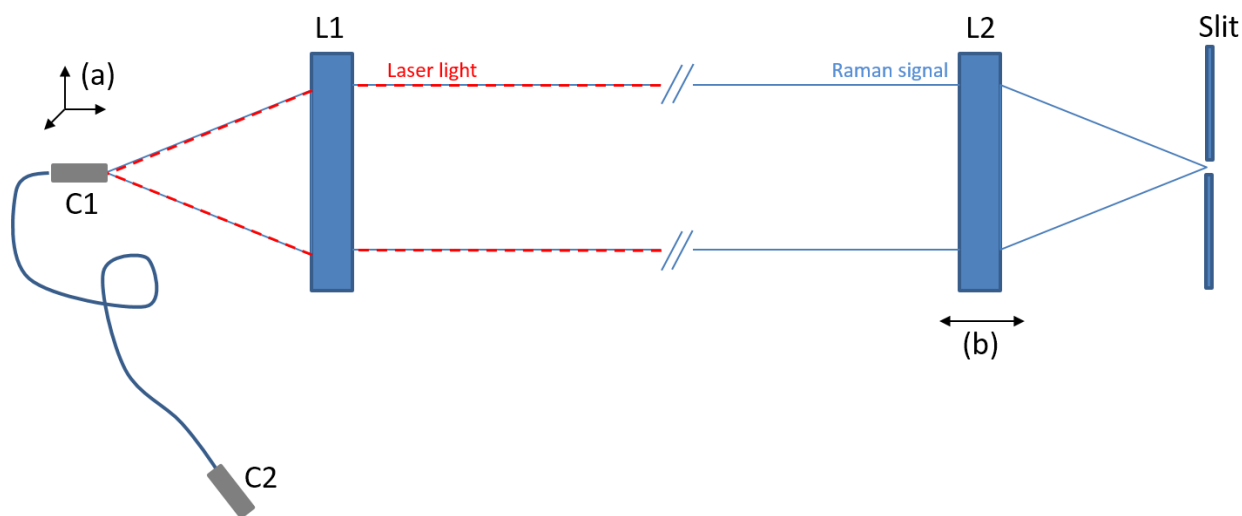


Figure 16, graphical representation of the collection path with lenses L1 and L2. L2 was moved along its Z-axis (b in the figure). C1 and C2 represent the fiber tips and the tilted lines are the filters that introduce the laser light from the laser.

To find the optimal location for L2, several measurements have been done using different locations. Measurements were done by collecting the signal of Ethanol (70% Vol.) and by comparing the intensity of the signal at the top peak in each case. Integration time used for these measurements was 15 seconds.

To finetune the alignment of L2, measurements were also done with a Neon-Argon lamp shining into the collecting end of the fiber, however this time the Full Width at Half Maximum (FWHM) of several peaks was considered instead of the signal intensity (peak height). The width of the peaks in pixels was used as a way to quantify signal quality, i.e. the wider the peak, the lower the quality of the signal. Measurements were done in several positions, where the FWHM of two peaks was considered, i.e. the left-most one (“Peak 1”) and the highest one (“Peak 2”), both are illustrated in Figure 17. Integration time was 5 seconds for Neon-Argon.

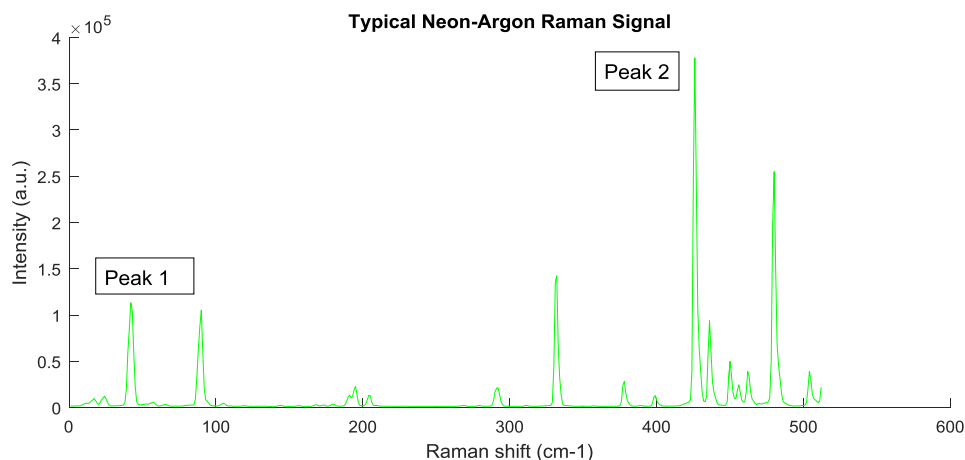


Figure 17, typical Raman spectrum of Neon-Argon light. The width in pixels of peaks 1 & 2 were used to quantify the signal quality, where a lower width means a higher quality.

4.3. Final Setup Signal Optimization

The new removable fiber adapter was introduced into the setup as soon as it was ready. To make sure it was working optimally, signal optimization and comparisons were done. This section will cover the steps that were taken to verify if the removable adapter was working optimally. Before placing and gluing the plate of the removable adapter, a placement and alignment protocol had to be formulated and followed. This protocol is shown in Appendix C.

4.3.1. Signal Optimization

Just like with the temporary adapter, signal optimization was required to get the best possible signal. However, unlike the temporary adapter, the removable adapter was designed to work side by side with the focused-beam measurements. This means that the setup, primarily lens L2, had to be aligned for both of them. Since L2 alignment was already done using the focused-beam ‘mode’ of the setup, the slit was already in focus of L2, which should not be touched again to align the removable adapter.

Because the lens in the removable fiber adapter L3 (see Figure 14) as well as lens L2 were unable to be moved, the only method to align the removable adapter was by using the 3-axis translatable fiber connector built into the adapter. To do and verify this, the same method was used as with the temporary adapter as explained in section 4.2.

First, the fiber core was moved into the true focus, or 'c' in Figure 18. This is necessary because, while optimal power may be achieved anywhere between points (a) and (b) in the figure due to the beam spot being smaller than the fiber core in this range, this is not necessarily the true focus of the beam. To find the true focus (i.e. the minimum beam waist), (a) and (b) were defined as points where fiber power started to drop (i.e. points where the beam waist size exceeded the fiber core size). The point in between (a) and (b) is the true focus c.

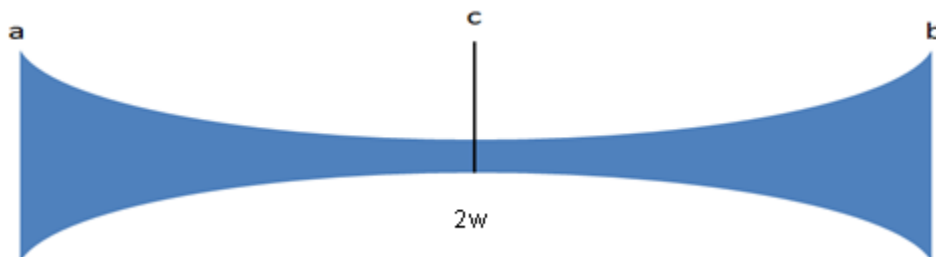


Figure 18, graphical representation of a diverging and then converging beam profile. 'a' and 'b' indicate the thresholds where the beam diameter exceeds the fiber core, 'c' indicates the center of the beam waist.

Then, the Z-axis of the fiber connector, which is the axis perpendicular to lens surface, was used to finetune the Neon-Argon signal. However, during the Neon-Argon measurements, it was found that the FWHM did not seem to change as the fiber connector Z-axis was changed. The intensity of the signal, however, did vary significantly. Thus, instead of using Neon-Argon, it was decided that a Raman signal produced from a liquid would produce more reliable results for intensity, because the Neon-Argon signal intensity is dependent on the position of the sample.

Measurements were then done using ethanol (70% Vol.) on a total of six different positions of the Z-axis of the fiber connector. Intensities were measured for all 3 ethanol peaks (see figure 6 in Appendix A), in order from left to right, for each fiber position. The fiber connector Z-axis was moved down slightly with each position. Again, integration time was 15 seconds for Ethanol.

4.4. Sampling Depth

An important piece of information that was needed to be able to accurately interpret spectra obtained from pigmented skin lesions using the fiber-optic probe is the sampling depth of the measurement. The sampling depth is defined here as the maximum depth from which signal can still be measured. This depth is influenced by absorption of signal as well as scattering of the signal.

Due to the very detailed and varying nature of the properties of human skin, it is difficult to impossible to determine the sampling depth accurately using skin. For example, the optical properties change depending on location of measurement, temperature, anatomy of the patient, blood flow and many other factors.^{[32][33]}

However, in order to get an estimation that better corresponds with the RASKIN setup, an experiment was done using a 20% intralipid emulsion (Fresenius Kabi BV, Netherlands). Intralipid emulsions are often used to simulate the absorbing and scattering properties of skin. Combined with it being a liquid, a simple experiment was devised to approximate what the maximum depth is that still yields a Raman Signal in skin. Before using the intralipid solution, this experiment was done using water to test its feasibility.

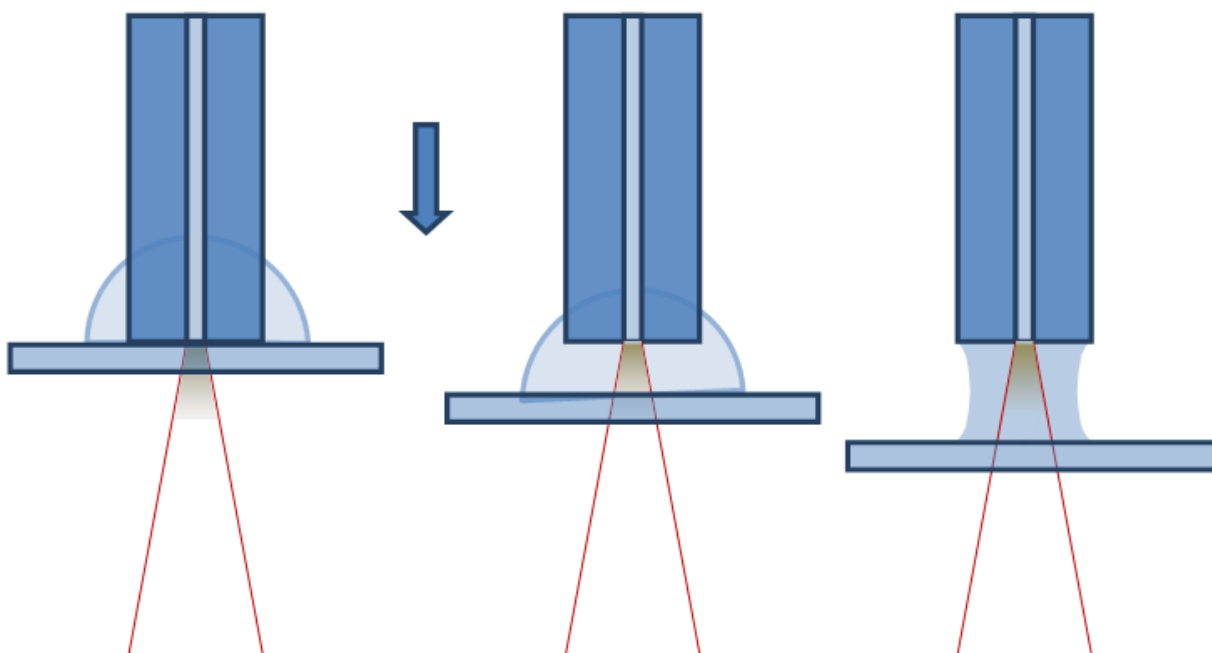


Figure 19, graphical explanation of the sampling depth measurement. The grayed part in the cone of light indicates the cone of unknown length that the spectrometer is able to get signal from. The cone is fully inside the liquid on the right, which is the point where signal intensity should stop increasing.

For both the intralipid emulsion and water, a few drops of liquid were put on a fused silica glass plate, as shown in Figure 19. Fused silica was used because of its lack of Raman signal in this wavelength range^[7], which means it can function as a surface that does not influence the experiment. The fiber was mounted in an accurate 1-Axis translatable micrometer stage. The initial position of the fiber tip was inside the liquid touching the silica glass, leaving no room between the glass and the fiber for the liquid.

It was then translated away from the glass, effectively increasing the measured signal intensity, in steps of 50 or 20 μm until a point where the signal intensity stopped increasing. This method is also graphically explained in Figure 19. Integration times were 30 seconds for each measurement.

The point at which the gray cone is fully inside the liquid should be the point at which the intensity no longer increases, since it will not 'see' an increasing volume anymore when it is moved further up. Due to the small steps the fiber was moved up with, the height of the cone was determined and an estimation was made of the maximum signal collection depth.

4.5. Repeatability

An important aspect of the performance of the removable fiber adapter is its repeatability. It has to be able to consistently produce the same results when being placed in or removed from the setup. In order to verify that the setup is consistent and results are reproducible, a simple experiment was done.

The distal end of the fiber was fixed inside ethanol (70% Vol.). Ethanol was chosen because of its strong peaks and because it is a liquid, making it stable and taking the variability of placing the distal end of the fiber on the sample out of the picture. While keeping the distal end fixed, the removable adapter was repeatedly removed and then replaced. Each time that it was placed back, three successive spectra were measured. The adapter was removed and placed back 5 times and 3 spectra were measured each time, resulting in 15 spectra in total.

Each set of 3 spectra was averaged to correct for any irregularities that might have occurred that had nothing to do with the removable fiber adapter. The 5 resulting averaged spectra were then scaled using MATLAB's "Extensive Multiplicative Scatter Correction" (emsc) function.^[34] These scaled spectra were then averaged after which each of the 5 individual spectra were compared to the average of the five to get a measure for deviation in shape.

4.6. Comparison

With all the calibrations and optimizations done, the fiber measurements had to be compared to the focused-beam measurements in order to prove that the concept could work.

To compare the quality of the spectra, measurements were done on excised skin lesions using both measurement methods on the same lesion. Several points were measured on each lesion with each method. The SNR will be used as a means of comparison, where a higher ratio could indicate a better performing method.

All spectra will first be processed and background subtracted. However, the spectra are not perfect after this as they still show a sloped offset. This slope originates from fluorescence of the skin. Figure 20 shows an example of such a sloped skin spectrum.

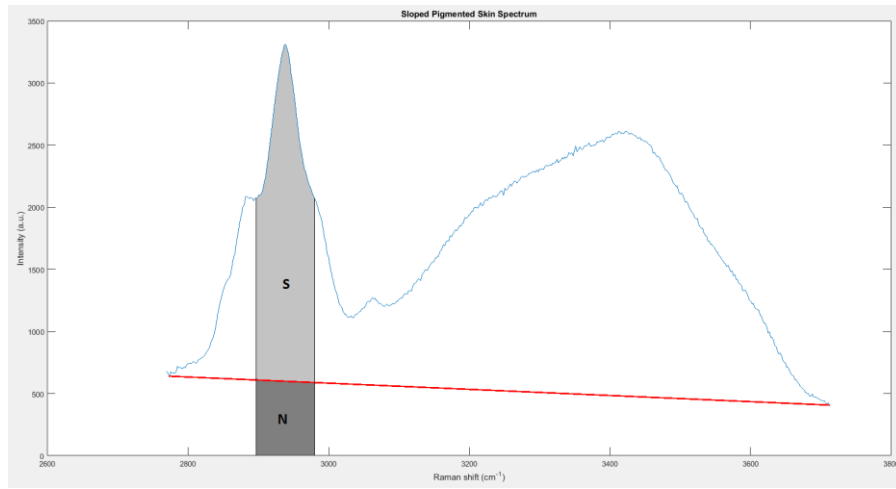


Figure 20, skin spectrum as used to determine the SNR. The sloped area is the fluorescence noise, the vertical blue lines indicate the area of interest for the SNR calculation.

The intensities in the area of interest, which is marked by the vertical lines, will be summed for each spectrum, resulting in the total signal S present in the area of interest. The fluorescence noise will be determined by summing the values of the slope along the area of interest. Shot noise will be approximated using the square root of the total signal S . The total noise N is then equal to the square root of the fluorescent noise and the shot noise. See equation 4.1. Note that in this calculation, the readout noise N_{READ} will be left out because it is considered negligible compared to the rest using this setup.^[5] The dark noise N_{DARK} is part of the processing procedure from raw to processed spectra so it does not need to be taken into consideration for this anymore either.

$$N_{TOTAL} = \frac{S}{\sqrt{S + N_{FLUOR}}} \quad (4.1)$$

The ratio of the total signal S and the total noise N will be the signal-to-noise ratio that can be used to compare the quality of the measurement methods. This method was applied to six different lesions and each of those lesions can then be compared between both methods.

5. Results & Discussion

This chapter will cover and discuss the results from the experiments that were detailed in chapter 4.

5.1. Setup Adaptation

This section will cover the results of designing the removable fiber adapter and testing the temporary fiber adaptation of the RASKIN setup.

5.1.1. Removable Adapter

Using equation 2.7, it was calculated that the maximum acceptance angle α_{max} of the fiber would be 12.7° , using 0.22 as the NA of the fiber. This means that the signal that travels back from the fiber towards the detector exits the fiber with the same angle, which geometrically limits the choice of lens and mirror.

It was calculated that, in order to have the lens and mirror fit in the existing setup, half inch ($\approx 12,5$ mm) optics had to be used. Furthermore, since the beam cannot be bigger than that either, the maximum focal length assuming a diverging angle of 12.7° was 35 mm. Lengths above this would result in light being wasted on the sides of the lens. See Figure 21 for a sketched representation. The results of all calculations that were done for the removable adapter are included in appendix D.

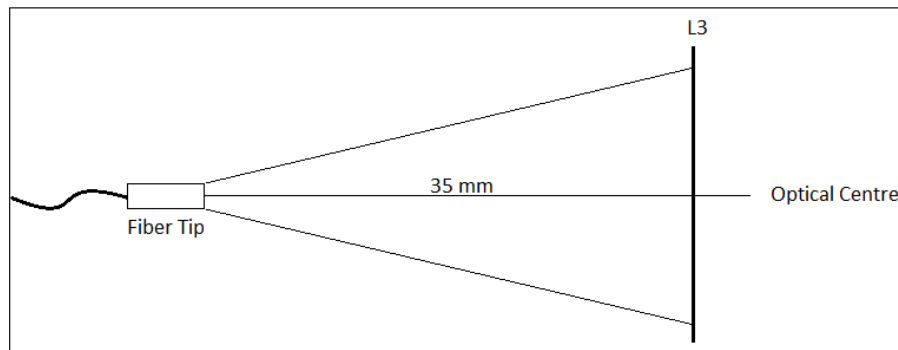


Figure 21, sketch of the signal cone exiting the fiber tip and entering lens L3.

After the calculations had been verified and the parts had been ordered, manufacturing began. The end result of the designed adapter is shown in Figure 22.

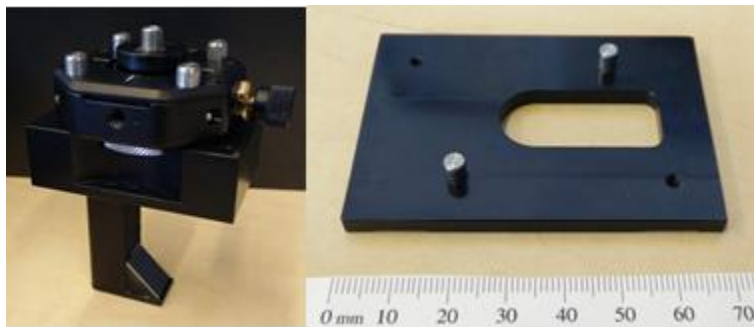


Figure 22, the finished removable adapter as it turned out to be (left) and the dowel-pin plate that ensures position reproducibility (right).

5.1.2. Testing the Temporary Adapter

Initial results of measurements with the temporary adapter were promising, because spectra was obtained of the measurements of among others cyclohexane (Figure 23), and background signal generated in the fiber proved to be minimal using this laser as opposed to using a 671 nm red laser, as is visible in Figure 24.

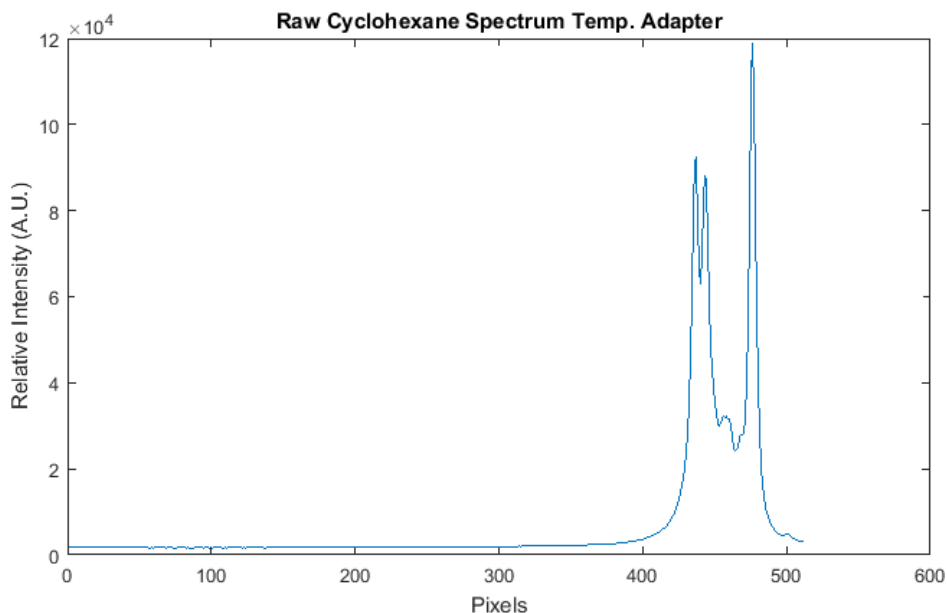


Figure 23, unprocessed cyclohexane spectrum using the temporary adapter in the RASKIN setup (976 nm).

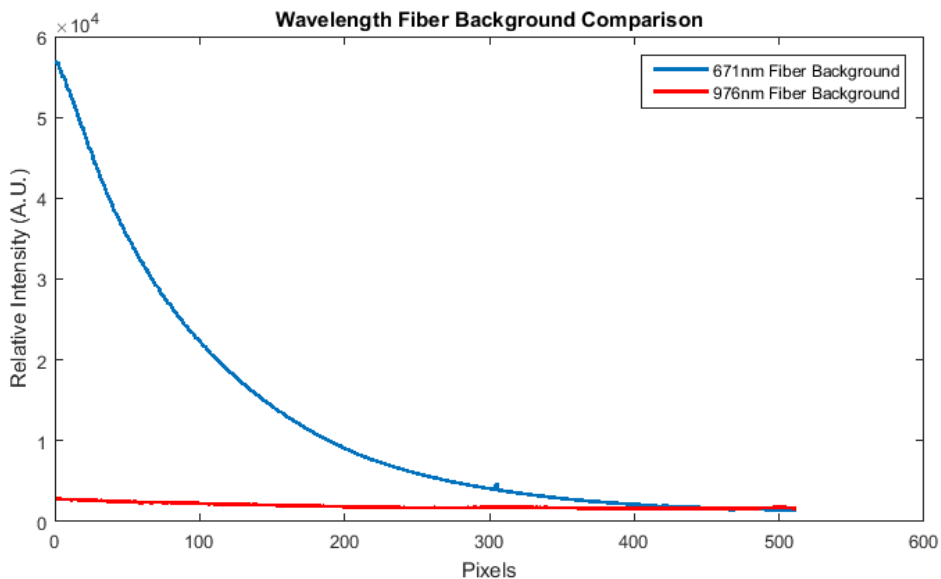


Figure 24, comparison of fiber background signal using different excitation wavelengths in the same fiber.

Previous research using the same fiber materials suggested a high background signal generated by the laser in the fiber core.^[7] However, Figure 24 shows that this is not true for 976 nm. The most likely explanation for this is that the background signal generated from the fiber is highly wavelength dependent. This result is significant, as it enables the use of commercially available fibers, over custom ones with specific materials, with this wavelength and in this wavenumber area. However, the spectrum of skin (see Figure 25) was noisy compared to focused-beam measuring, suggesting improper or imperfect alignment in the setup.

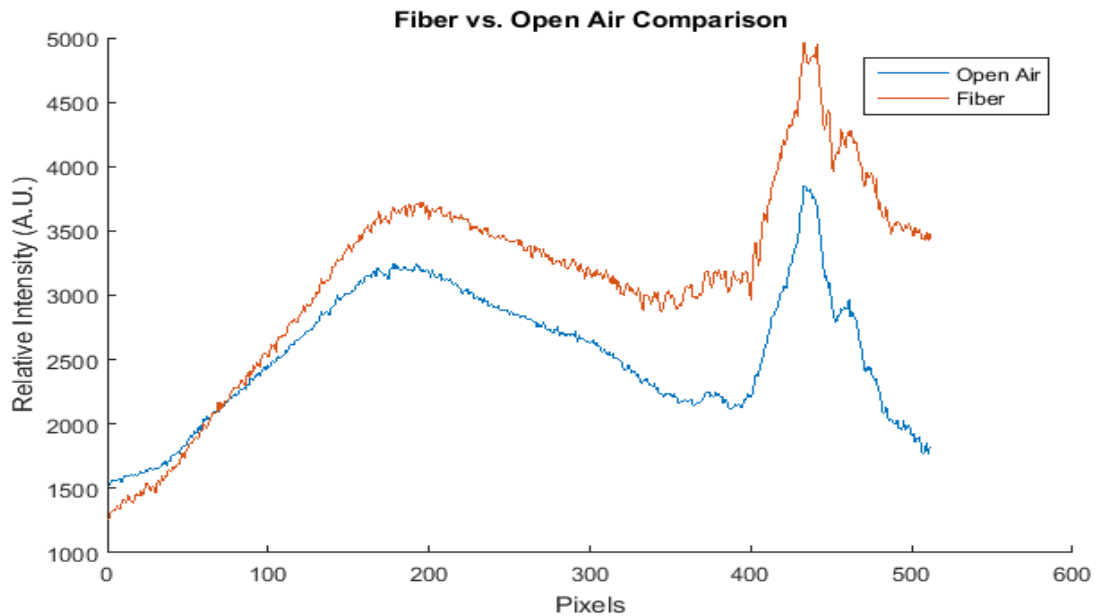


Figure 25, skin measurement comparison between existing focused-beam method and with a fiber using the temporary adapter.

5.2. Signal Optimization

This section will cover the results of the experiments done to optimize the collection path of the RASKIN setup.

5.2.1. Signal Intensity

A laser power of 90% was achieved at the distal tip of the fiber relatively quickly by translating the fiber core using the 3-axis stage to be in focus of the lens L1 (a in Figure 16). Power dropped on (rare) occasion, but this was solved by cleaning the fiber tip thoroughly. The loss of 10% power could be explained by inherent losses in the fiber and the couplings.

The results of the intensity measurement on Ethanol are shown in Figure 27 and the numbers at the peaks correspond with the numbers shown in Figure 26.

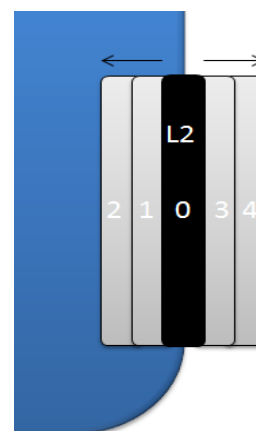


Figure 26, sketch of the locations of L2 during the signal quality experiment.

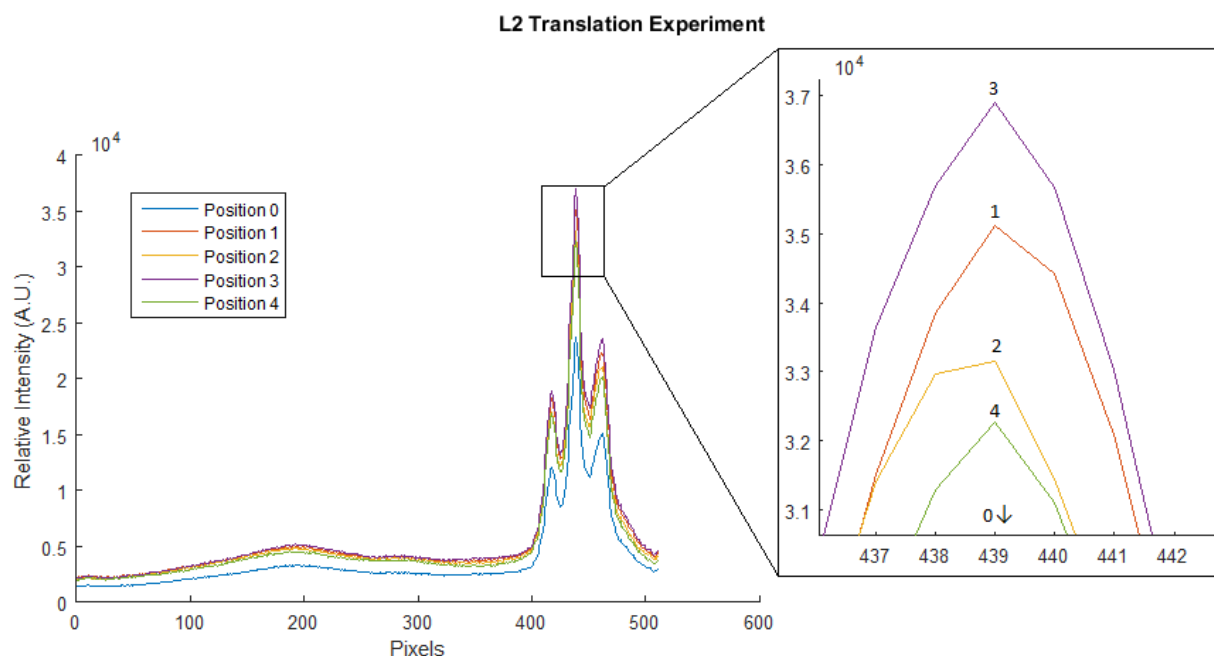


Figure 27, signal intensity comparison in ethanol Raman signal at different positions of L2. Position 3 shows the highest peak intensity.

Position 3 shows the highest intensity, and was thus chosen as the optimal location for L2, which was fixed in this location. The overall increase in intensity due to this optimization was about 55% with respect to the initial position (0) of L2, which showed a relative intensity of about $2.37 \cdot 10^4$. This big increase in intensity due to a relatively small change in the position of lens L2 reiterates the importance of proper and exact alignment.

Signal quality was also optimized using the FWHM of a Neon-Argon signal. The width of two peaks are the measure for signal quality (wider peak is lower quality). Spectra were measured at several (slightly) different positions of lens L2. Table 1 shows the FWHM values corresponding to each spectrum for their respective peaks. The peaks are indicated in Figure 17.

Table 1, FWHM numbers for both considered peaks in each measurement.

	Peak 1 FWHM (Pixels)	Peak 2 FWHM (Pixels)
Position 0	$5,5 \pm 0,2$	$2,9 \pm 0,2$
Position 1	$4,2 \pm 0,2$	$3,1 \pm 0,2$
Position 2	$4,8 \pm 0,2$	$2,9 \pm 0,2$

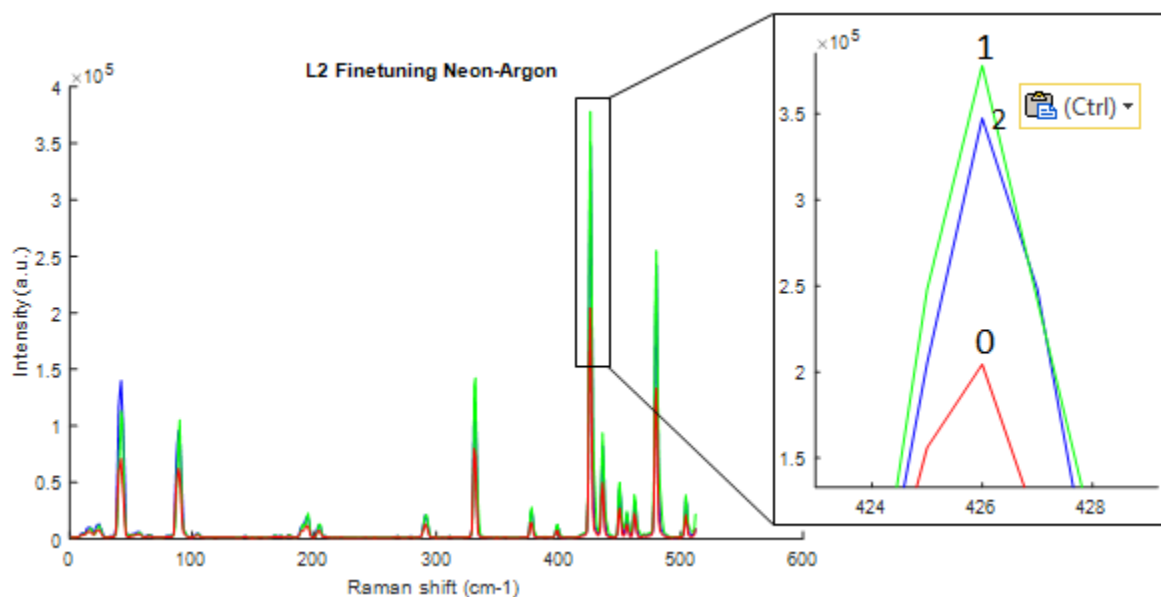


Figure 28, signal FWHM and intensity comparison in Neon-Argon spectra at different positions of L2. Position 2 shows the best average of intensity and FWHM.

It is important to note that the spectrum area of interest in this thesis, which is the CH-stretching band of skin, is located mainly in the higher pixel range of the detector. This corresponds roughly with the location of the more intense peaks of the Neon-Argon spectrum. Because of this, a thinner FWHM of the higher pixel peaks (i.e. peak 2) is considered a higher priority than a thinner FWHM of the lower pixel peaks (i.e. peak 1).

When both the figure and the table are then taken into consideration, position 2 shows the best ratio between (peak 2) FWHM and intensity. The FWHM improved slightly, mostly in the first peak, and the intensity increased significantly (about 75%) compared to the starting position. Therefore, position 2 was chosen to fix L2 at. Compared to spectra before the optimizations, signal intensity has increased to about $1 \cdot 1.55 \cdot 1.75 \approx 2.7 \approx 270\%$, meaning a total increase of 170%.

5.3. Final Setup

With the removable adapter ready, calibrations had to be repeated for the newly adapted setup. L2 had been adapted already, leaving only L3's (see Figure 14) intensity optimization to optimize the signal collection. Sampling depth has also been estimated and comparisons between focused-beam and the removable fiber adapter have been done. This section will cover the results of the experiments that were done to finalize, test and verify the modified RASKIN setup.

5.3.1. Signal Optimization

The only variable left to optimize with L3, the signal quality, was optimized using ethanol (70% Vol.) because it is stable and gives clear and strong peaks. Table 2 shows the results for the 6 measured positions. The individual spectra to go with the table are shown in Figure 29.

Table 2, intensity numbers for all considered ethanol peaks in each measurement on different positions of the Z-axis of the fiber connector.

	Peak 1 Intensity ± 8 (a.u.)	Peak 2 Intensity ± 8 (a.u.)	Peak 3 Intensity ± 8 (a.u.)
Position 0	$1,175 \cdot 10^4$	$2,218 \cdot 10^4$	$1,447 \cdot 10^4$
Position 1	$1,323 \cdot 10^4$	$2,508 \cdot 10^4$	$1,616 \cdot 10^4$
Position 2	$1,392 \cdot 10^4$	$2,620 \cdot 10^4$	$1,700 \cdot 10^4$
Position 3	$1,471 \cdot 10^4$	$2,772 \cdot 10^4$	$1,803 \cdot 10^4$
Position 4	$1,641 \cdot 10^4$	$3,106 \cdot 10^4$	$2,020 \cdot 10^4$
Position 5	$1,507 \cdot 10^4$	$2,807 \cdot 10^4$	$1,863 \cdot 10^4$

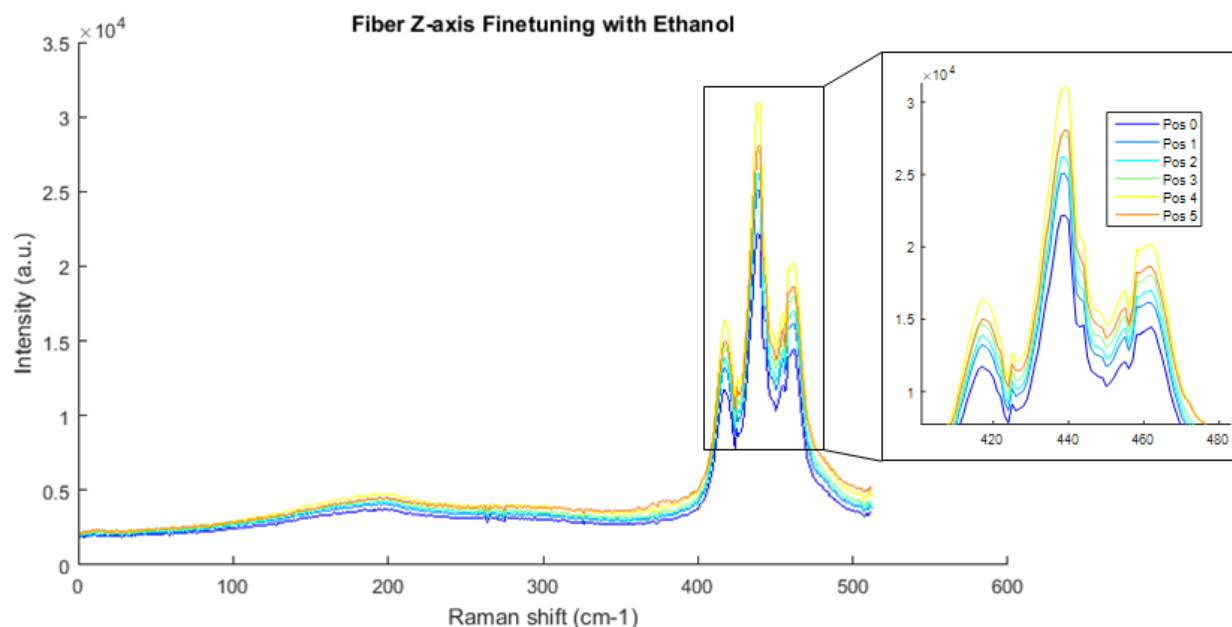


Figure 29, signal intensity comparison in ethanol signal at different positions of the Z-axis of the fiber connector. Position 4 (yellow) shows the highest intensity.

Position 4 showed the highest intensity in all peaks and was thus chosen as the optimal position for the Z-axis of the fiber connector. This position resulted in an overall increase in signal intensity of about 40%. The setup is now in the optimal position, with both the focused-beam path and the fiber adapter path fully aligned and optimized.

5.4. Sampling Depth

Sampling depth measurements were done using water first. The expectation is that signal is able to penetrate deeper into water due to its lack of scattering particles. Measurements done on water were done taking steps of 50 μm . The resulting spectra were integrated to be able to plot the area under the curve (of the OH band) as a measure of intensity at that distance from the silica glass. Results are shown in Figure 30.

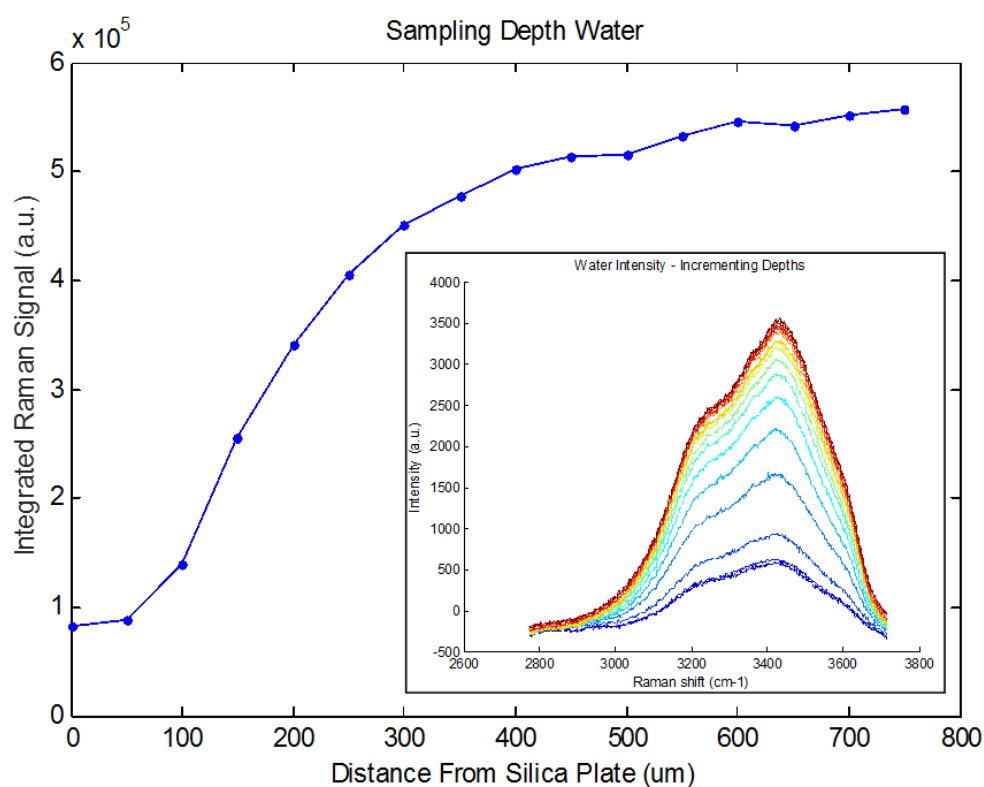


Figure 30, sampling depth results in water. The bigger figure shows the integrated intensity at each measured distance from the silica plate. The smaller figure shows the processed spectra from each measurement.

The curve in the figure shows an increasing integrated intensity up to 750 μm , which was the last point that was measured. However, the increase in signal intensity seems to slow down considerably after about 500 μm . Beyond that distance, the chance of scattered light reaching the detector gets small but is not zero, which could be an explanation for the slight increase in intensity until 750 μm . Since water does not have strong scattering properties like the intralipid solution or human skin, sampling depth in those is not expected to be above 500 μm based on the results of the measurements in water.

The intralipid solution (20%) was measured in smaller steps of 20 μm to provide more detail in the results. Again, the resulting spectra were integrated to get an area-under-the-curve intensity (of the CH band) instead of peak intensity. Results are shown in Figure 31.

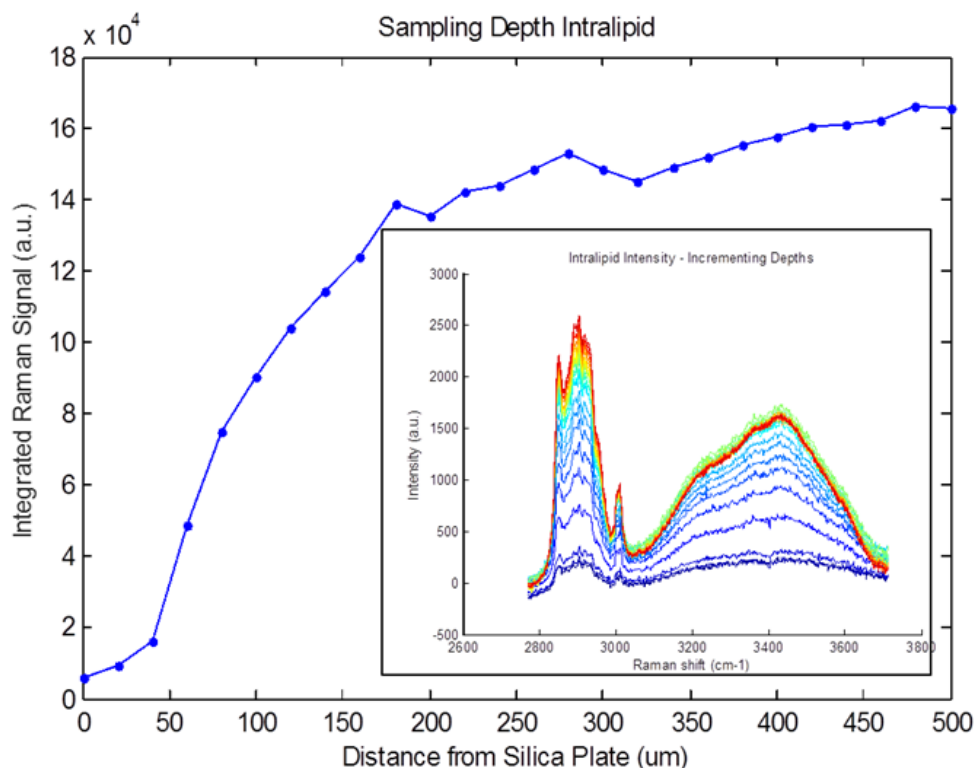


Figure 31, sampling depth results in the intralipid solution (20%). The bigger figure shows the integrated intensity at each measured distance from the silica plate. The smaller figure shows the processed spectra from each measurement.

Measurements were done from 0 to 500 μm distance to the silica plate. Again, signal does not really stop increasing, but it does stabilize slightly around 300 μm . As expected, sampling depth seems much shallower than in water, which is probably due to the strong scattering properties of the intralipid solution. The first 300 μm cover $1 - \frac{0.5 \cdot 10^4}{14 \cdot 10^4} \approx 96\%$ of the total signal intensity. This is a good result as it covers the area of interest for melanoma detection as explained in chapter 2.3. Irregularities in the curve could be due to varying concentrations of scattering particles during measurements.

5.5. Repeatability

First, three successive Ethanol spectra were taken for each time the removable adapter was removed and placed back. These spectra were emsc-scaled to be able to focus on difference in shape. The resulting scaled spectra can be seen in Figure 32.

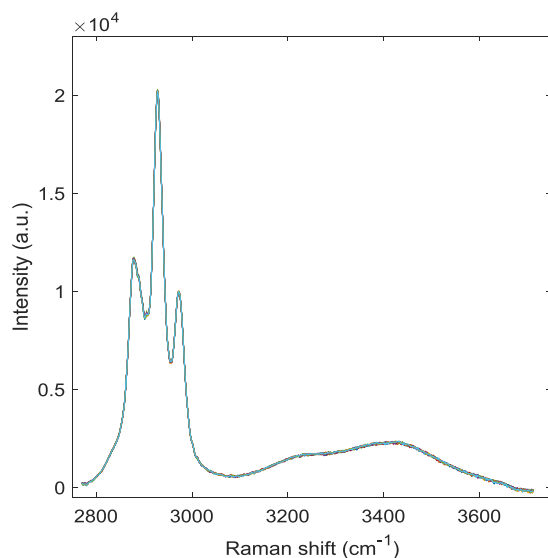


Figure 32, 5 emsc scaled spectra of ethanol (70%). Each spectrum is an average of 3 successive measurements.

Next, the difference between any of the five spectra with respect to the average of the five was calculated. This is an indication of the reproducibility of the removable adapter. The resulting difference graph is shown in Figure 33.

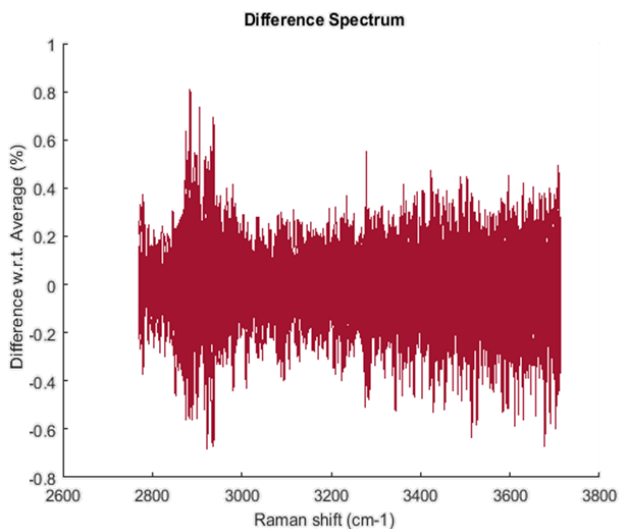


Figure 33, difference spectrum between the scaled ethanol spectra and their average.

The maximum deviation of a spectrum with respect to the average is 0.81%, which means that replacing the adapter will produce results that are repeatable within 0.81%.

5.6. Comparison

The first comparison as described in section 4.6 is shown in Figure 34. It shows the processed spectra for both measuring methods, as well as a distribution of the SNR that was calculated for each individual spectrum.

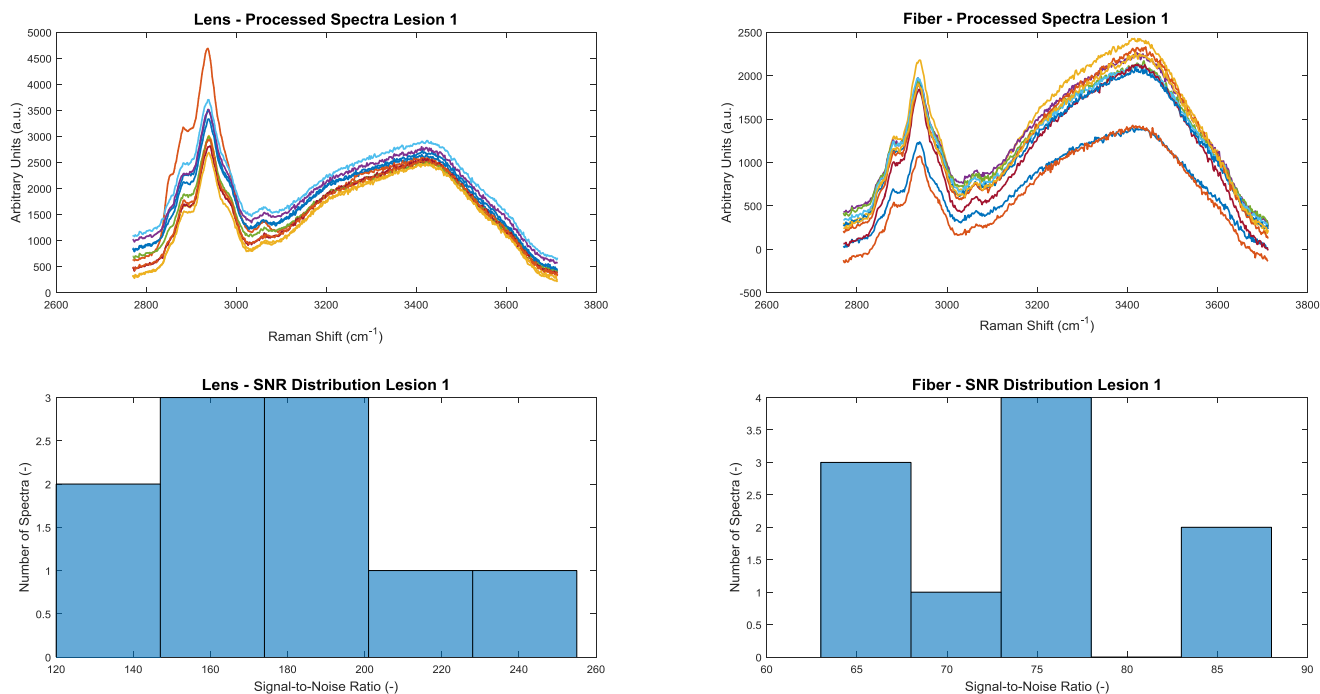


Figure 34, comparison between lens (focused-beam) and fiber measuring methods. The left side shows the spectra taken with the lens with a distribution of SNRs of each individual spectrum. The right side shows the same for the fiber spectra.

Figure 34 shows that the focused-beam setup seems to produce a much more consistent SNR in its measurements than the removable fiber adapter. The same steps have been taken for the other of the other six lesions. The signal-to-noise ratios were averaged for each lesion and listed in Table 3.

Table 3, average SNRs for the lens and the fiber adapter per lesion. The values are rounded to whole numbers.

Lesion Number	Average Lens SNR	Average Fiber SNR
1	182	74
2	109	56
3	79	95
4	214	56
5	186	44
6	427	43
Combined	219	59

The table shows that the SNR seems to be higher for the lens in almost every lesion. The average combined SNR of the lens is also considerably higher than that of the fiber.

However, SNR is not necessarily a good measure of how good a measurement method is. A high level of fluorescence noise will result in a lower SNR, while it does not necessarily make a spectrum bad.

Therefore, the average summed intensities and noise levels will be considered per lesion as well. The calculated values for all lesions are shown in Table 4.

Table 4, calculated average intensities for each lesion for both measuring methods. Uncertainty in the arbitrary units was determined using [5].

Lesion Nr.	Avg. Signal Fiber ± 8 (a.u.)	Avg. Signal Lens ± 8 (a.u.)	Avg. Noise Fiber ± 8 (a.u.)	Avg. Noise Lens ± 8 (a.u.)
1	15751	52647	212	290
2	13275	37517	236	344
3	19987	22512	210	285
4	10054	63272	181	295
5	14174	91068	320	490
6	15315	217387	354	509
Combined	14759	80734	252	369

Here, ‘Signal’ represents the integrated amount of photons per second S as shown in Figure 20 and ‘Noise’ is equal to the total noise N from the same figure. The table confirms that the focused-beam setup outperforms the fiber adapter when it comes to signal intensity. However, it is worth noting that the average noise in the spectra measured with the fiber is consistently lower.

While the spectra taken with the focused-beam setup show an almost 450% higher average intensity, they only show a 46% increase in average noise. From this it can be concluded that the lens still outperforms the removable fiber adapter by a significant amount. It is possible that the fiber measurements could still provide enough information to distinguish melanoma from other lesions and healthy skin, but more research is required to test this.

6. Conclusion & Discussion

In order to adapt the existing pre-clinical RASKING Raman setup for *in vivo* measurements, a removable fiber adapter was designed to be able to measure both with the focused-beam layout or with a fiber-optic probe. The choice of parts for this design were limited to the dimensions of the existing equipment, which left half-inch optics as the most practical possibility.

A temporary adapter was used during construction of the removable one in order to be able to get familiar with the process of fiber-optic Raman measurements and its complications. Measurements were found to be possible using a single fiber and the NIR laser, but low SNR proved to be a problem in skin spectra particularly. This meant optimization had to be done to improve the signal to be of sufficient quality to compare to the focused-beam measurements and to reduce the integration time per measurement.

To improve the collection path of the spectrometer using the temporary adapter, the distances between the slit and L2 and between the fiber tip and L1 were adjusted to be more precisely in focus. These optimizations resulted in improvements of signal intensity of 55% and 75% respectively, totalling an increase of about 170% from the starting position. This considerable increase in intensity meant that, in the same integration time, the SNR improved as well.

After the removable adapter was done, L1 and L2 were already fixed in their optimal positions, so L3 was the only lens that still needed aligning. This was done by fixing the distal end of the fiber in Ethanol, which was chosen for stability. After optimization of L3, signal intensity went up by about 40% compared to the situation before L3 optimization.

As part of the verification of measuring with a single fiber optical probe, the sampling depth of the probe was estimated using an intralipid solution to simulate the optical properties of skin. It was found that the bulk (>95%) of the signal originates from the first 300 μm of the intralipid solution. It is therefore reasonable to assume that the fiber does not measure deeper than 300 μm in skin either. This is a positive result, as this corresponds with the region of interest in skin.

To test the consistency of results when removing and replacing the removable adapter, a test was conducted using the signal of Ethanol, which was chosen because of its stability due to being a liquid. Measurements using the fiber adapter proved to be consistent to within 0.81%.

Finally, focused-beam measurements on excised skin lesions were compared to measurements on the same lesions with the fiber-optic probe. Signal-to-noise ratio was used as an indication of which method performs better. The focused-beam setup proved to be significantly better than the fiber adapted setup, showing signal-to-noise ratios of 219 and 59 respectively, although the fiber adapted setup showed slightly lower average noise compared to the focused-beam. The average noise levels were 252 and 369 (a.u.) respectively. However, this does not rule out the possibility of the fiber adapter being sufficiently good to be able to distinguish melanomas from other lesions. More tests are required to determine this.

7. Future Works

The RASKIN setup is adapted for use with a simple fiber-optic probe, but only few measurements have been done on excised skin lesions. In order to accurately evaluate its performance compared to the previous (focused-beam) method, more measurements on lesions are required using both methods.

The signal-to-noise ratios of spectra from the fiber were significantly lower than those from the focused-beam method. However, this does not mean that the fiber is unable to distinguish melanoma from benign skin lesions. Measurements done using the fiber-optic probe could be evaluated using the existing method that was used to evaluate the spectra taken with the focused-beam method before. The results can then be compared to histopathological findings which will give an indication of the ability of the fiber-optic probe to distinguish melanomas from benign lesions.

Regardless of that outcome, improvements can be made to the quality of the spectra that were taken with the fiber. Experimenting with an increase in radius of the fiber core is one option. While increasing the fiber core radius will also increase the relative amount of signal that is lost due to magnification, it has not been researched if it has an effect on the quality of the spectra.

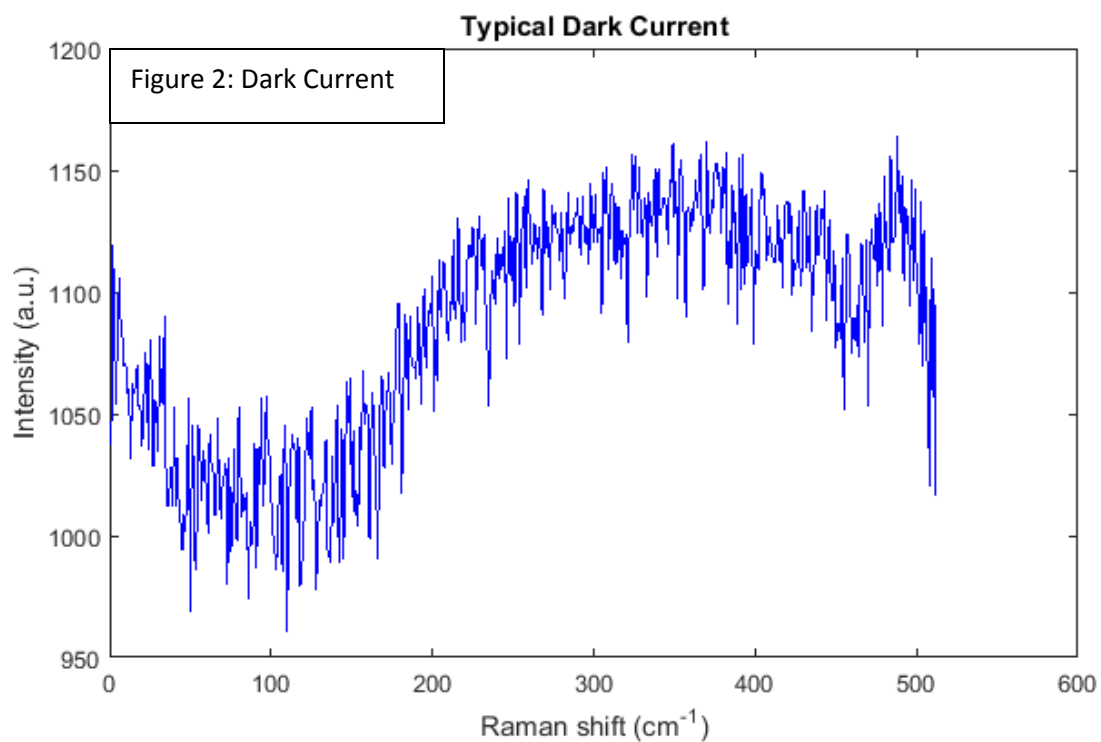
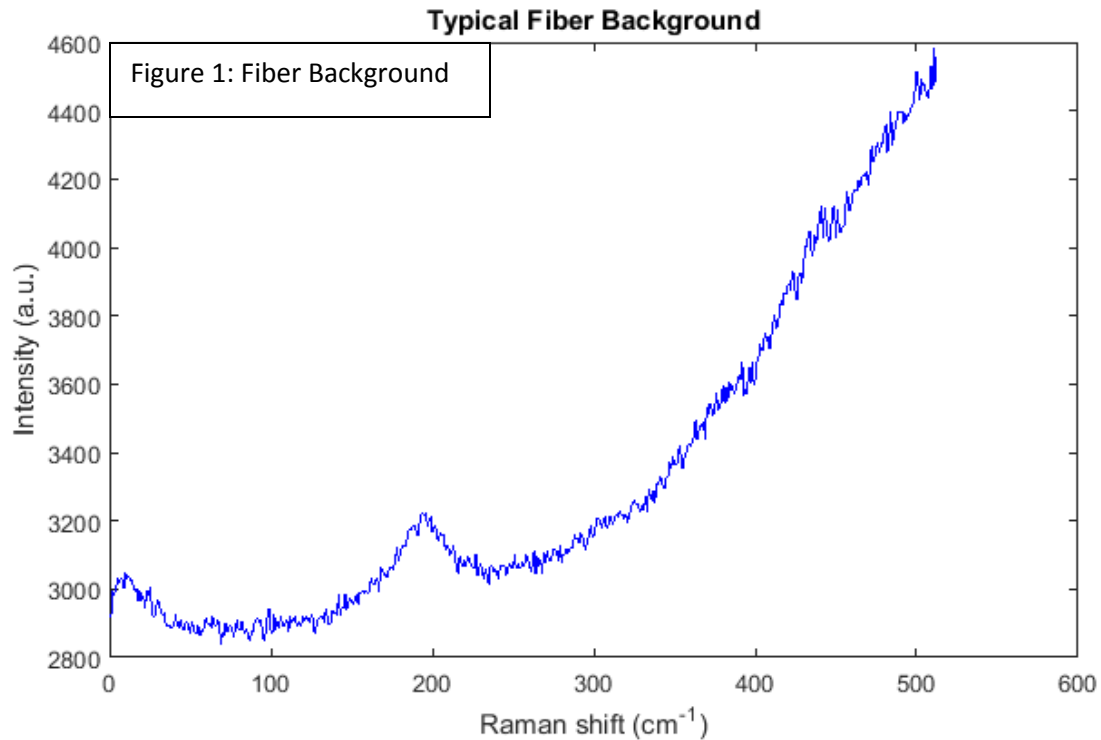
Furthermore, laser intensity has been kept at a stable level throughout all experiments. No experiments have been done to discover the effect of excitation intensity on the spectra and the quality thereof. While this was mainly due to the inability to vary laser power, it is something that needs to be considered before drawing conclusions.

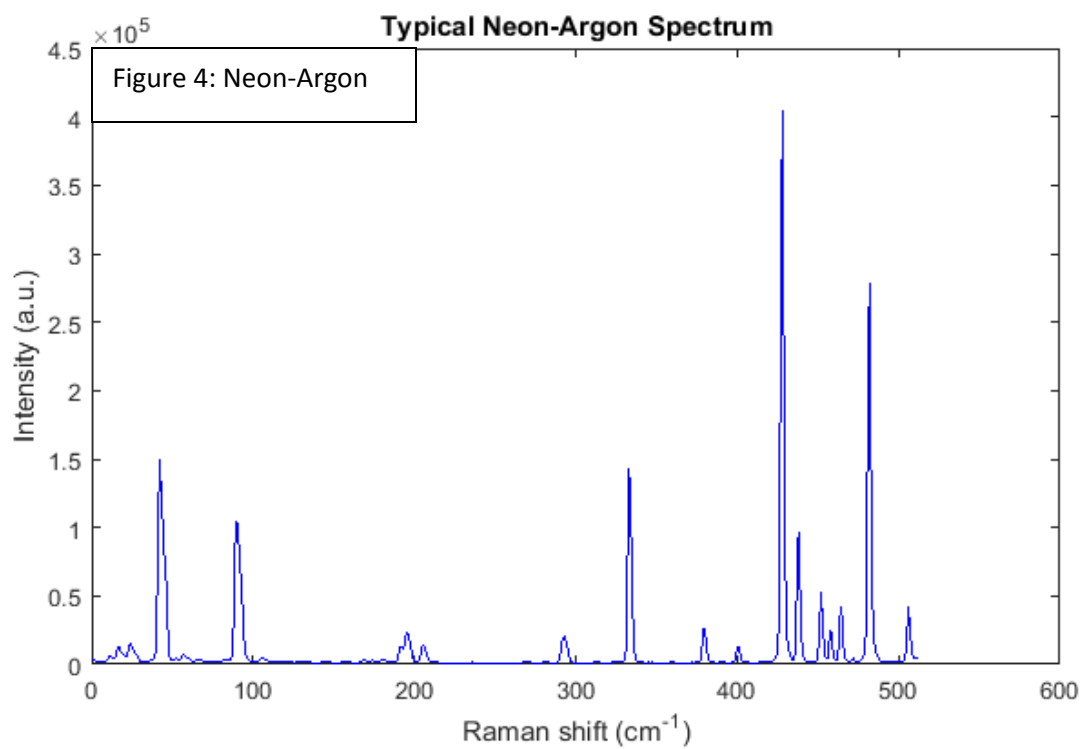
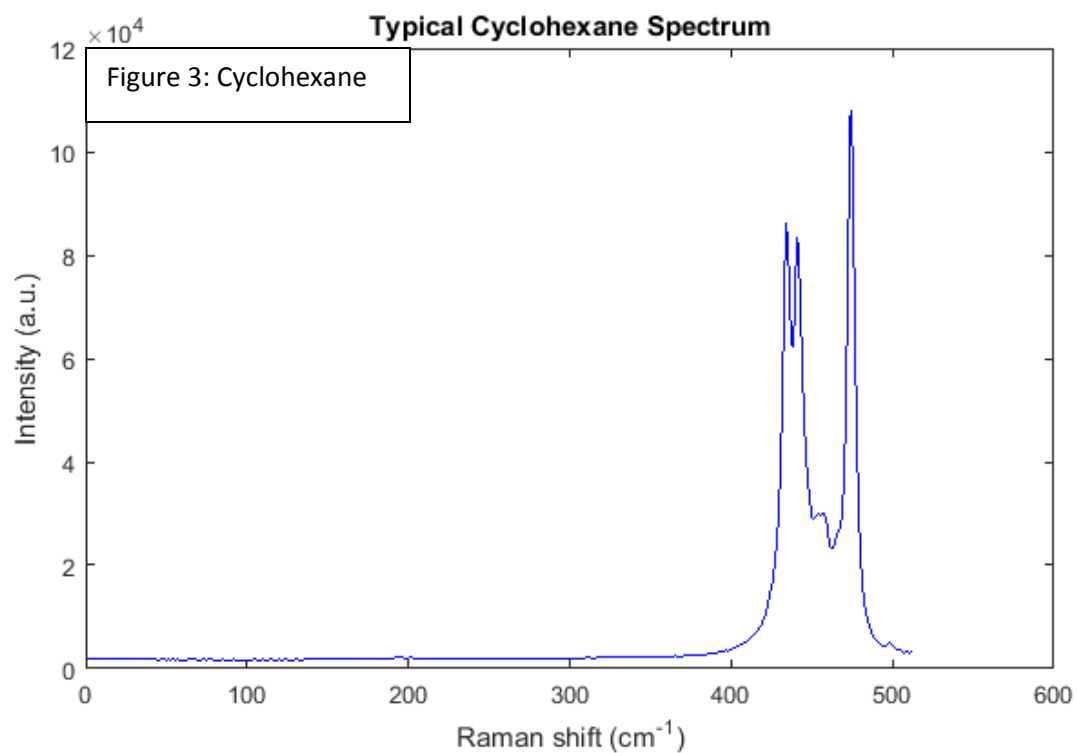
8. Bibliography

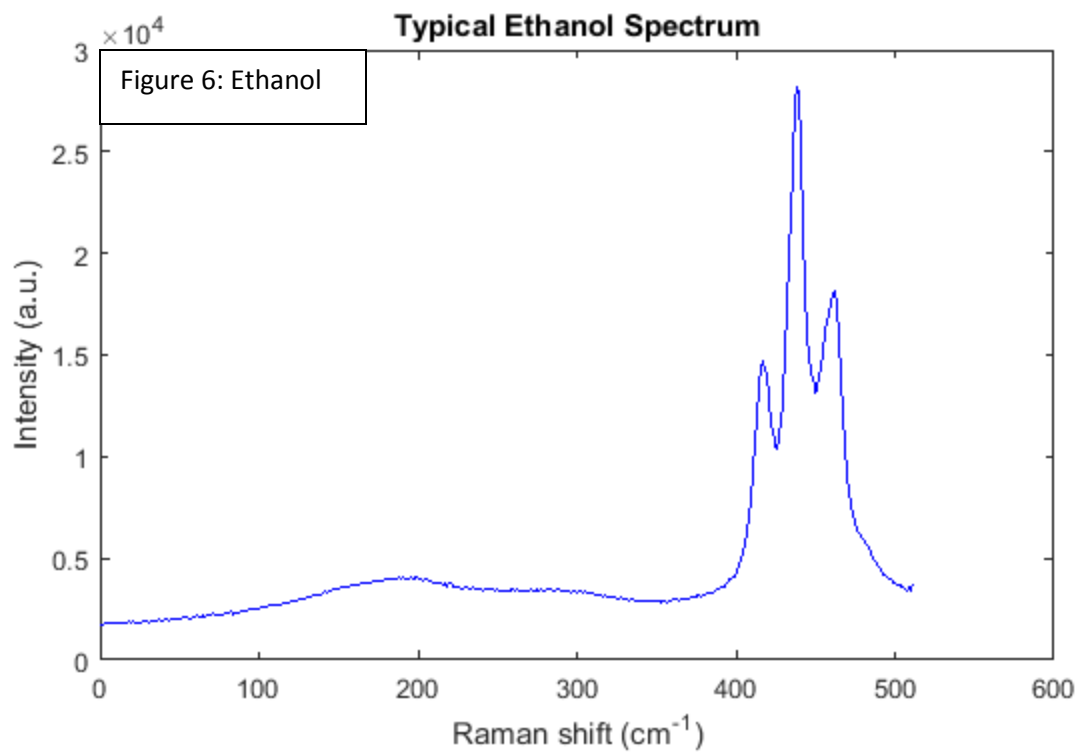
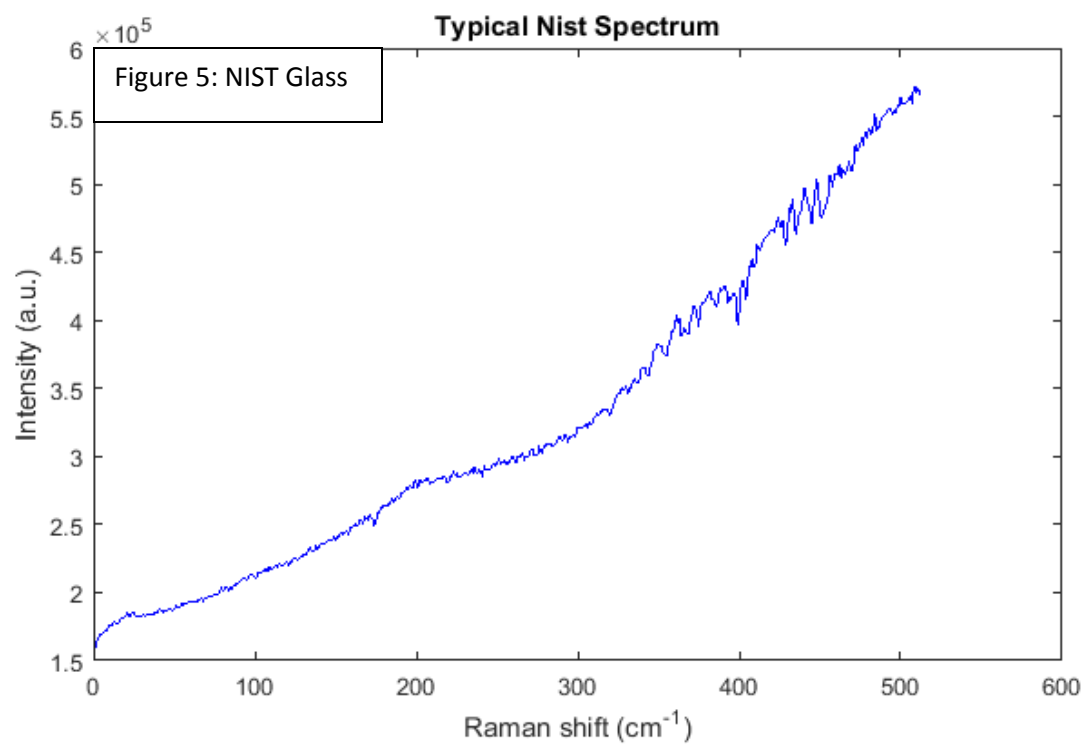
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Appendix A – Typical Raman Spectra of Several Materials







Appendix B - RASKIN Fiber Calibration Protocol

Starting situation: The computer, camera and laser are turned on and the shutter is blocking the laser. The fiber adapter with fiber is inserted and fixed. The fiber tip is fixed in the holder and pointing down. MATLAB, Xenics and power meter software (set to 976 nm) are running.

Xenics Software Settings:

- vRef voltage = 3.3 V
- Time per frame = 100000 μ s
- Width = 20 pixels
- Offset = 248 pixels

Step 1: Cool the camera using liquid Nitrogen. Use the Xenics software to check the temperature. The camera is considered cooled when the temperature is below 189 degrees Celsius. While it is cooling, perform step 2.

Step 2: Check the laser power. Hold the ThorLabs power meter under the fiber tip and open the shutter (wear goggles). Power is considered acceptable when it is 145 ± 2 mW at the end of the fiber. If the power is lower than that, use the X and Y axes to translate the fiber, but do not use the Z axis. If the power is still not sufficient, it might be due to dirtiness at the tip of the fiber. Clean the measuring fiber tip with Ethanol, then try again.

Step 3: Create a new folder in the RAW DATA folder in 'Documents', named after the date, e.g. "yyyymmdd". Optionally, create subfolders in here for each experiment or part of an experiment, like "yyyymmdd-1" and "yyyymmdd-2", etc.

Step 4: Open the 'Watchdog' script in MATLAB by typing "watchdog" in the command window.

Step 5: Set up a new measurement series in Xenics and use a previous measurement as a template for the naming convention. Adjust the date in the file name and name it something clear. Suggestions for common measurements will be given below.

Step 6: Dark Current (file name 'DARK')

- Shutter closed
- Measure for 2000 frames
- Measure 1 time

Step 7: Background with fiber (file name 'BACKfiber')

- Fiber adapter inserted
- Fiber pointing at floor of holder
- Measure for 2000 frames
- Measure 1-3 times

Step 8: Neon-Argon (file name 'NEAR')

- Shutter closed
- Neon-Argon lamp tip directly against fiber tip
- Measure for 50 frames (less if the camera saturates)

- Measure 1-3 times

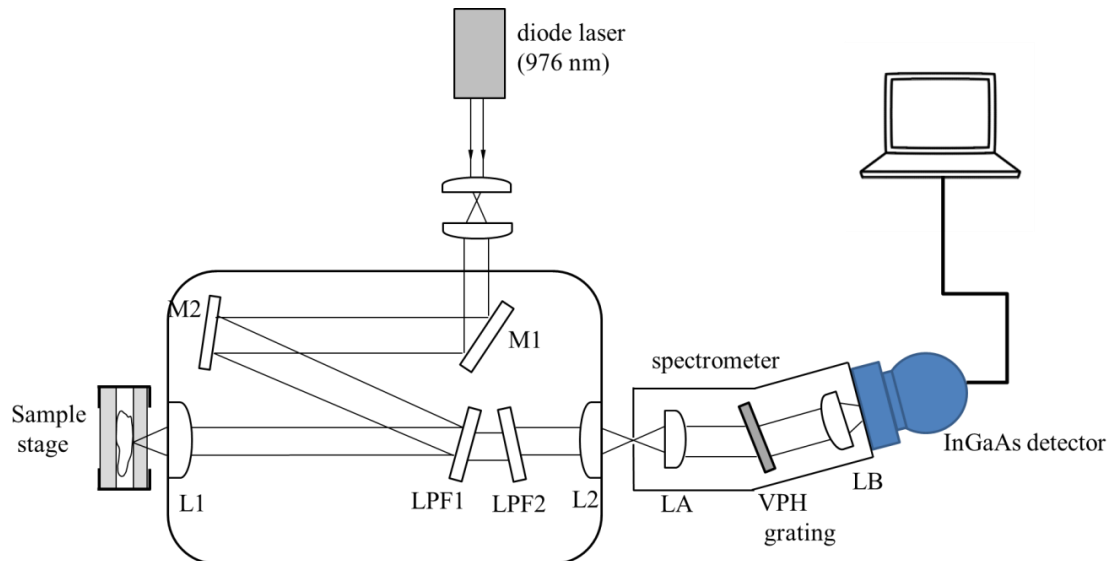
Step 9: NIST Glass (file name 'NIST')

- Shutter open
- NIST placed directly touching fiber tip (make sure it is perpendicular)
- Measure for 150 frames
- Measure 5-10 times

Step 10: Cyclohexane (file name 'CYCLO')

- Fiber tip slightly submerged in the Cyclohexane (use the stage to move it up then down)
- Measure for 100 frames
- Measure 1-3 times
- Clean the fiber tip with Ethanol afterwards

Appendix C - RASKIN Alignment & Adapter Placement Protocol



- 1) Starting Situation
 - a. Lens L1 is removed
 - b. Area behind L1 is clear (XYZ stage can be there, but no connectors)
 - c. Laser is on
 - d. Camera is cooled or cooling
- 2) Beam Collimation
 - a. Hold a fluorescent card in the beam after location of L1 (sample side) and mark the size of the beam
 - b. Place a mirror in the path of the beam after L1 to fold the beam (making it longer)
 - c. At a distance of at least 2m, hold the card in the beam again and check if the beam size is constant ($<0,5$ mm)
 - d. If it is not, adjust beam expander to compensate
 - e. End point: Beam is collimated (to within 0,02 degrees).
- 3) Ensure Beam is parallel to block and beam centering
 - a. Place a fluorescent card that fits exactly in the corridor right after LPF1 (direction L1) and mark the X and Y position w.r.t. the walls and floor of the corridor
 - b. Now place the same card right before L1 (LPF1 direction) and see if the X and Y locations of the beam are the same ($<0,5$ mm)
 - c. If they are not, adjust M1 and M2 to compensate
 - d. Close the laser shutter
 - e. Place temporary XYZ stage on its location right after L1 (sample side)
 - i. Note: XYZ stage was used instead of the fixed FC connector adapter because we can translate the fiber into focus using the stage instead of having to move L1 which can only be done by pushing from inside the box.
 - f. Place fluorescent card from 3) in the corridor right before L1 (LPF1 side)
 - g. Shine a white light through the connector (from sample side) and mark its exact location on the fluorescent card from 3)
 - h. Open the laser shutter (and white light off)
 - i. Hold the fluorescent card in the corridor (right after LPF1 and right before L1) and verify that the laser beam hits the marked center on the card both times (within 0,5 mm)

- j. If it does not, adjust M1 and M2 to compensate and re-do step 3
 - k. Remove the fiber holder with screws and insert and fix L1
 - l. End point: The beam is parallel to the walls of the block and is centered (optical center) within 0,15 degrees.
 - 4) Place fiber in laser focus to create a reference object point
 - a. Using Z-axis translation, find the spots where the beam waist exceeds the fiber core (power starts to drop on the power meter), both left and right of the waist center (see figure 1, spots *a* and *b*)
 - b. Either calculate the distance between these using stage resolution or by estimation, and translate so the focus is in the center of *a* and *b* (*c* on the first figure)
 - c. Repeat a) and b) for the X axis, and then for the Y axis, and repeat a), b) and c) iteratively to work towards position P in the second figure
 - d. The result should be a spot that is in the 3-dimensional center of the beam waist, at position *c* in the first figure and *P* in the second figure
 - e. Loosen screw holding L2
 - f. The focused light is on the center of the slit because the laser beam was aligned in respect to the reference disk
 - g. End point: the fiber is in the true 3D focus of the lens (within 10 μm).
-
- Figure 1, beam waist side view.
-
- Figure 2, beam waist front view.
- 5) Visually place the focus of L2 on the slit
 - a. with L2 out of focus, check if the spot (white light from reference source) is centered on slit (X and Y) – if not, move the spectrometer itself.
 - b. translate f2 and check if the focused light does not move sideways or up/down
 - c. Move L2 until spot falls within slit.
 - d. End point: L2 is roughly focused on the slit and ready for fine-tuning.
 - 6) Optimize Spot Position on Slit
 - a. Block the laser and shine white light from the collecting end of the fiber
 - b. Optimizing Focus on Slit
 - i. Use MATLAB script cougarimage.m to inspect the image of the light on the chip
 - ii. Iteratively translate L2 and check the image to find the best position
 - iii. The width of the image (in pixels) at the top should be equal to the width at the bottom (± 1 pixel)
 - c. Fine-tuning Focus of L2
 - i. Replace white light with Ne-Ar light at the collection end of the fiber
 - ii. Using the spectrum (after cougar2mat.m), optimize focus on the slit so that FWHM is minimal and intensity is maximal (CH band has priority)
 - d. Fix position of L2
 - e. End point: The slit is in the true focus of L2 (within 10 μm).
 - 7) XYZ Optimization of Fiber
 - a. Starting point: L1 and L2 are focused and the removable adapter is not inserted.
 - b. Install the XYZ adaptable fiber connector stage (or fiber adaptation unit)
 - c. Install a fiber in the connector and fix the other end to point at power meter
 - d. Iteratively adjust stage X, Y and Z to maximize power output on the power meter
 - e. Repeat 6) but move the Z-axis (up/down) of the fiber connector instead of L2.
 - f. End point: L3, as well as the rest of the setup, are completely focused and working optimally.
-
- Figure 3, fan effect in image on the chip.
-

Figure 4, optimal position of focus on slit.

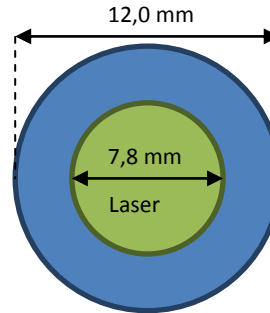
One-time dowel pin block alignment and gluing procedure

- 1) Initial placement
 - a. Turn on the laser (wear goggles)
 - b. Place the adapter block in the corridor
- 2) Optimize the position of the adapter block
 - a. Remove the whole adaptable fiber connector ring
 - b. Use/place a fluorescent card where the beam focuses, at the spot of the connector ring (cut it to the right size and place it in the ring)
 - c. Slide the unit back and forth and see if the location of the focused spot changes
 - i. If it does, visually align the spot as close to the center of the card as possible.
 - ii. If it does not, pick a convenient location and skip to 3).
 - d. Mark the position found to be optimal
 - e. Re-do procedure from a) to confirm found position
- 3) Gluing the adapter plate on the block
 - a. Apply glue to edges of plate
 - b. Put the plate in place and pressure it onto the block for a few seconds to minimize space between the plate and the block
 - c. Make sure it is in the right location, then let go and let it dry for at least 24h

Appendix D – Fiber Adapter Tolerance Calculation Results

Property	Value (Approx.)
Optics Size	½ in
Laser Beam Ø	7,8 mm
Spot size on fiber	4.0 µm (976 nm)
Fiber core Ø	50 µm
Fiber core NA	0.22
L3 - f; NA; Ø	25 mm; 0.24; 12 mm
Returning Beam Ø	11.3 mm
L2 - f; NA; Ø	35 mm; 0.36; 25 mm
Incoming NA	0.16
Spectrometer NA	0.20
Image size on slit	70 µm
Fiber Tolerance (x, y)	± 0.34 mm

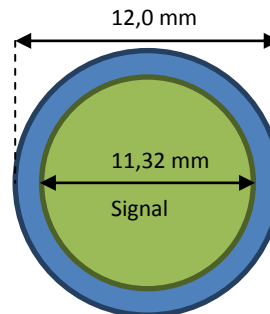
½ inch optics



2,1 mm tolerance on each side

$$\theta_{max} = \tan^{-1} \left(\frac{\text{Lens}\varnothing}{2} - \frac{\text{Laser}\varnothing}{2} \right)$$

$$\approx 2,8^{\circ} \text{ mirror offset}$$



0.34 mm tolerance on each side

$$\theta_{max} = \tan^{-1} \left(\frac{\text{Lens}\varnothing}{2} - \frac{\text{Signal}\varnothing}{2} \right)$$

$$\approx 0,78^{\circ} \text{ fiber offset}$$

Or an x/y translation of 340 µm

Appendix E – Explanation of Arbitrary Units

Arbitrary Units (a.u.) indicate a dimensionless relation between two quantities that were measured. They show the ratio between results that were obtained by measurements using the same equipment.

It is important to note that arbitrary units are based on a reference that is specific to the equipment that was used to measure with. I.e., it is wrong to compare arbitrary units taken from several different instruments, because their references are different.^[35]

Arbitrary units are used in this thesis as a means to compare spectra that were obtained using the same camera and processing method each time.

Appendix F – Detailed Internship Assignment Description

The Raman Spectroscopy Group currently has two distinct projects running at the same time. The first of these projects is attempting to help surgical guidance in distinguishing between healthy tissue and cancer tissue using the water content of the tissue as discriminant. The group found that the water content in cancer tissue is much higher than that in healthy tissue. Raman Spectroscopy can then be used to identify the water content in a specific location during surgery and to determine tumor margins, i.e. the margins where tumor tissue ends and healthy tissue begins.

This internship will take place in the second project, which focuses on identifying melanoma (a form of skin cancer) from benign melanocytic lesions (commonly called ‘skin moles’). The developed setup is different from that of the before-mentioned project. Melanin present in pigmented skin is a source of high auto-fluorescence background noise that occludes the weak Raman signal when using visible excitation light. For this reason, a new setup was developed using a near-infrared laser and a highly sensitive short-wave infrared (SWIR) detector to measure Raman spectra from skin lesions suspected of being melanoma.

Currently, the setup is used for ex-vivo measurements only. An excised skin lesion is placed into a holder that is then placed in the focus of the laser beam. The laser illuminates a specific spot on the sample, and part of the reflecting light is then captured by a spectrometer and imaged on the highly sensitive SWIR detector, producing a (Raman) spectrum.

The goal of this project is to create a practical spectrometer for *in vivo* measurements with a (near) perfect sensitivity. Practical means that it has to be able to measure a spectrum in a few seconds when held to a patient’s skin. To do this, the signal-collecting part of the setup has to be a hand-held probe attached to the light source and camera using an optical fiber.

Before this device can be built and used, research has to be done to find out if the spectrometer still delivers the same high quality measurements using a single fiber instead of a focused beam. This means that an adaptation has to be made to the existing setup to allow the use of a fiber-optic probe without disturbing the focused-beam setup. Having a method to easily switch between both methods (fiber-optic and focused-beam) means that both methods can more easily be compared and conclusions can be made regarding the performance of the fiber-optical probe.