Integrated Raman and electron microscopy

Correlative chemical specificity and nanoscale resolution

Frank Timmermans

INTEGRATED RAMAN AND
ELECTRON MICROSCOPY

CORRELATIVE CHEMICAL SPECIFICITY AND
NANOSCALE RESOLUTION

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Cover front: (Top) Photo of the integrated Raman - FIB - SEM microscope. (Left) Observation of specifically patterned whispering gallery modes. (Right) Overlay of SEM and SERS intensity images for silver plasmonic nanoparticles. Cover back: FIB cross section of a white blood cell presented in cyan-red 3D image.

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INTRODUCTION

1.1 Introduction

Microscopy is the field of imaging objects at a higher magnification and with a higher resolution than can be achieved directly with the human eye. The optical microscope is a ubiquitous laboratory instrument that shows a magnified image of the specimen under investigation. In the microscope setup a lamp might be placed below the specimen. Subsequently an image is obtained from the recorded intensity of light transmitted through the sample. This configuration, called bright field microscopy, reveals information with a resolution of a few hundred nanometer. However this system does not reveal detailed information on the specimen composition. Many optical microscope systems have been developed to improve the resolution and to increase contrast with chemical specific information.

One mechanism to increase contrast is through the detection of fluorescence emission from a sample. The increase in contrast in fluorescence microscopy is obtained by staining specific molecules in the sample with fluorescent reporter molecules. These fluorescent molecules are engineered to selectively bind to specific molecules in the sample, by selective binding a strong contrast is achieved for the labeled features. The principle of fluorescence emission is the absorption and subsequent emission of light by matter. The absorption excites molecules to a higher energy state. During subsequent relaxation of the molecule to the ground state a fluorescence photon is emitted. The emitted light has typically a lower energy, and thus a longer wavelength, in comparison with the absorbed photon. This energy difference occurs due to non-radiative losses when the molecule relaxes from the excited state.

Fluorescence microscopy provides an improved contrast in comparison with bright field microscopy, however it requires sample staining and contrast is only achieved for targeted molecules. Another microscopy technique, that is label free and provides chemical specific contrast, is Raman micro-spectroscopy. Raman scattering results in a signal that is spectrally unique for the molecular compounds

in the specimen. Raman spectrometer setups have been developed to detect the Raman scattered spectrum, enabling chemical characterization of compounds excited by the laser beam. A more thorough description of Raman scattering, and the required optical setup is provided in the next section. Raman micro-spectroscopic characterization of a specimen is performed by recording spectra for each pixel in a microscopic image. The resulting Raman image map contains chemical specific spectral information for each image pixel.

Raman microscopy is generally performed in a confocal microscopy setup. The development of confocal microscopy has resulted in an improvement of the resolution over wide field microscopy. This resolution improvement is achieved by using a pinhole in the detection path that eliminates light from out of focus sample regions. The resulting small depth of focus enables 3D analysis by imaging at different depths in the sample.[1]

The maximum resolution of an optical microscope is limited by the optical wavelength. This is stated in the Abbe limit, an optimal (diffraction limited) light focus has a resolution d defined in equation 1.1. Where λ is the light wavelength, n is the refractive index of the medium at the light focus, and θ the half-angle from the cone of the incident light. With dedicated microscopes at best a resolution of a few 100 nm can be achieved. For example with 405 nm excitation light and a numerical aperture (NA) objective of 0.95 the diffraction limited resolution is 213 nm.

$$d = \frac{\lambda}{2n \cdot \sin(\theta)} = \frac{\lambda}{2NA} \quad (1.1)$$

A huge improvement in microscopic resolution is achieved through the development of the electron microscope. The electron microscope employs a beam of accelerated electrons. The wavelength of the electrons reduces with a higher acceleration voltage to sub-nanometer values. As a result the electron beam can be focused to a spot of a few nanometers. Acquisition of the image information occurs by detection of scattered electrons as the beam is scanned over the image area. The theoretical diffraction limited focus of an electron beam is in the order of a few picometers, a 10 keV electron has a "de Broglie" wavelength of 0.012 nm. In practice the electron microscopy resolution is not limited solely to the electron wavelength but also to aberrations of the incident electron beam or aberrations caused in the sample. Using aberration corrected high resolution electron microscopes sub 0.1 nm resolution measurements have been achieved.[2-4]

Raman and electron microscopy provide a very different method of analysis. The first technique enables chemical specific characterization while the second provides a high resolution imaging method. It can therefore be advantageous to perform combined Raman and electron microscopy on the same sample location. Thereby combining high resolution imaging with chemical specific analysis. Through the development of an integrated Raman and electron microscope system correlative analysis is performed.

1.2 Raman spectroscopy

Raman spectroscopy is technique to measure molecule specific spectra by light scattering from matter. This inelastic scattering phenomenon had been predicted in 1923 by Adolf Smekal.[5] The scattering phenomenon is named after Sir C.V. Raman, who discovered a wavelength shift in scattered light when incident light traversed a transparent medium.[6] This discovery was made in 1928 by C.V. Raman and K.S. Krishnan. Raman was awarded the Nobel prize in physics in 1930 for his discovery.[7] Current Raman spectrometers typically consist of a continuous wave excitation laser that is focused on the specimen with an optical objective. The Raman scattered light is collected and dispersed in a spectrometer and recorded with a sensitive CCD camera.[8] A confocal optical system design offers a good optical depth of focus and enables 3D Raman microscopy. In confocal Raman microscopy spectra are typically collected in a point scanning geometry.

Figure 1.1: (A) Jablonski diagrams for Rayleigh, Stokes Raman, and anti-Stokes Raman scattering. (B) Example Raman spectrum measured of liquid toluene. (C) Schematic representation of the toluene molecule, hydrogen atoms on the benzene ring have been omitted.

A Raman scattering event can be represented by a Jablonski diagram. Figure 1.1A shows the Jablonski diagrams of several scattering events. The diagrams show the ground state E_g and a set of vibrational states E_{vib} of the molecule, light-matter interactions are presented using arrows. The most common event is Rayleigh scattering in which an elastic interaction between the photon and the material results in omnidirectional scattering with no change in optical wavelength. This is shown in the diagram as two arrows (up and down) interacting over a virtual state of the molecule. Since no energy transfer to the molecule takes place the final state coincides with the initial ground state energy. For Raman scattering both Stokes and anti-Stokes scattering can occur. In the first interaction an energy transfer from the incident photon to the molecule occurs resulting in a red-shift for the scattered light. The molecule undergoes a change in its energy state from the ground state E_g to the vibrational state E_{vib} . In the second interaction the molecule is already in an excited state before the scattering event. Energy is

transferred to the optical field leaving the molecule in the ground state E_g and the scattered light is blue shifted with respect to the incident light wavelength. Because at room temperature molecules are mostly restricted to their lower vibrational states and ground state, Stokes Raman scattering is more commonly applied than anti-Stokes Raman. In figure 1.1B the example Raman spectrum of toluene is shown, measured with an excitation laser wavelength of 647 nm. Multiple clear sharp Raman bands are observed that have a center frequency (Raman shift) and intensity that is specific for the molecule toluene. A schematic of toluene molecular structure is shown in fig. 1.1C.

1.3 Electron microscopy

The electron microscope uses a beam of accelerated electrons. The wavelength of electrons at 10 kV is as small as 12 pm which enables focussing of the electron beam in a much smaller spot than an optical laser beam. Electron beams are focused using electromagnetic lenses, that focus the charged particles using Lorentz force. A nanometer resolution can be obtained with modern systems, and aberration corrected electron microscopes can achieve a resolution better than 100 pm. The first electromagnetic lens was developed in 1926 by Hans Busch.[2–4] This led to the first electron microscope to be developed in 1931 by Ernst Ruska,[2–4] for which he was awarded half of the Nobel prize in physics in 1986.[9] Further work to electron microscopes has resulted in the development of scanning electron microscopes (SEM) and transmission electron microscopes (TEM). The SEM employs a focused electron beam that is raster scanned over a sample surface, of which the scattered electrons are detected. The TEM measures electron transmission through thin specimens, this results in less aberrations to the electron beam enabling a higher resolution with which even single atoms in materials are resolved.

The development in 1975 of a focused ion beam (FIB) has resulted in a system that uses a focused beam of ions, in a similar fashion to the SEM. Typically a beam of gallium ions is accelerated with an energy up to 30 keV. By scanning the beam over a sample surface and detection of electrons and ions emitted from the sample microscopic imaging can be performed. Additionally the FIB enables micromachining of structures through ablation or deposition of materials at the microscale. This enables the fabrication of micro-scale structures, and the inspection of deeper sample layers by ablation of surface material

The most important types of SEM electron scattering are secondary electron (SE) and backscattered electron (BSE) scattering. These events are characterized by the different energies of the scattered electrons. SE scattering occurs through an inelastic scattering of the excitation electrons with atoms in the sample, the energy of the scattered electron is at maximum approximately 50 eV.[10] BSEs are usually detected after multiple elastic events, these scattering events result in the escape of the electron out of the sample after which it is detected. Through the nature of elastic scattering the energy of the BSE's is much higher compared to SE scattering.[11] As a result of the different electron energies SE's are only detected from the sample surface with a depth of 5 to 50 nm depending on the material.

SEs generated deeper in the sample will interact with the surrounding material rendering them unable to travel out of the sample. BSEs are detected from a depth up to a few micrometers, depending on the acceleration energy of the excitation beam. The electron travel range in the material is limited to energy loss upon traversing the material, this energy loss is a result of the multiple scattering events and electron induced X-ray emission generated in the material. Figure 1.2 shows the SEM interaction volume for SE and BSE scattering. Additionally electron induced X-ray emission is shown which can be detected from deeper layers inside the material. This is limited to the penetration depth of the incident electron beam where the incident electron energy is sufficient for X-ray generation.[11]

Figure 1.2: SEM scattering interaction volumes. The volume depths are material dependent and described in more detail in this section.

1.4 Motivation for this thesis

The aim of this research is the development of an integrated system for correlative Raman and electron microscopy, and the investigation of applications of the integrated microscope. The integration of Raman and electron microscope enables combined high resolution electron microscopy with chemical specific Raman microspectroscopy.

Both Raman and electron microscopy enable sample characterization for many different applications. Several examples for which the combined techniques are of interest are discussed, not all these examples are covered in this thesis. For catalysis Raman spectroscopy has been applied for monitoring of the catalytic process while SEM has been applied for characterization of the catalytic nanostructures. In the field of pharmaceuticals both SEM structure observation and Raman characterization of the exact chemical composition have been used.[12] Structure observation and chemical specific analysis is also applied for carbon based nanostructures e.g. graphene and nanotubes. In solid state physics FIB - SEM is applied for micromachining and high resolution microscopy,[13] and Raman for material characterization with specificity for phonon modes and analysis of crystal

lattice orientations.[13] Analysis and identification of cells and tissue material can be performed by Raman chemical specific imaging,[8] such materials are also investigated with electron microscopes.[14] Finally of specific interest is the analysis of plasmonic nanomaterials, their light - matter interaction is strongly dependent on the nanostructure morphology as characterized with SEM.[15]

Both the physical integration of the microscope, proof of principle experiments demonstrating the capabilities of the integrated system, and research on plasmonic nanostructures and whispering gallery mode (WGM) resonators are presented in this thesis. We have taken it upon us to investigate the characteristics of plasmonic nanostructures and WGM resonators. Applications of these structures are not investigated however common applications are as sensors for biomolecules or for labels in cells and tissues.

1.5 Thesis overview

This thesis is organized in the following chapters.

Chapter 2 presents a literature overview of integrated correlative light and electron microscopy. This chapter discusses the advantages and challenges involved in employing such an integrated system. A comparison between optical and electron microscopy is made with the aim of providing an intuitive overview of the sample interactions that occur during microscopic imaging. An overview of both integrated fluorescence and Raman microscopes with different electron microscope systems is presented together with applications of the different systems. Using the right sample preparation protocols is often critical for successful experiments, a discussion on the different sample preparation techniques and their effect on the optical and electron microscopic measurements is provided.

Chapter 3 contains the theory of Raman scattering. The presented theory enables the calculation of the Raman signal strength. This is used for a calibration of the optical microscope detection efficiency, and quantitative Raman analysis enabling a measurement of the number of Raman scattering molecules. Next the theory of plasmonic field enhancement and surface enhanced Raman spectroscopy (SERS) is presented. SERS provides a Raman signal enhancement for molecules in the vicinity of noble metallic nanostructures. In this chapter the calculation of these signal enhancement factors, based on light - nanostructure interaction simulations, are discussed.

Chapter 4 discusses the physical integration of the Raman microscope with the FIB-SEM. The calibration procedure for the Raman microscope, and the SEM to Raman sample translation is presented. After this a practical discussion on the operation of the integrated system is provided. The chapter presents multiple proof of principle experimental results in which correlative SEM-Raman and FIB-Raman is demonstrated on various sample materials. Finally a demonstration of correlative SEM-Raman analysis of red and white blood cells shows the capabilities for cell type identification with Raman spectroscopy and SEM analysis of the cell morphologies.

Chapter 5 covers the characterization of plasmonic nanostructures with correlative SEM - SERS experiments. SEM observations of nanostructure morphologies

are correlated with the measured Raman signal enhancement from the structures. This research enables a quantitative analysis of the signal enhancements for different nanostructure morphologies. Quantitative experiments are made possible by a careful calibration of the Raman microscope detection efficiency and measurement of the Raman scattering cross section of the used reporter molecules. Measured enhancement factors are compared with simulations that are performed based on the SEM observed structure morphologies.

Chapter 6 covers the characterization of whispering gallery mode optical resonators. Optical resonances are observed in the Raman spectrum after a SEM treatment of transparent microspheres. The resonances are characterized as WGMs that only become visible through the deposition of hydrocarbon materials performed during SEM imaging. During Raman imaging a photoluminescent light is excited from the deposited material, the excited light couples with the optical resonator for permitted mode wavelengths which is in turn detected in the Raman spectrum. In this chapter this phenomenon is characterized and the effect of different microsphere sizes and FIB and SEM treatment procedures are investigated.

Chapter 7 presents the development of a new stand-alone Raman microscope. This microscope features a blue (405 nm) excitation laser, this short wavelength provides a strong optical focus and thus a good optical resolution of approximately 300 nm. The strong optical resolution enables an improved correlation of Raman with electron microscopic images. The system facilitates in bridging the resolution gap between electron and optical microscopy. Finally applications specific for high resolution (resonance) Raman microscopy with blue excitation light are investigated for the analysis of red blood cells and silver plasmonic nanostructures. Correlative SEM experiments are performed by translating the sample to a separate SEM and locating the region of interest (ROI) using sample markers.

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CORRELATIVE LIGHT AND ELECTRON MICROSCOPY

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Abstract

Correlative microscopy is an emerging field employing combined optical and electron microscopy for specimen analysis. This chapter discusses the integration of optical microscopes, such as Raman and fluorescence microscopes, with electron microscopes. To provide some insight into the comparison between optical and electron microscopy a discussion on the differences between the two different techniques is presented based on the involved physical photon and electron interaction processes. Correlative microscopy has been used for the combination of Raman chemical specificity, or the localization of fluorescent proteins to aid electron microscopy, with high resolution electron microscopic morphological or ultrastructural information. The integration into a single system of the optical microscope with the electron microscope results in new challenges in for example sample preparation, but also enables new opportunities such as an improved workflow. The complementary information from electron microscopy and light microscopy present an opportunity for correlative investigation of a broad range of sample properties. We report on developments in the field of correlative light and electron microscopy (CLEM) such as available sample preparation techniques, commercial integrated light and electron microscopes, and recent publications reporting on applications of integrated microscopy.

2.1 Introduction

The last decade has witnessed an enormous growth in research aimed at improving and applying both correlative light and electron microscopy, and integrated correlative light and electron microscopy (iCLEM). This has resulted in a steady increase in the number of publications in this field. Microscopic imaging with CLEM or iCLEM renders information by applying both optical microscopy (OM) and electron microscopy (EM) to the same sample area.[1–3] The abbreviation iCLEM is used to distinguish integrated systems of light and electron microscopy to perform correlative imaging. Correlative microscopy provides additional insight that cannot be obtained or is more difficult to obtain with a single microscope. New insights into, for example, biological processes and cellular working mechanisms are expected from iCLEM.[3]

Generally, fluorescence microscopy (FM) and Raman micro-spectroscopy (RM) are optical spectroscopic methods that provide chemical information at the diffraction limit. Conversely scanning electron microscopes and transmission electron microscopes are used to achieve a vision capability with a much higher resolution far below the optical diffraction limit. This resolution depends on the microscope system, but can be between nanometer and picometer values. The practical difference between SEM and TEM is that the first provides high resolution images from the surface of larger samples,[4] while the second is used to make high resolution transmission images from thin sample slices.[5] Sample characterization performed using combined light and electron microscopy modalities facilitates new techniques for sample analysis and paves the way for future discoveries in the biological and material sciences. This chapter provides a review of the most important recent technological developments in this field.

2.2 Comparison between optical and electron microscopy

In this section, a quantitative comparison for optical and electron microscopy will be presented. This will be illustrated by comparing some general values of, for example, the particle energy and scattering coefficient, a summary of this comparison is given in table 2.1. The reader is provided with insight into the major differences between these imaging techniques. These differences originate from the different interaction mechanisms of electrons and photons with matter. OM depends on the scattering or absorption and emission of photons from molecules, while EM depends on the interaction through the coulomb forces between the electrons in the excitation beam and the atomic nuclei or bound electrons. As such, the scattering cross-sections for electrons are very high, as compared to the efficiency of photon scattering, see table 2.1.

	Electron microscopy	Optical microscopy
particle energy	SEM: 1 - 30 KeV TEM: 100 - 300 KeV	1.6 - 3.1 eV
Particle flux	$10^{14} \text{ s}^{-1} \mu\text{m}^{-2}$	$3 \cdot 10^{16} \text{ s}^{-1} \mu\text{m}^{-2}$
Scattering coefficient	0.1 - 0.5	FM 0.2 - 0.8 RM: $10^{-13} - 10^{-12}$
Ratio Inelastic / elastic scattering coefficient	SEM: 2 - 3 [6] TEM: 0.1 - 1.0 [7]	This ratio is not used for FM and RM. Since these techniques rely on inelastic interactions.
Energy deposited per inelastic event	20 eV [6, 8]	0.1 - 0.4 eV

Table 2.1: Summary of the most important characteristics for both EM and OM. Providing a fast comparison between both imaging techniques and to note the large differences in photon and electron characteristics. Calculations and motivations for the values are provided in the text within this section.

In materials science, a TEM can be used with an acceleration voltage of typically 100 - 300 kV.[9, 10] The higher energy enables the researcher to image thicker specimens and to obtain a potentially higher resolution. These high particle energies do present a risk for knock-on damage, in which nuclear displacement occurs after interaction with an electron. For this reason lower voltages, such as 100 kV, are used to limit knock-on beam damage. However the main source of damage is through inelastic scattering, which causes energy to be deposited into the sample. The main mechanisms which cause inelastic scattering are emission of secondary electrons and X-ray emission. This damage generally occurs in the form of radiolysis, which is a process in which chemical bonds in the material are broken, or in the form of heating, which is a major issue in polymers and biological specimen. Therefore it is important to know the ratio of inelastic / elastic scattering events. In a SEM this ratio is in the range of 2 - 3 and is mainly independent of the electron energy,[6] for TEM the ratio ranges from 0.1 to 1.0, in TEM Inelastic scattering is mainly due to X-ray emission. This depends strongly on both the particle energy and sample.[7] The inelastic / elastic scattering ratio does change significantly for particles with a larger atomic number where elastic scattering starts to dominate.[11]

The scattering cross-section for both elastic and inelastic scattering are inversely proportional to the electron velocity.[6, 12] Biological specimens have, as a result of the abundance of atoms with a low atomic weight, a low contrast which makes it advantageous to use a much lower acceleration voltage ranging from 5 kV to 30 kV[9, 10, 13] thus generating higher signals. These energies are comparable to the energy used in SEM imaging and the resulting lower penetration depth improves the image contrast. Typical SEM acceleration voltages range from 2 to 30 kV[14, 15] although in the biological sciences voltages down to 300 V[15] are also used to obtain suitable contrast. In SEM similar specimen damage phenomenon occur as in TEM, i.e. knock-on damage, radiolysis, and sample heating. The use of slower electrons with SEM compared to TEM does increase the scattering

cross-section and reduces knock-on damage, however it increases the damage caused by radiolysis.[7] The inelastic scattering process mostly occurs through interaction with plasmons and the outer electron shells. As a result inelastic events deposit on average an energy of 20 eV, which is roughly independent of the electron particle energy.[6, 8]

In comparison, the energy from a typical photon with a wavelength of 400 nm to 800 nm is between 3.1 eV and 1.6 eV. These values show that the difference in particle energy is a factor of 10^3 to 10^5 . Radiation damage by light microscopy is generally also caused by inelastic processes such as in FM, or direct light absorption as in infra-red absorption, as shown in figure 2.1. Mostly these processes cause heating of the sample, only when using short wavelengths in the UV range can direct chemical alterations be caused by photon interactions. When optical micro-spectroscopy is used, a typical interest exists in inelastic events, such as fluorescence or Raman microscopy, since these are the events that offer sample specific chemical information, unlike elastic events such as Rayleigh scattering. The rate of energy deposition depends strongly on whether the sample has a high or low fluorescence yield, or whether absorption bands are close to the excitation energy. The energy that is deposited per event ranges from 0.1 - 0.4 eV, which can be obtained from the wavelength difference between the excitation and emitted light.

Knowledge of the elastic and inelastic scattering coefficients is important to assess potential beam damage, as well as to obtain insight in the expected signal strength. For EM the scattering coefficient depends on both characteristics of the sample, atomic number, and beam characteristics, particle energy and angle of incidence. The (in)elastic scattering coefficient is determined as the ratio of elastic or inelastic scattering events per total number of incident electrons. Generally, beam settings are chosen to achieve high scattering coefficients for the signal of interest. For SEM the secondary and backscattered electrons are commonly detected and typical scattering coefficients for these signals are in the range of 0.1 to 0.5.[16, 17] Forward scattering electrons in TEM have a scattering coefficient comparable to the backscattered electrons in a SEM, so also in the range of 0.1 to 0.5.[12]

Figure 2.1: Jablonski diagrams describing Fluorescence, Raman and infrared spectroscopy

These values are very different compared to Raman spectroscopy, which has notably low scattering cross-sections. A calculation of the Raman scattering intensity I_R compared to the excitation intensity I_0 renders the RM scattering cross-section, which can be compared with the EM scattering coefficient. The Raman scattering cross-section depends on the excitation wavelength and compound of interest, but a typical value for a Raman band is 10^{-30} cm^2 . [18, 19] Making some estimates the molecular density ranges from 10^{21} to 10^{22} cm^{-3} and the axial resolution L of an optical microscope is around $3 \text{ }\mu\text{m}$. Using these values results in a Raman scattering coefficient of $3 \cdot 10^{-13}$ to $3 \cdot 10^{-12}$. It shows a difference of 11 to 12 orders of magnitude compared to EM. It is important to note that many fluorophores for FM, with quantum yields between 0.2 and 0.8, do approach the scattering coefficients achieved with EM, while also Raman scattering coefficients under SERS conditions may be comparable.

Another key parameter for EM is the probe beam current. The current is set on the basis of several factors. A higher current provides a stronger signal; however, it also results in a larger beam diameter which decreases the resolution and may also result in more sample damage and surface charging. An electron microscope can be used with a probe current of several pico amperes up to several nano amperes. [9, 14] However, in typical situations a current of 5 to 50 pA is used, which corresponds to $3.1 \cdot 10^7$ to $3.1 \cdot 10^8 \text{ e}^- \cdot \text{s}^{-1}$, or when assuming a 1 nm^2 focus area $3.1 \cdot 10^{13}$ to $3.1 \cdot 10^{14} \text{ e}^- \cdot \text{s}^{-1} \mu\text{m}^{-2}$. This is a much lower number compared to OM in which a typical excitation power of 5.0 mW with a 600 nm wavelength contains a photon flux of $1.5 \cdot 10^{16} \text{ s}^{-1}$, or roughly $3 \cdot 10^{16} \text{ s}^{-1} \mu\text{m}^{-2}$. Different probe currents or excitation powers can be used, and also the chosen electron or photon energy will influence the choice of particle flux, but a typical situation results in a flux that is a factor $10^2 - 10^3$ smaller for EM compared to OM.

2.3 Integrated CLEM

The combination of light and electron microscopy offers the opportunity to study specimens over a large range of resolutions. Therefore, images can be made both from large areas at a lower resolution with light microscopy and subsequently electron microscopy images can be made from specific regions at a much higher resolution or vice versa. Light microscopy techniques encompass detailed spectroscopic imaging techniques such as Raman micro-spectroscopy and fluorescence imaging. These techniques enable acquisition of chemical information or to locate specific areas with the excellent contrast provided by labeling as is common in FM light microscopy.

However, correlative microscopy with separate instruments for light and electron microscopy needs to meet several challenges. Firstly, it is required to recognize and locate a region of interest with both types of microscopes. This requires tagging of these regions with specific light and electron microscopy labels. [20–22] Secondly, when a sample is moved between the different microscopes there is a risk that changes occur in the sample, whether caused by damage or reorientation. As a result, the relocation process can become challenging and very time consuming.

These challenges can be overcome by integrating both microscopes in a single system. This provides a specific and well known correlation of the coordinates between the different imaging modes. A schematic representation of an iCLEM system is shown in figure 2.2. With the fixed distance between EM lens and OM objective, the region of interest is preserved when switching between the microscopes.[3, 23] Using this procedure the correlation of coordinates is limited to the accuracy of the stage which can be kept below 1 μm . To obtain an improved coordinate correlation, the specific ROI can be found by wide field microscopy, or through the use of fiducial markers, which improves the correlation to the diffraction limit. Using an integrated correlative microscope generally removes the need for fiducial markers.[22] These markers could interfere with specific samples and often it may not be allowed to add markers, for example in product quality analysis. Furthermore potential sample changes are avoided because the specimen does not leave the vacuum chamber while switching imaging modes.

Figure 2.2: Schematic of a SEM vacuum chamber with incoupling optics for correlative light microscopy.

2.3.1 Fluorescence and electron microscopy

In FM contrast results from the fluorescence emission of molecules with a sufficiently high fluorescence quantum yield. Usually the natural presence of such molecules is low, which is why addition of molecules with an increased fluorescence quantum yield is carried out.[23] The fluorescence signal is obtained by excitation of the fluorophores at or near the electronic absorption band. The collected fluorescence signal has characteristically a larger wavelength than the excitation light, which makes it possible to separate the signal with a dichroic mirror or filter from the

excitation beam. FM can be applied for imaging of both organic and inorganic compounds. It is currently an important technique in the application area of imaging biological samples.

Fluorescence microscopy enables to monitor dynamic behavior of molecules in living cells. This is usually performed by making time-lapse microscopy images of labeled cells.[24] The excellent contrast in FM together with the ultra-structure information in EM, makes it a promising technique for integration with EM. Electron microscopes offer high spatial resolution images of cells, but require fixation (for TEM), and fixation and conductive coating (for SEM) of the specimen. These preparation methods kill the cells thus preventing imaging of its dynamic behavior. A combination of these modalities offers both dynamic behavior and high resolution images to be obtained. This requires the researcher to first make time-lapse images with the FM after which the sample is placed in the EM for high-resolution analysis.[25]

Several systems for integrated fluorescence microscopy and electron microscopy have been developed and optimized over recent years.[23, 26, 27] These systems enable rapid image acquisition of large areas using FM followed by high-resolution EM imaging of specific regions selected on the basis of the fluorescence images.

2.3.2 Raman and electron microscopy

RM is a label-free spectroscopic imaging technique which is based on in-elastic light scattering of monochromatic (laser) light from the electronic polarizability in molecules that is also modulated by molecular vibrations. In Raman spectroscopy the frequencies of the molecular vibrations are detected and provide a compound specific signal, i.e. a molecular fingerprint of the molecules present in the measurement volume. RM is widely applied in material science and biomedical applications to analyze the molecular composition as well as to obtain detailed physical - chemical information of molecular components. The relatively small Raman scattering cross-section (10^{-30} cm^2) requires carefully designed confocal Raman microscopes that are optimized for efficient signal collection.

As previously stated, FM finds many applications in the medical and the biological research fields. RM is, next to these fields,[28] also used in analysis of inorganic compounds and therefore finds applications in material sciences[29] and solid state physics.[29] Correlative RM and EM, too, is applied in both the biological[30] research fields and in the material sciences.[30] The integration of RM with EM combines the high resolution information obtained through EM with the sample's molecular composition obtained through RM. This technique is not limited by potentially damaging effects of the electron beam on the fluorescent labels. RM is a label free technique, and as such it is easier to switch multiple times between the Raman imaging and electron imaging modes. This enables additional freedom to investigate regions of interest multiple times and the potential to combine RM analysis with sample modification techniques as FIB milling.

Of specific interest is an integrated correlative Raman micro-spectroscope with FIB-SEM. The FIB - SEM is a valuable tool for micromachining with applications mainly in the materials science, but also for biological sciences. The combina-

tion of chemical specific Raman spectroscopy with FIB micromachining enables characterization of a specimen at selected intervals during FIB machining. This also enables investigation of the influence on material properties by FIB ions that may contaminate the sample during milling. FIB removal of material can be used to help collect suitable Raman spectra from layers in the sample below a non-transparent surface material, additionally specific features can be isolated from surrounding background material. Furthermore the FIB is used for fabrication of sample lamellas for TEM, this method additionally enables Raman analysis of deeper layers in a sample.[31, 32] The FIB is also a useful tool in the fabrication and modification of SERS substrates.[33] The integration of techniques has achieved improvements in the workflow and the efficiency of the experiment time.

Using the FIB, layers from a specimen can be removed, which allows for high resolution SEM imaging of every consecutive surface layer. With this procedure, the z-resolution is limited to the layer thickness removed with the FIB, which can be as low as 10 nm.[34–36] As a result of this resolution a low penetration depth of the electron beam into the sample, and therefore a low electron energy (1 to 5 kV) is sufficient for SEM imaging.[34–36] This 3D characterization of typically small sample features can benefit from correlative 3D optical microscopy for the localization of ROIs. Correlative protocols combining light microscopy data with 3D FIB-SEM imaging have been developed.[37, 38]

2.4 Sample preparation techniques

Due to the high temperature required for the electron source to operate and the need to prevent beam broadening during electron propagation towards the sample, it is necessary to perform EM experiments under vacuum conditions. This requires the use of a vacuum chamber with pressures of typically 10^{-5} to 10^{-10} mBar. These vacuum levels cause changes in several types of samples. Organic materials experience degassing which may affect the structural properties. Biological cells and tissues will dehydrate which alters their morphology. The potential changes in morphology varies greatly over different samples, even when comparing specimens within a specific category such as life sciences. There are several techniques and sample preparation methods to prevent this, such as cryogenic sample preparation[39] and atmospheric electron microscopy.[40, 41] Furthermore, there is the potential to perform low-vacuum SEM or wet SEM. In this method a low vacuum, around 30 mBar, is used and water vapor is present close to the sample. This preserves better the sample's native environment with the trade-off that the electron beam will undergo more broadening under these conditions.

Drying of biological samples before they can be placed in the SEM vacuum chamber is required to ensure controlled drying in favor of degassing in the vacuum. SEM sample preparation protocols for dehydration aim to preserve the sample morphology. Since the preparation protocols do not aim to stain the specimen with any compound they are in practice compatible with optical microscopy. The most common sample preparation technique is critical point drying, in which the water in biological tissue is replaced with a different inert fluid. This fluid,

typically liquid CO_2 at its critical point pressure approximately 80 Bar, is brought to its critical point temperature, which for CO_2 is approximately 35 °C. At the critical temperature the fluid changes to vapor without a change in density, so surface tension effects will not alter the sample during dehydration.[42, 43] An alternative dehydration method is hexamethyldisilazane (HMDS) drying. For sample preparation the water is replaced with HMDS, which is then air dried. HMDS has a reduced surface tension and cross-links proteins, which reduces sample deformations during the drying. This drying procedure requires more manual handling and less expensive equipment, it results in similar quality results.[42, 44] The effects of drying on the optical microscopy measurement depend on changes in the sample morphology, additionally for Raman microscopy it is expected that Raman bands will shift up to a few wavenumbers because the molecules are no longer surrounded by a water medium.

Other demands for sample preparation arise from charging effects caused by the electron beam impact. To prevent charging effects, the sample has to be well connected with a conducting surface. For SEM this is ensured by coating samples with a thin conductive layer such as gold or carbon. The use of such coatings greatly increases the EM resolution; however, they do have a potential negative effect on the correlative OM signal. Most integrated microscopes employ top down excitation light and collect the backscattered or emitted signal, both the excitation and collection light have to travel through the coating which results in signal losses. Aksenov has evaluated the effect of gold and carbon coatings of various thicknesses on a collected Raman signal.[45] The results are shown in figure 2.3 and show generally a low light loss for carbon coatings up to several nanometers and a near transparency of the optical signal for thin gold coatings up to a nanometer. In other works the investigation of conducting carbon coatings has shown only an emerging amorphous carbon background on the Raman signal.[46]

Figure 2.3: Effect of carbon (left) and gold (right) coatings on the optical signal. Figure reproduced from Aksenov.[45]

For both SEM and TEM techniques samples are placed on conducting substrates such as aluminium, silicon or gold to further prevent electric charging.[45] The surface on which the samples are mounted does not interfere with the collection and excitation of light in a top - down illumination microscope. However the selection of substrates should not be taken lightly as it could contribute a significant background signal for OM, which is an important issue for Raman spectroscopy. Furthermore, optical microscopy could induce heating and thermal damage in samples which might require to select proper surfaces for heat dissipation. This is also important as there is less heat dissipation due to the vacuum. Metal surfaces quartz or mineral surfaces, like e.g. CaF_2 and MgF_2 , are good heat conducting substrates. Besides the reduced heat conduction, a positive effect of the vacuum is that, as a result of the absence of oxygen, photo-oxidation processes are minimized, thus decreasing this type of sample damage.

An important preparation method for biological samples is fixation. The goal in fixating a sample is to stop cellular activity and to preserve the samples structural properties. It is therefore often the first applied sample preparation step, to fix the state of the specimen before any other treatment is performed. Fixation is often performed through the addition of chemical components such as aldehydes or oxidizing agents. Using chemical fixatives provides a more stable sample during electron microscopy, however a drawback is the loss of some structural properties due to the chemicals. Furthermore these chemicals may contribute a signal to light microscopy techniques that may obscure other desired spectroscopic properties of the sample. Although it is noted that fixation is also commonly applied for stand-alone Raman and fluorescence microscopy. An alternative preparation method uses cryo-fixation, which freezes the sample rapidly, thus ensuring a native structure for EM analysis.

TEM imaging requires samples to be prepared in thin slices. This can be attained by ultramicrotomy. Cutting of biological materials requires that the sample is more rigorously fixated. For this purpose the specimen is embedded with chemical compounds such as epoxy - or acrylic resins. These embedding chemicals may again obscure both RM and FM signals. Sample preparation protocols that combine embedding and labeling with fluorescent proteins have been demonstrated.[47]

In biological samples, fluorescence staining is another important preparation technique. The aim of staining is to enhance contrast for specific molecules, which otherwise would provide no contrast. For TEM staining with heavy metals, such as lead, osmium tetroxide or uranyl acetate is also applied to provide contrast in the biological sample, that otherwise would show very limited contrast in TEM. Such compounds contain atoms with high atomic numbers to increase the electron scattering signal. These stains have significant consequences for correlative light microscopy. The heavy atoms near fluorescent molecules partially quench their fluorescence signal.[26, 48] Furthermore the heavy atom stains contribute an optical background signal that obscures both the Raman and fluorescence data.

	SEM	TEM	RM / FM
Sample dehydration	+	+	Dehydration of biological samples may result in shrinking and changes in morphology.
Conductive coating	+	-	The coatings result in a significantly lower collection efficiency.
Fixation	+	+	Fixatives may result in a background on the optical signal.
Embedding	-	+	Embedding resins may result in a background on the optical signal.
Staining	-	+	Heavy metal stains induce quenching on fluorescent dyes, and results in a background on the optical signal.
Cryo fixation	+	+	Potential frost formation results in a lower collection efficiency

Table 2.2: This table summarizes sample preparation methods for SEM and TEM and their effect on correlative optical microscopy, an expanded description is provided in this section. Whether the specific preparation technique is applied for SEM or TEM is indicated with a "+" or "-". Not all presented preparation methods are always essential for electron microscopy, not using a conductive coating will still allow SEM imaging but with the risk of charging effect, and cryo fixation is used as an alternative to sample fixation and embedding. Thus indicating that in practice the required sample preparation has to be suitably chosen for each individual situation.

Given the important role of sample preparation for both EM and OM and the different practical reasons to implement them, we will present in somewhat more detail cryogenic sample preparation and atmospheric electron microscopy.

2.4.1 Cryogenic sample preparation

EM generally requires specific chemical preparation methods for staining and fixation of the sample. Samples also become dehydrated by the reduced pressure in the EM chamber. The chemical preparation methods and sample dehydration alter the chemical composition and structure of mainly organic samples. An alternative to chemical sample preparation is cryogenic freezing. This involves freezing of the sample at temperatures of typically -160 to -180 °C[39] and during imaging the sample must be kept below the devitrification temperature of ice at -137 °C.[49]

The goal of using cryo-electron microscopy is to preserve the sample's native structure. Since dehydration in vacuum is an issue for instance in biological samples, but also in food analysis, cryo-electron microscopy is mainly used for such specimens. Currently, correlative FM with cryo-TEM, and cryo-electron tomography has been applied,[50, 51]. Also cryo super resolution microscopy with the aim for correlative EM has been performed. Although the fluorophore properties under cryo-conditions require investigation.[52] Correlative RM with cryo-electron microscopy has been

demonstrated in proof of principle experiments.[53] Alternatively cryo-freezing is applied as a sample fixation technique after which freeze substitution with an embedding medium is applied to prepare before EM. For such techniques, retaining the fluorescence signal after freeze substitution has been demonstrated.[47, 54]

Besides the considerable advantages of cryo-techniques in EM sample preparation, there are challenges to consider as well. First, the specimen must be kept below the devitrification temperature of ice during the experiment. Second, an ice layer forming on the sample may lower the EM signal, and this is especially the case for tilted samples as the ice cross-section will increase in these situations. Third, the freezing process has to be performed carefully to prevent vitrification, i.e. the formation of ice crystals, which may damage or alter the sample structure. This leads to a maximum sample thickness of 20 μm for plunge freezing, and a maximum thickness of 200 μm for high pressure freezing.

2.5 Electron beam induced deposition

The developments of integrated light and electron microscopy have revealed a complication emerging from correlative microscopy. This arises from the deposition of molecules in the vacuum chamber induced by the incident electrons, called electron beam induced deposition (EBID). Material deposited onto the substrate from the vacuum can originate from dissociation from the sample substrate, degassing of O-rings and other elements in the vacuum chamber, or potentially from oil used in vacuum pumps. During the deposition process the gaseous molecules are decomposed leading to a deposition of mostly amorphous material, called hydrocarbon contamination.

Hydrocarbon deposition has always been an issue in SEM, since it changes contrast in the SEM images and can obscure fine details of the sample. With an integrated Raman microscope the contamination becomes a bigger issue since the EBID material shows up as a background in the Raman spectra. Furthermore thicker deposited contamination layers can also result in light absorption thus lowering the Raman signal from the sample. Observations with correlative SEM - Raman of this contaminant has been reported using a 514.5 nm excitation wavelength by Wille et al.[46] and using a 785 nm excitation wavelength by Guerra et al.[55] Both articles show that the Raman spectra of the contaminant contains amorphous carbon and also a very broad photoluminescence contribution. The carbon deposition spectrum appears to be substrate dependent, showing mostly photoluminescence after deposition on carbon based substrates and an amorphous carbon signal after deposition on other substrates.

To limit the effect of hydrocarbon contamination on the experiments a high quality vacuum is required. Therefore contaminations in the vacuum chamber need to be prevented and an increased vacuum pumping time before starting an experiment might be desired. Tools that improve the vacuum quality are available, for example using a load lock enables an exchange of the sample without venting of the chamber. Additionally plasma cleaners can be used to remove contamination from the vacuum chamber and sample, as a result hydrocarbon contaminants

are removed from the chamber providing an improved vacuum pressure after the cleaning procedure. Finally we have found it can be advantageous to perform Raman imaging before a high resolution SEM image is made from the sample. In this procedure the SEM can be used to navigate the sample and identify regions of interest, this is performed with a low magnification or low electron current to limit electron irradiation per surface area. Next a Raman image is made of the identified regions of interest, and finally SEM images with a high resolution performed at an increased magnification are obtained. EBID contamination will occur in the final SEM image scan, however it does not affect the Raman image made before EBID.

2.6 Dedicated microscopy techniques

2.6.1 Atmospheric electron microscopy

Another method to perform EM without altering the native sample structure and material distribution is by separating the vacuum chamber from the sample. Electron microscopy has a long history in using such closed cells.[56] For atmospheric scanning electron microscopy (ASEM), the vacuum chamber and sample are separated by a thin membrane which is mostly transparent for the electron beam. Although, the secondary electrons will often be absorbed by the membrane, backscattered electron detection is possible. This preserves part of the systems imaging capabilities[40, 41]. In atmospheric scanning transmission electron microscopes (STEM) the sample is placed between two membranes. This creates a separate atmospheric pressure chamber within the vacuum chamber where the electron beam travels through the sample and both membranes.[57, 58]

The environmental SEM allows for the analysis of wet samples. It can therefore be used for SEM imaging of live cells, although a high electron dose irradiation with high electron energy is not beneficial for the cell survival. This system does provide an interesting combination for correlative light microscopy in the analysis of live cells. ICLEM systems with the sample at atmospheric pressures have been developed and applied for live cell analysis.[59–61] A schematic picture of an ASEM integrated with an optical microscope is shown in figure 2.4. The integration of Raman microscopy with an environmental STEM system has been reported by Picher et al.[62] In this system Raman scattering is excited and collected using a parabolic mirror focused on the sample. The membranes that separate the sample from the vacuum are generally made from silicon nitride SiN and have a thickness of 50 to 150 nm.[57, 63]

Figure 2.4: Schematic of an ASEM integrated with a light microscope. A membrane separates the EM vacuum chamber from the sample. The optical microscope is entirely outside the vacuum chamber.[59]

2.6.2 Electron tomography

Tomographic imaging is a method to analyze the 3D structure of materials and specimens. Electron tomography provides a high 3D resolution, 1 nm³ is possible for tomographic imaging using a high resolution TEM. To perform tomographic analysis, generally a TEM is used to record a series of 2D images from a sample, with each image taken for a different specimen tilt position. This series of images is used to reconstruct the original 3D structure of the object.[64–66] The maximum tomographic resolution depends on the number of recorded slices, but also on the particle size and symmetry.[65]

A maximized resolution can be obtained by recording the sample over an as broad a range of tilt levels as possible.[64–66] The limiting factor for this range is usually the decrease in signal from the TEM due to higher specimen thickness at greater tilt angles. Therefore, it is important to use sufficiently thin samples for TEM and especially for electron tomography.

Applying ultramicrotomy is a method to obtain suitably thin slices.[67] An ultramicrotome can prepare slices with a thickness between 30 and 100 nm[67] for TEM applications, and when transmission of electrons is not required thicker slices are possible. Another sample preparation method for TEM is through FIB milling of the specimen. FIB milling has become a common preparation method in material sciences,[68, 69] and is increasingly applied in life sciences.[67, 70] With the FIB material slices can be selected with a precision of 10 to 20 nm,[67] which is a significant improvement over ultramicrotomy. This is crucial to achieve imaging

over a sufficiently broad range of tilt angles for successful ET. Often samples are being prepared as lamellas.[71] This allows for imaging over a larger region of tilt angles. Furthermore developments over the last decade have made it possible to prepare samples in a small needle shape using a FIB.[72] This would allow for a larger or potentially a full 180° sample rotation with which improved 3D reconstruction and resolution can be obtained.[72]

When analyzing, for example, biological structures in their frozen hydrated state there is a risk of damaging the sample due to its sensitivity to radiation. The consequence is that the excitation dose in electron tomography should preferably be as low as possible. This may significantly lower the desired signal-to-noise ratio (SNR) of a single TEM slice.[50, 51, 66, 73] However, the reconstruction process with the multitude of TEM slices will compensate for part of the SNR loss. Due to a single TEM image having a low SNR, it becomes more difficult to locate regions of interest for ET. Furthermore, because the full tomographic reconstruction only becomes available after the full data collection, a large portion of the reconstructions may not provide the desired structures.[50, 51] Fortunately, this limitation of electron tomography can be overcome by applying correlative optical microscopy.

Correlative microscopy makes it possible to locate ROIs using the optical microscope before electron tomography.[23, 26, 50, 74] Generally, FM is used to locate the regions of interest. FM is favored in this procedure due to its fast imaging capabilities and its ability to search for specific sample features that are potentially very small using target-specific labels. With this method, the coordinates of organelles and proteins can be mapped before the more time consuming TEM and tomographic analysis. Protocols have been developed where, depending on the techniques applied, the sample was fixated, dehydrated or fast-frozen and then stained and prepared into thin slices.[39] Locating the region of interest with correlative fluorescence microscopy is often performed during sample preparation, when a TEM sample slice is obtained. This can be either integrated with the FIB that is used for preparation of the lamella, or on a separate microscope before the sample is moved to the ultramicrotome or FIB.[50, 75]

2.6.3 Super resolution microscopy

The last two decades have seen the development of many super-resolution microscopy techniques. These techniques are often based on the localization, with high precision, of individual fluorophores by identifying the centroid position of the fluorescence light spot. Several of these techniques are: stimulated emission depletion (STED) microscopy, stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM).[76] These high-resolution imaging techniques are being applied to bridge the resolution gap between light and electron microscopy. Localization of specific targets e.g. proteins and mitochondria is performed by fluorescence super resolution, with high precision. Next EM can be used to analyze each specific region. These optical microscopes can achieve a spatial resolution above 20 nm for STORM and PALM, and up to 40 nm for STED which improves the correlation between OM and EM images.[77,

78] Correlative super-resolution with electron microscopy performed on separate systems is demonstrated for STED and Palm protein localization with electron microscopy,[47, 78, 79] and for STORM sub-diffraction resolution with TEM.[80] Correlative super resolution with electron tomography is especially powerful for resolving small molecular systems in cells.[81]

In this context, it is worthwhile to describe another correlative super-resolution imaging technique namely tip enhanced Raman spectroscopy (TERS). To perform TERS, a silver or gold coated tip is used akin to those used in atomic force microscope (AFM) or scanner tunneling microscope (STM), as such correlative AFM and TERS is performed. Through the SERS effect, Raman signals of molecules in the tip's vicinity will be significantly enhanced. This results in a spatial resolution for TERS microscopy of roughly the size of the tip apex, and spatial resolutions down to 10 nm are often achieved.[82] Remarkably even sub-nanometer TERS microscopy has been demonstrated for single molecule imaging.[83]

2.6.4 X-ray - EDX and cathodoluminescence

There is another combination of OM and EM which has not yet been discussed, termed X-ray fluorescence (XRF) spectroscopy. In XRF, the sample is excited using X-ray or gamma ray radiation. Subsequently, the material will emit X-ray fluorescence that can be detected using energy dispersive analysis. The XRF emission wavelength depends on the electronic orbitals of the present compounds. This allows for the discrimination of individual compounds through the detection of the spectral differences. In energy dispersive X-ray spectroscopy (EDX) the detector provides the energy of the emitted photons, thus enabling analysis of spectral characteristics. In another method of performing XRF, the sample is excited using the electron beam in a SEM or TEM. The X-ray fluorescence is emitted and detected in the same way as for X-ray induced fluorescence. However, the electron induced XRF does have some limitations. First it suffers from a continuum background radiation called Bremsstrahlung,[84] and second electron induced XRF in a SEM can only be used as a surface sensitive technique due to the limited penetration depth of the electrons, as shown in figure 1.2. XRF is mainly used for the elemental analysis of materials such as metals, semiconductors, microelectronics and ceramics.[85] However, XRF is limited in application to organic compounds because the signal rapidly decreases for compounds having an atomic number lower than carbon.[86]

A second light emission process following excitation with an electron beam is cathodoluminescence. Cathodoluminescence is the emission of light that occurs after a recombination event from an electron hole pair; these electrons and holes having been excited with an electron beam.[87] Signal collection of cathodoluminescence is often performed with a light guide or optical fiber and incoupling with a spherical mirror. In SEM correlative nanoscale cathodoluminescence has been demonstrated using a light guide with a spherical mirror.[88, 89] One can however also use an in SEM on axis optical microscope objective to collect the cathodoluminescence signal. This alignment can be used to perform iCLEM while additionally obtaining the correlative Cathodoluminescence signal.[90, 91] Cathodoluminescence is generally

applied in the study of electronic bands of insulators and semiconductors.[87] Furthermore cathodoluminescence in combination with EM has been employed to study the plasmon modes in metallic nanostructures.[88]

Recognizing that the XRF and cathodoluminescence signal are excited through an electron beam or X-ray radiation, it is clear that a resolution can be obtained far below the normal OM diffraction limit. High resolution X-ray scanning microscopy (XRM) has been performed with a 10 nm spatial resolution.[92] For cathodoluminescence, resolutions of 20 nm have been obtained.[93] This provides the opportunity to use XRM or cathodoluminescence to bridge the resolution gap between optical and electron microscopy. These techniques can be used for integrated optical microscopy, or it is possible to use both a conventional OM and EM with XRM where the XRM resolution bridges the OM and EM resolution gap.

2.7 Commercial systems

With the advances in and increasing use of (i)CLEM commercial CLEM systems have become available. These products feature either RM or FM integrated with a SEM or TEM. Other products facilitate the sample transfer, between individual microscopes, with conservation of coordinates for a region of interest, such as the Zeiss Shuttle & Find. These products are regularly developed by collaborations of manufacturers with an expertise in optical and electron microscopes. Other developed systems are optical microscopes build to be suitable as add-on modules for integration with electron microscopes. A short overview of several commercial correlative microscopy systems is presented.

Several integrated fluorescence microscopes with SEM and TEM are available.[94] Delmic offers an inverted FM integrated underneath the sample and on axis with the SEM column,[27, 90, 95] this configuration enables the additional collection of cathodoluminescence light excited with SEM imaging.[90] Such configurations have also been used in combination with an atmospheric SEM system, commercialized as JEOL's ClairScopeTM. [59, 60] This enables live cell imaging with correlative FM and SEM backscattered electron detection. Another integrated system featuring ASEM and correlative optical microscopy is B-nano's airSEMTM, in this system the sample is separated from the membrane and placed on a translation stage. In this configuration the imaging area is no longer limited to the size of the ASEM membrane, the correlative optical microscope is placed parallel next to the SEM column.[96, 97] FEI's Icorr is a wide field fluorescence microscope integrated in FEI's Tecnai TEM. The system is based on the prototype presented in Ref.[3] Correlative FM imaging is performed by tilting the sample 90°, from its TEM position, to the iCorr light microscope.

Similarly to FM there are multiple Raman microscopes integrated with EM commercially available.[98] The integration of these microscopes is typically performed with SEM systems to obtain combined Raman chemical specificity and SEM nanoscale resolution. Renishaw's structural chemical analyser (SCA) is an integrated SEM - EDX - Raman spectroscope. The Raman excitation is provided with a parabolic mirror that focuses the light onto the sample, on-axis with the

electron beam. Which enables simultaneous Raman and SEM measurements. The combination of EDX and Raman spectroscopy further enables both molecular and atomic specificity. The system however does not enable a Raman imaging capability and only point measurements can be performed on locations selected with the SEM stage movement. The design of the system is presented in ref.[55] A fully integrated Raman microscope with SEM is obtained in the RISE microscope from WITec and Tescan. In this configuration the optical objective is placed parallel and adjacent to the SEM lens. Correlative imaging is performed by a stage movement of the sample between the SEM and Raman positions. This is a fully integrated non-upgradable system that enables 3D confocal Raman microscopy with SEM.[99] The system used in this thesis is HybriScan's HSCMM Raman microscope integrated with a dualbeam FIB - SEM.[100] The HSCMM is a Raman microscope that functions as an add-on module that is compatible with FEI and JEOL electron microscopes. The optical objective is placed inside the vacuum chamber parallel and next to the SEM lens, enabling 3D confocal Raman microscopy correlative with SEM. Because the microscope can be added on different FEI and JEOL systems integration can be performed with a variety of techniques such as FIB, SEM, and EDX.

2.8 Applications

Several applications, mostly in the biological sciences, have already been discussed in this chapter. However, to give an impression of the opportunities for correlative microscopy several recently published applications will be discussed. Currently correlative FM and EM is mostly applied in the biological sciences. An application of correlative FM and EM is with live cell fluorescence imaging, and correlative high resolution electron microscopy of specific events in cell dynamics. This method typically requires the use of separate light and electron microscopes, time laps fluorescence imaging is used to select a convenient time for cell fixation, after which additional sample preparation before translation to the electron microscope is performed. This has been demonstrated for investigation of the Golgi apparatus during cell mitosis,[101] and for analysis of the structure of transport intermediates from the Golgi apparatus to the plasma membrane.[102] More recently the combination of live cell imaging and electron microscopy has been demonstrated in an integrated fluorescence - ASEM system, with the sample in liquid environment. This system enables optical observation of cell dynamics and subsequently SEM analysis at a selected time and location for high resolution investigation of selected events. This has been demonstrated for the observation of cellular uptake of quantum dots.[61]

FM is used to quickly identify regions of interest after which EM is performed on these ROIs. Additional analysis can be performed by applying electron tomography, or by cryogenic sample preparation. A strong focus is given to the development of suitable preparation techniques, limiting the fluorescence reduction by sample preparation for electron microscopy. Reports of example application are used to demonstrate the developed protocols, such experiments are performed on for example mammalian cells,[95] and endothelial cells.[48] Research for cryo fluorescence - electron microscopy is aimed at developing suitable protocols for

correlative microscopy, for example optimizing preservation of the fluorescence signal. Mostly example applications to demonstrate correlative microscopy of cell structures at cryogenic condition are reported,[23, 103] also in combination with electron tomography reconstruction of such structures.[103] Correlative super resolution and electron microscopy performed on separate systems has shown promise for the nanoscale protein localization in cell structures. As such the localization of nuclear pore complexes and variations in their symmetrical structures has been shown.[104]

Raman spectroscopy integrated with SEM has been applied for multiple applications. The Renishaw structural chemical analyser (SCA) provides Raman spectroscopy on any point on the sample integrated with SEM and EDX, it however does not enable microscopic Raman imaging.[98] Applications of this system have been demonstrated for: discrimination of bacteria,[105, 106] characterization of particulate matter in air or soil to detect harmful compounds,[107, 108] characterization of uranium microparticles,[109, 110] analysis of forensics samples,[111] and characterization of the state of historical paintings.[55] In other works Picher et al.[62] used an integrated Raman - environmental STEM to monitor the growth of nanotubes, with the Raman spectroscope providing a local temperature measurement. In several other works not integrated systems have been applied for the correlative microscopic characterization of the state of historical paintings,[112] and the effect of FIB induced changes in boron doped diamond electrodes.[113] For the commercial WITec RISE microscope an initial publication has only demonstrated analysis of several example samples, such as graphene.[99]

2.9 Current developments

There are multiple developments for CLEM aimed at improving the microscopes hardware. A development of new technologies such as integrated super resolution optical microscopy, and the collection of combined optical microscopy with cathodoluminescence and electron microscopy is ongoing. The consortium Microscopy Valley involves 6 research groups in the Netherlands that in collaboration with the industry is developing such techniques. In another European consortium, universal SEM (UnivSEM), several companies and research groups work on the development of a multimodal SEM. This consortium aims to combine SEM with scanning probe microscopy (SPM), time of Flight secondary ion mass spectrometry (TOF-SIMS), EDX, cathodoluminescence and Raman microscopy.

Other developments of the microscope hardware, and software aim at automating the image acquisition to improve the data collection speed. Large field of view and 3D EM imaging requires a long measurement time up to several days of continuous measurement for a single sample. The localization of ROIs is facilitated by fluorescence microscopy, however software developments for correlative data analysis are required.[94] The use of semi-automated integrated microscopes will provide the high content data required for large specimens such as whole organisms and neuronal circuits.

Similar for time consuming Raman experiments, software that enables auto-

mated Raman imaging on locations observed in a large field of view SEM image will significantly improve the data collection by having the system perform continuous acquisition. Since Raman microspectroscopy has many applications in both materials and biological sciences it is expected that integrated microscopes will also be applied for similarly diverse fields. Of particular interest is the field of plasmonics. The interaction of light with the plasmonic system depends strongly on the nanoparticles composition, size and shape. The optical light interaction and nanoparticle morphology can both be investigated by correlative light and electron microscopy.

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RAMAN SPECTROSCOPY AND SERS

Abstract

Raman light scattering forms the basis for Raman spectroscopy. It relies on interaction of light with molecular vibrational resonances, providing compound specific information. This chapter introduces the fundamentals of Raman scattering and of confocal microscopy for micro-spectroscopic analysis. Next to conventional Raman spectroscopy, attention is given to surface enhanced Raman spectroscopy and quantitative analysis of the Raman signal intensity. A quantitative analysis of the Raman spectrometer is performed which is useful to correlate measured signal amplitudes to the number of molecules, enabling analysis of the molecular density and the Raman scattering cross section for any compound. Furthermore a quantitative calibration provides the opportunity to compare the performance of the different Raman microscopes used in this thesis and allows us to compare results measured with separate microscopes. Our interest in SERS is explained and illustrated with detailed simulations. The simulations are used to visualize the near field light - plasmon interaction strength and to calculate the SERS enhancement factors, this introduction to simulations is relevant for the quantitative SERS study presented in chapter 5.

3.1 Raman spectroscopy

3.1.1 Introduction

Interaction of light and matter is a fundamental concept that takes many forms. Incident light irradiation on matter can lead to multiple processes, most prominently absorption and scattering of light. An incident photon can be absorbed by the material, resulting in an attenuation of the transmitted light intensity and a transfer of energy to the sample. This energy transfer can result in heating of the material, or fluorescence emission of the absorbed light. Scattering processes can be divided in elastic scattering such as Rayleigh scattering, and inelastic scattering such as Raman scattering.

Raman scattering was first discovered by C.V. Raman in 1928,[1] who observed that after irradiation with monochromatic light a portion of the scattered light changed in wavelength. The frequency shifts between the incident and scattered light is specific for the compound under investigation. When investigating the scattered light in a spectrometer the Raman bands can be observed. This enables spectroscopic analysis which has been applied for the analysis of gasses, liquids, and solids in diverse fields such as: crystallography,[2, 3] cytometry,[4, 5] catalysis,[6–8] and semiconductors.[9, 10]

This chapter discusses the fundamentals of Raman spectroscopy, starting with a description of the scattering process and a discussion on the Raman scattering interaction strength. Typical Raman micro-spectrometers employ confocal detection to eliminate out of focus light and to obtain a good optical microscopic resolution. The diffraction limited resolution is calculated and a measurement of the real optical resolution is presented for one of our home build Raman microscopes. The theory presented in this chapter is required to compare the different microscopes presented in this thesis, based on their resolution and detection efficiency while taking excitation wavelength and objective type into consideration. Furthermore, quantitative Raman studies presented in chapter 5 and 7 are based on the theory presented in this chapter.

3.1.2 Raman scattering

Raman scattering is an inelastic scattering process in which photon scattering in interaction with a molecule leaves the molecule in a vibrational excited state. Due to the energy transfer to the molecular vibration the scattered light becomes redshifted from the excitation wavelength. The molecular specificity of Raman spectroscopy thus originates from molecular vibrations, which allows specific Raman bands to be assigned to vibrational states of the compound molecules. The vibrational response of a molecule can be described by a mass-spring system. The vibrational resonance frequency ω_R is then modeled as a function of the atomic masses m and their bonding strength k using formula 3.1.

$$\omega_R = \sqrt{\frac{k}{m}} \quad (3.1)$$

This equation is valid for a simplified model assuming there is no influence from neighboring molecules and no cross-coupling between vibrations. With these approximations a model for the vibrational modes can be made. The molecular resonances are described as damped harmonic oscillators, driven by an excitation light field, originating from the laser. The damped harmonic oscillator equation is described in equation 3.2. The equation describes the Raman intensity I_{Raman} using a sum of vibrational resonances R as a function of the resonance frequency ω_R , the damping factor γ_R , and the amplitude A_R . The Raman spectrum $I_{Raman}(\omega)$ is a function of the frequency ω , which is the laser minus the Stokes frequency $\omega_0 - \omega_S$.

$$I_{Raman} \propto \sum_R \frac{A_R}{\omega_R^2 - \omega^2 - i \cdot \gamma_R \cdot \omega} \quad (3.2)$$

The Raman interaction strength depends on the interaction of the electric field of the excitation light with the molecules electron cloud. The polarizability α of the molecule induces the dipole moment $\vec{P}$ responsible for light scattering. This is described in equation 3.3 where in the second line the oscillation of the optical oscillating electric field is introduced.[11]

$$\begin{aligned} \vec{P} &= \vec{\alpha} \cdot \vec{E} \\ P &= \alpha \cdot E_0 \cdot \cos(\omega_0 \cdot t) \end{aligned} \quad (3.3)$$

The vibrational modes R contribute to the polarizability, as a result the polarizability can be written as a sum of contributions of vibrational modes, equation 3.4.[11, 12] In this equation the term α_0 results in Rayleigh scattering, the polarizability term in the sum over vibrations R results in Raman scattering for the respective vibrational modes with frequency ω_R . The differential $\frac{\partial \alpha}{\partial q_R}$ represents the change in polarizability α during the transition from the ground to vibration state. Vibrations with a high change in α give a stronger Raman response and are called Raman active, vibrations with a zero change are called Raman inactive.

$$\alpha = \alpha_0 + \sum_R \left(\frac{\partial \alpha}{\partial q_R} \right) \cdot q_R^0 \cdot \cos(\omega_R \cdot t) \quad (3.4)$$

Combining equations 3.3 and 3.4 the oscillating dipole terms are described, equation 3.5.[11, 12] Three frequency components are observed in this equation: ω_0 corresponds to Rayleigh scattering at the excitation light frequency, $\omega_0 - \omega_R$ corresponds to Raman scattering the scattered light is red-shifted with ω_R , and $\omega_0 + \omega_R$ corresponding to anti-Stokes Raman scattering with a blue-shift of ω_R . Where Raman scattering originates from molecules that transition from the ground state to a vibrational state, anti-Stokes Raman scattering originates from molecules in a vibrational state that transition to the ground state during the scattering event. The anti-Stokes Raman signal thus increases for compounds with a higher temperature, since more molecules are present in a vibrational state.

$$\begin{aligned}
 P &= \alpha_0 E_0 \cos(\omega_0 \cdot t) + \sum_R \left(\frac{\partial \alpha}{\partial q_R} \right)_0 q_R^0 \cos(\omega_R \cdot t) E_0 \cos(\omega_0 \cdot t) \\
 P &= \alpha_0 E_0 \cos(\omega_0 \cdot t) + \frac{1}{2} \sum_R \left(\frac{\partial \alpha}{\partial q_R} \right)_0 q_R^0 E_0 [\cos((\omega_0 - \omega_R)t) + \cos((\omega_0 + \omega_R)t)]
 \end{aligned}
 \tag{3.5}$$

The polarizability is further influenced by the molecule's electronic state energies and the frequency of the excitation light field. The influence of the vibronic states r on the polarizability α is described in equation 3.6.[13, 14] A correct analysis requires that a sum is taken over the effect of the vibronic states however an approximation can often be made in which only the dominant term is taken into account, this is the term with the smallest $\Delta\omega = \omega_r - \omega_0$. This effect is of particular importance in resonance Raman spectroscopy where the excitation frequency ω_0 is close to an electronic absorption state ω_r . In resonance Raman spectroscopy the value for $\frac{A_r}{\hbar\omega_r - \hbar\omega_0 - i\Gamma_r}$ is mostly limited to the damping constant Γ_r and can increase up to a factor of 10^3 compared to off-resonance Raman spectroscopy. Also for the off-resonance situation an increase of the polarizability for a decreasing $\Delta\omega$ is observed. For dye molecules that have an absorption state in the visible spectrum the $\Delta\omega$ is considered low compared to typical molecules. This makes the resonance effect an important parameter when measuring and comparing the Raman scattering intensity for multiple excitation wavelengths. The A_r and B_r terms represent coupling between the ground - vibronic and vibration states.[15] The Raman scattering cross section is dependent on the $\frac{d\alpha}{dQ}$ term, which is proportional to α as presented in equation 3.6.

$$\alpha \propto \sum_r \frac{A_r}{\hbar\omega_r - \hbar\omega_0 - i\Gamma_r} + \frac{B_r}{\hbar\omega_r + \hbar\omega_0 + i\Gamma_r}
 \tag{3.6}$$

To directly calculate the Raman signal the Raman scattering cross section is used. The scattering cross section σ for a vibrational resonance r is calculated using equation 3.7.[12] The equation calculates the differential Raman scattering cross section $\frac{d\sigma}{d\Omega}$ with Ω the 4π solid angle. The expression $g_R \times (\alpha_R^2 + \frac{7}{45}\gamma_R^2)$ is known as the scattering activity, g_R is the degeneracy of the vibration and α_R and γ_R stand for the isotropic and anisotropic part of the polarizability. The strength of the local field factor L is included in the calculations, this differs from the macroscopic field due to the polarization of molecules in the medium. L is expressed in equation 3.8, where n_s and n_l are the refractive indices for the Stokes and laser wavelength respectively.[12]

$$\frac{d\sigma}{d\Omega} = \frac{h}{8 \cdot c \cdot \epsilon_0^2} \cdot \frac{(\tilde{\nu}_0 - \tilde{\nu}_R)^4}{\tilde{\nu}_R \cdot (1 - e^{(-\hbar c \nu_R / kT)})} \cdot g_R \cdot (\alpha_R^2 + \frac{7}{45}\gamma_R^2) \cdot L
 \tag{3.7}$$

$$L = \frac{n_s}{n_l} \frac{(n_s^2 + 2)^2}{3^2} \frac{(n_l^2 + 2)^2}{3^2}
 \tag{3.8}$$

Equation 3.9 is used to calculate the total Raman scattering intensity I_{Raman} based on the differential cross section, the excitation source intensity I_0 , and the number of molecules N in the laser path. The factor 4π assumes isotropic scattering over the full sphere solid angle. The combination of equation 3.7 and 3.9 enables the calculation of Raman scattering for a single vibrational resonance R , the calculation of Raman scattering for a complete molecule is obtained with the sum over all the molecules vibrational modes.

$$I_{Raman} = 4\pi \frac{d\sigma}{d\Omega} \cdot I_0 \cdot N \quad (3.9)$$

In practice the Raman intensity depends on the morphology of the sample and excitation beam. Both the molecular density and the excitation source irradiance have a spatial distribution so are a function of (x, y, z) . This is covered in more detail with information on confocal microscopy.

3.1.3 Confocal Raman micro-spectroscopy

The number of molecules in the confocal voxel is determined by the axial and lateral microscope resolution. The diffraction limited focus, calculated for plane wave illumination with a microscope objective, provides a beam radius in focus r_0 as presented in equation 3.10.[16] The axial beam propagation is illustrated in figure 3.1, showing the beam radius $R(z)$ as a function of the distance z from the laser focus. The beam radius is calculated using equation 3.11, in this equation r_0 is the beam radius in the laser focus and Z_r is the Rayleigh length defined in equation 3.12.[16, 17] Using these equations the laser irradiance can be calculated as a function of r and z , as presented in equation 3.13.

Figure 3.1: Beam profile along the axial direction in the laser focus. The beam radius $R(z)$ is calculated from the beam radius in focus r_0 and the Rayleigh length Z_r

$$r_0 = \frac{0.61\lambda}{NA} \quad (3.10)$$

$$R(z) = r_0 \sqrt{1 + \left(\frac{z}{z_R}\right)^2} \quad (3.11)$$

$$Z_r = \frac{\pi r_0^2 n}{\lambda} \quad (3.12)$$

$$I(r, z) = \frac{I_0}{1 + \frac{z^2}{Z_r^2}} \cdot e^{\frac{-2r^2/r_0^2}{1+z^2/Z_r^2}} \quad (3.13)$$

The confocal microscope in fact increases the optical resolution beyond the focus of the laser spot. The collected Raman scattered light is focused through the confocal pinhole, that functions as a spatial filter. The spatial filter decreases the Raman voxel volume by suppressing out of focus light.[16, 18] By suppressing light from out of focus sample regions removes background from the collected signal, provides a higher lateral resolution, and enables 3d imaging of the specimen. The increased diffraction limited resolution in confocal microscopy depends on the confocal aperture size. It is calculated using a convolution of the excitation laser beam path and the Raman detection optical path as projected onto the pinhole. In practice an approximation is made to calculate the lateral confocal resolution based on eq. 3.10 and $\frac{0.61}{\sqrt{2}} = 0.4$, resulting in equation 3.14. The axial resolution is similarly approximated with the conventional Abbe limit for axial resolution $2\frac{n\cdot\lambda}{NA^2}$, [19] dividing with $\sqrt{2}$ results in equation 3.15

$$r_{lateral} = 0.4 \frac{\lambda}{NA} \quad (3.14)$$

$$r_{axial} = 1.4 \frac{n \cdot \lambda}{NA^2} \quad (3.15)$$

When measuring or calculating the axial resolution the size of the sample has to be taken into account. For best practice the axial resolution is measured with a Z-scan on a sample consisting of either a thin layer or a particle smaller than the lateral resolution. The width of the Z-scan intensity is different for these situations, because the small particle will interact with a smaller portion of the excitation light when the sample is moved out of focus.

The excitation beam profile is furthermore influenced by the truncation of the laser beam on the objective opening diameter.[16, 18] A higher filling ratio of the laser beam diameter over the objective opening diameter will increase the optical resolution, at the cost of excitation light losses at the objective truncation. The microscope lateral resolution can be precisely determined with a measurement of a sub-diffraction size sample. The resolution measurement of a home-build Raman microscope is shown in figure 3.2. The microscope employs an excitation laser operating at 647.08 nm, and uses a 0.95 NA objective suitable for samples in water with coverglass correction. A 200 nm polystyrene sphere is used for the lateral resolution measurement, the particle is imaged with a scanning stepsize of 50 nm.

Principal component analysis (PCA) is performed for the Raman image data, the scores and loading of the first principal component are shown in Figure 3.2A and B. Figure 3.2C and D show respectively the x and y cross section of the scores intensity from figure A, the FWHM of the cross sections is measured to be 0.51 and 0.47 μm . In a second experiment a Z-scan is measured over a 25 nm thin layer of polysulfone, the measurement is performed with a stepsize of 50 nm. The normalized Raman intensity of the Z-scan is shown in figure 3.2E, and the polysulfone spectrum at maximum intensity in figure 3.2F. The measured axial resolution FWHM is 3.0 μm . With the measured microscope spot size, and when the molecular density is known, it is possible to calculate the precise number of Raman scattering molecules, an important parameter in quantitative Raman spectroscopy.

Figure 3.2: Measurement of the resolution of the Raman microscope employing a 647.08 nm excitation wavelength. (A) PCA scores from a Raman image map of a 200 nm diameter polystyrene bead. (B) First principal component loading corresponding to the scores presented in A. (C and D) Normalized x and y cross section (single row / column) respectively of the Raman image map presented in A, the resolution measured in full width at half maximum (FWHM) is 0.51 and 0.47 μm respectively. (E) Normalized intensity profile from a z-scan of a thin (25 nm) polysulfone layer. The measured FWHM resolution is 3.0 μm . (F) Raman spectrum of the polysulfone layer in the laser focus.

3.2 Quantitative Raman spectroscopy

The theory for Raman scattering spectroscopy, including the effects of the excitation wavelength and the confocal voxel size, has been described in chapter 3.1. In quantitative Raman spectroscopy the goal is to interpret the number of molecules in the confocal voxel and their Raman scattering cross section. Because the measured Raman spectra are a product of both these properties additional measurements with

reference samples are required for quantitative analysis. Performing quantitative SERS spectroscopy will introduce the enhancement factor as an additional variable. In quantitative SERS the goal is typically to determine first the exact number of molecules and their Raman scattering cross sections, before the SERS enhancement factor is determined from the observed Raman signal strength. In this section a quantitative calibration of the Raman microscope is provided. First the standard spectral calibration of the spectrograph and the relative optical detection efficiency as a function of detection wavelength are presented. Next a quantitative calibration of the Raman microscope detection efficiency is discussed.

3.2.1 Raman microscope calibration

Calibration of a Raman microscope is presented for the homebuild system which employs 647 nm excitation light. A calibration routine for Raman microscopy is required to obtain a wavelength calibration for the observed spectrum, and to measure differences in light transmission and detection over the full spectral range so these effects can be excluded from the measured Raman spectra. The spectrograph wavelength calibration is performed through a measurement of the spectral lines from a mercury-argon (ArHg) lamp and the Raman spectrum from toluene. The wavelengths of the ArHg spectral lines and Raman shifts from the Toluene Raman spectral lines are accurately known from literature, as such they are used to obtain the wavelengths for the CCD pixels on which the bands are recorded. Figure 3.3A shows the normalized spectra for ArHg (black) and toluene (red), the spectral lines used for wavelength calibration are indicated with blue crosses. Figure 3.3B shows the obtained Raman frequency vs. CCD pixel number curve, the positions of used spectral lines are indicated in red, the black line is obtained with a 4th order polynomial fit through these data points.

Figure 3.3: (A) Normalized argon - mercury (ArHg) gas discharge lamp spectrum (red) and toluene Raman spectrum (black), both spectra are used for spectral calibration of the Raman spectrometer. (B) Calculated spectral calibration based on the measured spectra shown in A. The red crosses correspond to the indicated bands for ArHg and toluene.

For the microscope relative detection efficiency a white light calibration routine is performed. A lamp emitting a calibrated white light spectrum is used, the lamp is placed below the objective and the observed spectrum is compared with the calibration spectrum. Figure 3.4A shows the observed white light spectrum, and figure 3.4B the spectrum emitted from the lamp. Figure 3.4C shows the microscope response curve normalized to 1, this curve is calculated as the ratio of the measured and original spectra. It is observed that light transmission is lower for near infrared (NIR) light compared to light with a shorter wavelength, this can for a large part be attributed to a lower detection efficiency of the CCD camera in the NIR spectral region. Further a very low detection efficiency is observed below 200 cm^{-1} which is a result from the notch and dichroic filters that are used to suppress Rayleigh scattered light from the Raman spectrum. The oscillations observed in the measured spectrum originate from optical interference in the thin CCD detector plate. Through the flat surfaces of the detector plate effectively a Fabry-Perot interference pattern is created.

Figure 3.4: (A) Spectrum measured from the calibrated white light lamp (B) Emitted spectrum of the calibrated white light lamp. (C) Calculated Raman microscope transmission curve. The transmission curve, normalized to 1, is used for relative intensity correction of Raman measurements.

3.2.2 Quantitative detection

Quantitative Raman spectroscopy requires a calibration of the Raman microscope spectral response, i.e. the relation between the number of Raman photons emitted from the sample and the number of detected counts.[20, 21] The photon to count ratio is obtained for the 647 nm Krypton ion based Raman microscope using two independent methods. In the first method the transmission curves from all optical elements in the setup and the CCD camera detection efficiency are obtained, which combined gives the relation of emitted photons to detected counts. In the second method a Raman measurement from a compound with known Raman response is used to calculate the detection efficiency.

Using this method the first element to consider is the objective, depending on the objective numerical aperture a portion of the isotropically scattered light is collected. The collection aperture depends on the NA as described in equation 3.16. Collected photons are then transmitted through the objective, this introduces further losses mainly for light collected with the higher NA portion of the objective

as these have an oblique angle interaction with the lens system. Light transmission through the objective was measured using the 647 nm Krypton laser line for varying beam diameters. Using this method the transmission as a function of scattering angle is obtained, enabling calculation of the transmission of isotropically scattered light at 647 nm. Next the objective (Olympus, U-Plan-Apochromat, 40x / 0.95 NA, coverglass correction 0.11-0.23) spectral transmission curve provided by the supplier was used and scaled to the measured light transmission at 647 nm. Resulting in the spectral transmission curve for Raman scattered light, the plot is shown in figure 3.5A.

For the commercial optical filters and mirrors in the setup the transmission and reflectance curves are obtained from their suppliers. Light transmission through the home-build spectrograph is measured with the laser using the same incident beam width as the collected Raman scattered light. A full spectral transmission curve is obtained using a calibrated white light source. The measured transmission curve from the white light source is scaled at the point of 647 nm to the absolute transmission measured with the laser. The resulting curve is presented in figure 3.5B.

Figure 3.5: Optical transmission curves for components of the 647 nm Raman microscope, (A) the Olympus optical objective and (B) the spectrograph. The curves show the full Raman light transmission $\frac{I}{I_0}$. The spectrograph confocal pinhole and CCD camera detection efficiencies are not included.

$$d\Omega = 4\pi \cdot \sin^2 \left(\frac{\sin^{-1}(\frac{NA}{n})}{2} \right) \quad (3.16)$$

Light transmission through the confocal pinhole depends on the distance of the scattering molecule from the microscope focal plane. This has been covered in section 3.1.3 "Confocal Raman micro-spectroscopy" with the transmission as a function of focal distance presented in figure 3.2E. Finally Raman spectra are detected on an Andor CCD camera over an array of 1600 pixels, the camera spectral detection efficiency is obtained from the supplier. Photons detected with the CCD camera are converted to electrons, which are in turn converted to digital counts. The camera is operated at a 0.8 electron per count gain, meaning that on average 100 electrons transfer to 125 counts. The transmission curve of Raman photons

emitted from the specimen to detected counts, as determined from the specifications of all independent parts in the setup, is shown in figure 3.6(red curve).

A second method for calibration of the microscope detection efficiency relies on a measurement of the Raman signal strength from a specimen with a known Raman scattering cross section. Toluene is chosen as medium with a known Raman cross section, for Raman excitation at 632.8 nm a toluene molecule has a differential cross section of $3.53 \cdot 10^{-30} \text{ cm}^2 \text{sr}^{-1}$ for its band at 1001 cm^{-1} . [22] The Raman cross section $\frac{d\sigma}{d\Omega}$ for 647 nm excitation wavelength is calculated using equation 3.7 with new values for the excitation $\tilde{\nu}_0$ and scattered $\tilde{\nu}_r$ optical frequencies, resulting in $3.22 \cdot 10^{-30} \text{ cm}^2 \text{sr}^{-1}$ Raman scattering cross section. The number of Raman scattered photons is calculating using equation 3.9. The number of molecules is determined using the molecular density of toluene, the microscope resolution as presented in figure 3.2, and considering the confocal detection efficiency for scattering molecules outside the focal plane figure 3.2E. The microscope transmission efficiency is obtained for the 1001 cm^{-1} band as the ratio of the 'measured number of counts' over the 'calculated number of Raman scattered photons from the sample'. The microscope detection efficiency for the full spectrum is then determined using the white light calibration presented in figure 3.4B. The white light calibration curve is scaled to the measured transmission value at the 1001 cm^{-1} point, the resulting curve is presented in figure 3.6 (black curve).

The detection efficiency of the Raman microscope is considered to be good. An efficiency of approximately 3 % is achieved, but it is important to consider where most of the losses originate. First the fraction of collected Raman light with the collection angle $\frac{\Omega}{4\pi}$ is in toluene 11.6 %, next the transmission efficiency through the objective is approximately 36 %. This leaves a more than 60 % detection efficiency of Raman light from the objective through the confocal spectrograph and including the CCD camera detection efficiency. On the accuracy of the quantitative calibration it is noted that there are many aspects involved, such as the measurement of the focal volume size, the accuracy of the literature values of the toluene Raman scatter cross section, and the measurement of the performance of the individual components in the microscope. It is observed in figure 3.6 that the detection efficiency curves, measured using two independent methods, agree well for the efficiency averaged over the full spectral region. They do however deviate in the response in the near infrared spectral region. A possible explanation for this is that the light transmission for the optical objective and confocal pinhole are measured using 647 nm laser light, potentially their light transmission is lower than specified in the near infrared. Furthermore on the measured transmission curve a fringing effect can be observed, this originates from interference in the thin CCD detector plate. For these reasons the calibration routine based on the toluene signal strength and white light calibration is expected to be the most accurate for quantitative Raman analysis with this microscope.

Figure 3.6: Transmission curve showing the detection efficiency of Raman photons emitted from the sample to detected counts. The values are obtained for Raman emission from toluene. Transmission curves are shown for measured (black) light transmission and light transmission determined from the combination of optical components (red).

3.3 Surface enhanced Raman spectroscopy

3.3.1 Introduction

Surface enhanced Raman Spectroscopy is a technique in which the Raman signal of molecules in the vicinity of a metallic nanostructure is enhanced. Through this process molecules adsorbed on the metallic surface provide an enhanced signal that is orders of magnitude higher from their typical Raman response. The achieved signal enhancement is strongly dependent on the metallic nanostructure shape, size and material, for ideal structures SERS signal enhancement factors have been reported up to 10^{10} . [23–25] High enhancement factors have been applied for sensing applications enabling single molecule detection with an enhancement factor of $10^7 - 10^8$. [24, 26] In other applications metallic nanoparticles with adsorbed Raman reporter molecules are used as Raman labels in biomedical applications. Typical metallic structures used for SERS applications are gold nanoparticles. Gold exhibits a surface plasmon resonance peak in the visible spectrum around 550 nm, resulting in a maximum SERS enhancement factor for an excitation source near this resonance. The resonance wavelength of the nanoparticle is tunable using nanoellipsoids or nanorods, shifting the plasmonic resonance up to the near infrared for these elongated particles. The influence of the nanostructure morphology on the signal enhancement is studied in chapter 5 of this thesis, correlative SEM - Raman experiments and simulations are performed to obtain the plasmonic properties of the studied structures.

3.3.2 Plasmonic field enhancement

The SERS signal enhancement originates from surface plasmon resonances. These are oscillations of the free electrons in the metallic structure, driven by the incident light field. The free electrons are driven with maximum efficiency when the light wavelength matches with the oscillation resonance. Electron oscillations cause an accumulation of charges at the nanostructure surface as represented in figure 3.7A. The surface charge accumulation results locally in an electric field, which is effectively enhancing the electric field component of the excitation light. Through the nature of the surface plasmon resonances the properties of the plasmonic structure are determined by the structure shape, size and the used compound. It is therefore useful to first consider a gold nanosphere as a simple structure commonly used in the field of SERS and plasmonics. Figure 3.7B shows the calculated enhancement of the local electric field component $\frac{E}{E_0}$ around a 60 nm gold sphere, the simulation is performed for 532 nm excitation light, close to the plasmon resonance wavelength. The nanostructure is further characterized through its far field scattering properties, presented in Figure 3.7C.

Figure 3.7: Scattering properties from a 60nm diameter gold nanoparticle. (A) Schematic representation of free electron oscillation under influence of an incident light field. (B) Local electric field enhancement E/E_0 calculated for excitation with 532 nm light. (C) Far field scattering spectrum of the gold nanosphere showing the resonance band near 530 nm.

In the far field scattering spectrum the structures plasmonic resonance wavelength can be clearly observed as a peak around 530 nm. The extinction and scattering spectra are found to be proportional to the Clausius - Mossotti relation, as presented in equation 3.17.[27] The g factor is maximum for a resonance situation where $\epsilon(\lambda) = -2\epsilon_m$, with $\epsilon(\lambda)$ the dielectric function of the gold obtained from "Johnson and Christy"[28] and ϵ_m the dielectric constant of the surrounding medium which for this situation in vacuum is 1. A maximized coupling between the free electrons and electromagnetic excitation field is achieved for approximately 530 nm excitation, however also for off-resonance excitation a significant enhancement of the local electric field is observed.

$$g = \frac{\epsilon(\lambda) - \epsilon_m}{\epsilon(\lambda) + 2\epsilon_m} \quad (3.17)$$

In this work SERS experiments are performed with 785 nm excitation light,

simulations with this wavelength of the near field enhancement for the nanosphere is shown in figure 3.8A. Higher field enhancement can be achieved by adapting the nanostructure morphology, a dimer structure consisting of two coupled nanoparticles with a small separation gap enables a much higher field enhancement in the gap hot spot. Figure 3.8B and 3.8C show field enhancement simulations for coupled structures with a gap of 3 and 6 nm.

Figure 3.8: A comparison of the local electric field enhancement for different plasmonic nanostructures. The calculations are performed for 785 nm excitation light which coincides with the laser wavelength in the SEM - Raman integrated system. (A) Single gold 60 nm diameter nanoparticle (monomer). (B and C) Two coupled 60 nm diameter gold nanoparticles (dimer) with a gap of 3 nm and 6 nm respectively. The local field enhancement varies significantly for the different structures, especially in the dimer gap (hot spot) location.

The local field enhancement increases the polarizability of molecules present near the nanostructure. The SERS enhancement factor (EF) is calculated as a function of the field enhancement at the excitation laser wavelength $\frac{E_L}{E_0}$ and at the Stokes shifted wavelength $\frac{E_S}{E_0}$, as shown in equation 3.18. Often an approximation is used in which the enhancement factor is calculated using only the laser wavelength assuming $\frac{E_L}{E_0} \approx \frac{E_S}{E_0}$, called the E^4 approximation. For the example situation in figure 3.8B the field enhancement in the dimer hot spot is approximately 25.5, using the E^4 approximation this results in a local Raman enhancement factor of $4.2 \cdot 10^5$.

$$\begin{aligned}
 I_{SERS} &= EF \cdot I_0 \\
 I_{SERS} &= \left(\frac{E_L}{E_0} \right)^2 \cdot \left(\frac{E_S}{E_0} \right)^2 \cdot I_0 \\
 I_{SERS} &\approx \left(\frac{E_L}{E_0} \right)^4 \cdot I_0
 \end{aligned} \tag{3.18}$$

3.3.3 Near field simulations

Calculations of the plasmonic local field enhancement and far field scattering properties of a nanostructure require the use of a suitable simulation program. The calculations solve the Maxwell equations for an incident light field in interaction with the nanostructure of interest. Results obtained from two simulation programs have been compared for calculations of the nanostructure scattering properties. The first analysis software DDSCAT uses the discrete dipole approximation (DDA) for the calculation of scattering and absorption from structures approximated as arrays of polarizable dipoles.[29–31] Modeling of a nanostructure with discrete dipoles gives a large amount of flexibility in that any geometry can be modeled. Furthermore calculations can be very accurate using a sufficiently small interdipole spacing d on the condition that sufficient computation power is available. Solving of the structures optical properties is performed through a calculation of the response of the dipoles to the incident light field the dipoles then interact with each other through their induced electric fields, this principle has introduced the term coupled dipole approximation. An example structure of a gold nanosphere modeled as 119009 dipoles is shown in figure 3.9A.

Figure 3.9: Structure builds used for simulations in (A) DDSCAT and (B) MNPBEM. For DDSCAT the 3D construction of a spherical particle build with discrete dipoles is shown. For MNPBEM the spherical particle is approximated with discrete boundary elements.

The second investigated simulation software program is the "Metallic nanoparticle boundary element method" (MNPBEM) toolbox.[32, 33] The toolbox solves the optical response of metallic nanoparticles using a boundary element method (BEM) approach. It approximates the plasmonic structure as a homogeneous structure separated from the dielectric environment with an abrupt interface. The particle boundary is discretized with a finite number of boundary elements, the simulation accuracy is improved by choosing a large number of boundary elements. The Maxwell equations are solved with the provided structure morphology and dielectric function of the metal and the medium, for gold the dielectric function from "Johnson and Christy" is used.[28] MNPBEM is implemented as a toolbox in

MATLAB, it has the advantage that calculations are generally faster due to the approximation of the structures as homogeneous media and discretization of the particle boundary. An example structure of a gold nanosphere modeled with 576 surface elements is shown in figure 3.9B.

The near and far field scattering properties from a gold nanosphere of 60nm diameter, calculated with both MNPBEM and DDSCAT, is shown in figure 3.10. The near field simulations for DDSCAT 3.10A and MNPBEM 3.10B show a significant difference in field distributions and amplitude. The far field scattering spectra in figure 3.10C are comparable for both simulations. Considering near field enhancement calculations it is clear that DDSCAT shows a distorted field distribution. This arises from the discretization of the structure with too few dipoles (fig. 3.9A) which causes morphological features at the particle boundary in the near field calculations. The model structure therefore does not represent a complete spherical particle. The irregularities at the surface then introduce a higher electric field enhancement. Using MNPBEM simulations a spherical particle can be better defined without the need for a number of dipoles or boundary elements that is too large for computation. The simulations show a good calculation of the electric field distribution, they compare well with other scattering calculation programs, and require a short computation time.

Figure 3.10: Comparison of simulation results performed with the DDSCAT and MNPBEM toolboxes. (A and B) local field enhancement for a 60 nm diameter gold nanosphere calculated with DDSCAT and MNPBEM respectively, both are represented with the same intensity color scale. (C) far field scattering (blue), absorption (red) and extinction (black) spectra calculated for the 60 nm gold sphere with DDSCAT (dashed line) and MNPBEM (straight line).

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INTEGRATED - RAMAN - FIB - SEM

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Abstract

This chapter presents the integration of a confocal Raman microscope with a focused ion beam - scanning electron microscope. The integrated system enables correlative Raman and electron microscopic analysis combined with focused ion beam micromachining on the same sample location. This provides new opportunities, for example the combination of nanometer resolution with Raman advances the analysis of sub-diffraction sized particles. Furthermore direct Raman analysis of FIB engineered samples enables in situ investigation of modified samples. The Raman microscope is an add-on module to the electron microscope. The optical objective is brought into the vacuum chamber, while the laser source and spectrometer are placed in a module attached onto and outside the chamber. We demonstrate the integrated Raman FIB SEM functionality with several experiments. First, correlative Raman and electron microscopy is used in particle counting and combined spectroscopic analysis. Combined chemical specific and high resolution analysis is used for the investigation of (sub) micrometer sized crystals and crystal polymorphs, and for the characterization of graphene flakes. Analysis of sample changes made with the ion beam is performed on diverse samples. Raman analysis of patterns milled in a silicon substrate reveals changes in the crystal lattice, and analysis after milling with the FIB on an organic polymer reveals amorphization of the surface materials. Finally a correlative SEM - Raman study for red and white blood cells is presented. Cell type characterization based on Raman spectra is performed, and correlated to cell morphologies observed by SEM.

4.1 Introduction

Correlative light and electron microscopy is typically performed using two different methods. First using separate microscopes in which the sample is transferred between the independent systems and the region of interest is relocated with the help of fiducial markers. Second using an integrated microscope in which the optical objective is placed in the electron microscope vacuum chamber. This provides an easy transfer between both systems, removing the need for sample markers, and enabling a faster workflow. As a result there is an increasing interest in the use of integrated light and electron microscopes for the CLEM field. In chapter 2 a more thorough discussion is provided on the developments of iCLEM, the new strategies for microscopic analysis it enables, and potential applications.

Details of the integration of the microscopes may vary. The light may enter the vacuum chamber through direct coupling[1], through an optical fiber to connect to a remote spectrometer[2–4], or with a rod and a parabolic mirror for optical focusing.[5] In this chapter we present Raman microscopy directly integrated in a dualbeam FIB - SEM. The integrated combination of FIB and Raman allows for chemical specific characterization of FIB modified samples with confocal Raman microscopy. Furthermore this combination potentially enables new strategies for Raman analysis by preparatory FIB treatment, such as the removal of non-transparent surface layers, or the isolation of specific structures from surrounding materials. The correlative SEM and Raman microscope enables the opportunity to study samples at a high resolution level and to combine this structural and morphological information with chemical specific spectral information. A discussion on other applications of the FIB - SEM - Raman is provided in chapter 2.3.2.

In this chapter the method of integration will be discussed, it preserves all functionalities of the FIB-SEM and enables an easy exchange between the FIB-SEM mode and the Raman mode. Furthermore experiments will be presented and discussed as a proof of principles of the capabilities of integrated correlative Raman-FIB-SEM. Finally the first experimental results to the correlative SEM - Raman analysis of red and white blood cells are presented. The integrated microscope presented in this chapter is applied for the analysis of plasmonic nanostructures (presented in chapter 5) and the observation of whispering gallery modes in dielectric microspheres (presented in chapter 6).

4.2 System integration

4.2.1 NOVA600 FIB-SEM

The dualbeam FIB-SEM Nova 600 NanoLab developed by FEI is shown in figure 4.1. It employs a high resolution field emission scanning electron microscope (FESEM) column, which can achieve a resolution of 1.1 nm for electron microscopy at 15 kV electron energy and imaging with the through the lens detector (TLD) of secondary electrons. Electron beam energies between 200 V and 30 kV can be chosen with a probe current up to 20 nA adjustable in 21 steps. The ion beam features a MagnumTM ion column with a Ga liquid metal ion source, the achievable FIB

resolution is specified at 7 nm. The FIB acceleration voltage is adjustable between 5 and 30 kV, with a probe current of 1.5 pA to 21 nA adjustable in 12 steps. The NOVA 600 Nanolab contains two TLDs for SE and BSE detection, an Everhart-Thornley detector (ETD) for secondary electron detection, and a channel detection electron multiplier (CDEM) direct ion detector. Furthermore the NOVA 600 features a gas injection systems, a 5 axis motorized stage, and a micromanipulator. The dualbeam system is most commonly applied for micromachining of structures such as: gold plasmonic nanostructures, photonic crystals, the preparation of AFM tips, and TEM sample preparation.

4.2.2 HybriScan Raman microscope

Figure 4.1: The HybriScan HSCMM Raman microscope mounted as an add-on onto the FIB-SEM vacuum chamber. The Raman microscope is seen as the black - white box on the left side of the dualbeam FIB-SEM.

The Raman microscope functions as an add-on module to the FIB-SEM. The main part of the microscope, containing most of the optical components, is placed onto the SEM vacuum chamber as shown in figure 4.1. The microscope employs a 785 nm excitation laser with an optical power onto the sample adjustable from 0.1 to 37 mW. The optical objective is vacuum compatible, features a long working

distance of 2.3 mm, with a NA of 0.65. 3D-Raman imaging is performed with an integrated piezo-driven xyz-stage. The stage has a travel range of 21 mm for the lateral directions and 11 mm for the axial direction, the stage movement repeatability is specified at 25 nm with a resolution of 1 nm, which is well below the optical resolution. A spectral range from 330 to 2625 cm^{-1} is available, the spectral resolution is measured at 4.9 cm^{-1} FWHM.

Figure 4.2: Measurement of the resolution of the Raman microscope employing a 785 nm excitation wavelength. (A) PCA scores from a Raman image map of a 500 nm diameter polystyrene bead. (B) PCA loading corresponding to the PCA scores presented in A. (C and D) Normalized x and y cross section respectively of the Raman image map presented in A, the resolution measured in FWHM is 0.9 μm . (E) Normalized PCA scores intensity profile of a z-scan from the same polymer bead through the microscope focus. The measured FWHM is 4.4 μm . (F) PCA loading corresponding to the PCA scores presented in E.

A measurement of the optical resolution has been performed, the results of which are shown in figure 4.2. A Raman image map of a 500 nm diameter polystyrene bead is made with a low scanning step-size (100 nm). The PCA scores (fig. 4.2A) and loadings (fig. 4.2B) are used for analysis of the Raman spot size. An X and Y cross section of the scores intensity map is presented in figures 4.2 C and D respectively showing a lateral resolution of 900 nm FWHM. The axial resolution is measured with a Z-scan over the same polymer bead. The PCA analysis scores (normalized) (fig. 4.2E) and loadings (fig. 4.2F) show a 4.4 μm FWHM resolution.

4.2.3 System integration

The Raman microscope is placed onto the port above the door to the FIB-SEM vacuum chamber, as shown in figure 4.1. Light enters and exits the vacuum chamber

through an optical window that seals the vacuum. An alignment mirror and the objective are placed inside the chamber with the objective positioned parallel to and next to the SEM column. The mirror and Raman objective are mounted in a compact system in such a way that contact with components in the SEM is avoided and that the ability of a necessary stage tilt of 52° for FIB milling is preserved. Correlative imaging can then be performed by moving the sample from the SEM probe position to the Raman probe position with the SEM x-stage over a distance of approximately 50 mm. The exact stage movement is determined through a calibration in which a specific sample region is observed with both the SEM and the video camera in the optical microscope. Both observations with the corresponding stage position gives the relative stage position difference between the SEM and Raman objective. A schematical representation and photo of the system integrated in the vacuum chamber is presented in figure 4.3.

Figure 4.3: (Left) Schematic of Raman microscope objective integrated in the FIB-SEM vacuum chamber. In the real situation the FIB column is placed at a 90° configuration with respect to the Raman - SEM plane. (Right) Photograph of the Raman objective and 3D scanning stage integrated in the vacuum chamber.

The versatility of the FIB-SEM may require the presence of a large number of tools and detectors in the vacuum chamber, e.g. gas injectors, ion detectors and an Everhart-Thornley detector. The integration of the Raman microscope with the FIB-SEM therefore requires a compact, mechanical solution for the mounting head of the optical objective. In the final embodiment the Raman objective head is placed at a height above the SEM lens, while the Raman focal plane is below the SEM lens. This alignment provides a safe distance of the objective to the sample table and still enables analysis of, for instance, 6 inch wafer samples with a radius larger than the SEM to Raman distance of 50 mm. 3D-Raman imaging is performed with an integrated xyz-stage. The communication with the stage

requires a cable feedthrough, which is mounted in a vacuum flange and placed above the vacuum chamber load-lock port.

4.2.4 Calibration procedure

A calibration of the Raman spectrometer spectral detection range and relative detection efficiency over the full spectrum is performed. The procedure is in principle similar to the one presented in chapter 3.2.1. The spectrograph wavelength calibration is performed through a measurement of the spectral lines from a Neon - Argon gas discharge lamp, and subsequent interpolation of the obtained wavelength vs. CCD pixels. Figure 4.4A shows the spectrum observed from the neon-argon lamp, and figure 4.4B shows the obtained relative wavenumber axis vs. CCD pixels.

Figure 4.4: (A) Gas discharge spectrum from a neon - argon lamp. The well known positions of the spectral bands are used for the calibration of the spectrograph. (B) Spectral calibration of the spectrograph showing the relative wavenumber axis vs. pixels of the CCD detector.

The calibration of the microscope relative detection efficiency is performed with a white light calibration routine that is slightly different from the routine used for the 647 nm Raman microscope, shown in figure 3.4. As white light source an ion doped glass structure is used that emits a broadband fluorescence spectrum when excited with the Raman laser. The doped glass (SRM 2241) has a calibrated emission spectrum measured by the supplier the National Institute of Standards and Technology (NIST). Figure 4.5A shows the observed white light spectrum, and figure 4.5B the calculated normalized transmission curve. It is observed that the detection efficiency decreases significantly for the higher wavenumber region (~ 900 nm wavelength), this is a consequence of the lower detection efficiency of the CCD camera in the NIR region.

Figure 4.5: (A) Fluorescence spectrum recorded from ion doped glass with a NIST calibrated response. (B) Normalized intensity calibration for the spectral response of the Raman microscope, obtained through a measurement of the NIST glass.

The use of an integrated light and electron microscope requires a calibration for the movement of the sample between microscopes, to ensure that analysis is performed on the same sample location with both systems. The sample movement is performed with the SEM stage and it is therefore required to know the exact distance between the Raman and SEM focal points in x-y coordinates of the stage. For this procedure a recognizable structure etched in a silicon substrate is located with both the optical and SEM microscope. Observation of the structure is shown in figure 4.6A for the light and figure 4.6B for the SEM microscope. To obtain the precise distance in x and y coordinates of the SEM stage and overlay of the two images is made, this is done directly with the HybriScan measurement software. The overlay image is shown in figure 4.6C. This overlay is obtained based on software image recognition where rotation, positions shift, and magnification are adjusted to match the figures. The validity of the overlay is verified with the difference intensity between the video and SEM images, the difference intensity image is presented in figure 4.6D. Here it should be considered that contrast is different between the optical and electron microscope. Structures that are bright in the video image might be dark in the SEM.

Figure 4.6: SEM - video image overlay performed using structures etched in a silicon wafer. (A) Video image of the etched pattern. (B) SEM image. (C) Overlay of the video (green) and SEM (purple) images. (D) Difference intensity between the video and SEM images. The figure colorscale is not used as an indication for figure overlay because of the differences in contrast between the video and SEM images. Should sample features be seen mirrored in the image this would be an indication of a wrong overlay, for the presented situation a good overlay of the figures is achieved.

4.2.5 Operation of the integrated microscope

Operation of the integrated microscope is typically performed by first using the SEM to identify and locate a ROI, the SEM is used for this purpose as it is much faster than the optical microscope. Next Raman microscopy is performed on the identified ROI. For optical microscopy the appropriate considerations for excitation power, integration time, and image scanning step size have to be made. The decision on these settings are based on an excitation power low enough to not damage the sample, an integration time for a sufficient signal, and a scanning step size typically around half the optical resolution. After Raman microscopy new high resolution SEM images are made of the investigated area. High resolution SEM images are only obtained after Raman spectroscopy to limit the effect of electron beam induced deposition of hydrocarbons that may contaminate the sample. Hydrocarbon contamination can become very high during high resolution EM imaging with a long integration time and high magnification. Sample contamination through EBID becomes visible in Raman spectroscopy as a broadband background contribution on the signal, and potentially decreases the Raman signal itself through absorption of excitation and Raman scattered light.[6, 7]

For operation of the integrated microscope the operation settings, such as

electron beam energy and laser power, are suitably chosen for each specific sample. Organic and polymer compounds typically have a low conductivity, which can result in surface charging. For this reason typically a low electron acceleration voltage of 2 to 5 kV and a low probe current of 2.5 to 13 pA are used. SEM imaging is performed with SE detection using either the Everhart-Thornley detector for general imaging, or with the through the lens detector which is used during operation of the SEM in immersion mode for a higher resolution. Operation of the FIB is performed with a probe current of 46 to 98 pA and at 30 kV ion energy, the low probe current enables accurate FIB milling with limited sample charging. The FIB milling progress can be monitored, this is done with the emission of secondary electrons from the sample. As working distance the sample is always set at the eucentric distance of 5 mm below the SEM lens. Raman imaging is typically performed with around 10 mW laser excitation power but can be chosen anywhere between 0.1 mW and 37 mW. The Raman pixel integration is generally set at 1 second, the image step size is always set below the Raman resolution generally a 500 nm step size is chosen.

4.3 Sample materials

Substrates for Raman experiments are performed on: Calcium-fluoride Raman-grade windows obtained from the company Crystran, and on μ RimTM substrates obtained from the company BioTools, inc. Polystyrene beads of 0.2 μ m, 0.5 μ m and 1 μ m are obtained from Polysciences, inc.

The samples containing calcium carbonate and calcium sulfate crystals are made using solutions from analytical grade calcium chloride CaCl_2 , and sodium bicarbonate NaHCO_3 , sodium sulfate Na_2SO_4 obtained from Sigma Aldrich. Cyanobacteria *microcystis aeruginosa* (strain 145) were obtained from Culture Collection of Algae and Protozoa, Oban, Scotland. Calcium-fluoride Raman-grade substrates from the company Crystran were used for these experiments.[8]

Analysis of graphene flakes is performed on samples fabricated through chemical vapor deposition (CVD) on a nickel substrate. The process fabricates single- and multi-layer graphene, with multi-layers visible as darker areas in SEM analysis.[9]

4.3.1 Data treatment

To extract the maximum information from the Raman analysis a suitable treatment of the data is required. First the microscope calibration is used to correct the raw data for relative microscope efficiency and to include the spectral frequency axis, as discussed in chapter 4.2.4. Next multiple pretreatment steps can potentially be applied before the data is analyzed with either univariate or multivariate analysis. The used data pretreatment and analysis steps are specifically chosen for each measurement, they are accurately reported with the reported results.

As data treatment a noise reduction method can be applied by using singular value decomposition (SVD), this factorizes the dataset in the U, S, and V matrices.[10] Next the Raman dataset is reconstructed using the first selected S-components, the chosen S-components are specific for real spectroscopic data and

the remaining S-components that correspond to noise contributions in the dataset are discarded. Noise reduction with SVD works best for larger datasets, since specific Raman components are more easily separated from noise contributions. For verification that only noise contributions in the dataset are discarded a reconstruction of these discarded components can be made and is checked for potential Raman data components. A second data treatment procedure is a filter that removes smooth background contributions from the dataset.[11] This procedure removes the contribution of autofluorescence light from the measured signal while preserving the Raman contributions. The final step before data analysis is the selection of a spectral region of interest, this is usually the entire Raman fingerprint region or several specific Raman bands.

Multiple experiments presented in this thesis are analyzed using multivariate analysis, the used techniques are principal component analysis and subsequently hierarchical cluster analysis (HCA). PCA reduces the datamatrix to a set of orthogonal principal components (loadings) and coefficients (scores). Each measured Raman spectrum in the original dataset is transformed to an array of scores which, when multiplied with their respective PCA loadings, reconstruct the original spectrum. The transformation is such that the first principal component (PC) contains the largest possible variance in the dataset. The following PCs are orthogonal to the first PCs and also contain the largest possible variance.[12] This enables to present the strongest contributions to the dataset by making a Raman image of the first PC scores.

Next HCA can be performed.[13] HCA is applied on the most important or potentially all PC scores. HCA groups the measured spectra in clusters based on the similarity of their PC scores, as a result similar spectra are grouped in the same cluster. This data analysis method further reduces the dataset to a number of clusters chosen by the user, enabling a more efficient presentation of the data. Pixels in the Raman image are colorcoded based on their respective cluster, as such a colorcoded image is obtained that presents locations with similar spectra in the same color. Finally for each cluster the averaged spectra are presented.

4.4 Integrated correlative microscopy proof of principles

Experiments have been performed on samples selected to demonstrate the integrated Raman - FIB - SEM functionality. In this section also the results of projects performed in collaboration with other researchers are presented. Results presented in this section are divided in three parts, correlative SEM - Raman, correlative FIB - SEM - Raman, and a separate project on correlative analysis of red and white blood cells.

4.4.1 Correlative SEM - Raman

iCLEM experiments typically use the SEM for tasks that require a higher resolution than available with optical microscopy, such as localization or identification of particles and particle counting. Here it will be demonstrated that the combination

of high resolution SEM with Raman microscopy enables an unequivocal relation between Raman light intensity and the number of particles. Inversely, the amplitude in Raman microscopy can be used to count numbers of particles in un-resolved optical images, or in particle mixtures with a low SEM contrast, but a strong Raman contrast. For this purpose first locations with particles are identified. The locations contain either single particles or clusters of particles. Next the Raman signal intensity on each location was determined. The recorded intensity depends discretely on the number of particles at each location of interest.

This experiment has been performed using 500 nm polystyrene beads (fig. 4.7). Two correlative SEM (figure 4.7 A and D) and Raman images (figure 4.7 B and E) have been recorded for this purpose. Figure 4.7C shows the Raman spectrum from 800 to 1250 cm^{-1} , the measurement points (black crosses) show the spectral region from 970 to 1015 cm^{-1} of which the integrated signal is used in figures B and E. The measurements were conducted with 37 mW excitation power, a 1 second integration time per pixel, and a scanning step size of 250 nm. The intensities from 11 single particles and from clusters of 2, 3, and 6 particles are recorded. The signal strength is determined for each particle location through both spatial and spectral integration of the Raman signal. The recorded amplitudes have been plotted against the number of particles as determined by SEM in figure 4.7F. A linear relationship is obtained, as expected, and a fit through the recorded points is used to extract the average spatially and spectrally integrated intensity of a single bead. The standard deviation measured over 11 single beads is found to be 14% of a single bead intensity, indicating that the SNR at these settings is high enough for individual particle counting in optically unresolved clusters.

Figure 4.7: (A and D): SEM images of 500 nm polystyrene beads with indicated in red the region selected for Raman imaging. SEM imaging is performed with SE detection, 5 kV electron energy, the scale bar is 10 μm and 5 μm for figs A and B respectively. (B and E) Raman images of the indicated regions showing the intensity of the 1001 cm^{-1} band after spectral integration over the range from 970 - 1015 cm^{-1} , indicated by the black crosses in (C). (F) The spatially integrated Raman intensity, shown in B and E, for every single or cluster of polystyrene particles. With the current experimental setting, 37 mW and 1 second integration time, 4467 counts are measured per particle in the indicated band.

A demonstration of correlative SEM - Raman analysis of a sample containing different crystals, and crystal polymorphs is presented in Figure 4.8. SEM imaging is performed with 5 kV acceleration energy and 53 pA probe current, Raman imaging is performed with and 10 mW excitation power, 1 second spectral integration time, and 300 nm step size. The sample contains crystals of calcium sulfate and calcium carbonate and bacteria (*microcystis aeruginosa*). In figure 4.8A a region of interest is selected for Raman imaging from the SEM image. The correlated Raman image is shown in false-color coding figure 4.8B after HCA based on PCA of the data, as explained in chapter 4.3.1. Before PCA the data is pretreated by removing broadband background fluorescence. The imaged region consists of many different compounds that require a close spectral analysis. For this purpose locations of individual particles are chosen based on the SEM image, as shown in figure 4.8C. The correlative Raman spectra of these locations are selected and presented in figures 4.8D, 4.8E and 4.8F.

Figure 4.8: (A) SEM image showing a region with multiple different particles of interest, the red square indicates the region analyzed with correlative RM. SEM imaging is performed with SE detection, 5 kV electron energy, the scale bar denotes 10 μm . (B) 12 level cluster analysis image of the recorded Raman data. (C) Locations indicated in the SEM image corresponding to individual particles of interest. (D) Raman spectra averaged over the indicated regions, the spectra correspond to calcium sulfate crystals as recognized by the spectral peaks at 1006 and 1136 cm^{-1} . (E) Raman spectra 4, and 5 correspond calcium sulfate. Spectrum 6 corresponds to vaterite, a polymorph of calcium carbonate, recognizable from the 1088 cm^{-1} band. Spectrum 7 contains components of both calcium sulfate and calcite, calcite is another calcium carbonate polymorph recognizable from the 711 and 1086 cm^{-1} bands. (F) Spectra corresponding to several highly fluorescent bacteria. The spectra corresponding to the following locations have been offset for clarity 1, 3, 4, 5, 7 with 150, 150, 200, 200, 450 counts.

The spectra show the presence of multiple gypsum crystals composed of calcium sulfate, two polymorphs of calcium carbonate, namely calcite and vaterite and multiple locations with a high fluorescence signal that originates from the photosynthetic bacteria *m. aeruginosa*. The Raman spectra observed from the crystals are compared with literature for the identification of the crystals based on their Raman spectra.[14, 15] The figure demonstrates the application of correlative microscopy obtaining both high resolution SEM morphological information combined with Raman spectroscopic identification of many different particles in the sample.

Raman microscopy is an important technique for the investigation of graphene and other carbonaceous materials fabricated through CVD. Fabrication with CVD enables the fabrication of large area graphene films on Nickel substrates, the sample consists of single- and multi- layer regions which can be identified with Raman spectroscopy.[16] Raman analysis of graphene flakes enables analysis of the 2D, G and D graphene Raman bands. The graphene D band is often an indication of disorder in the graphene structure,[17] specific locations with high D-band intensity can thus be further investigated with high resolution correlative electron microscopy after Raman identification. Further the Raman intensity maps of the G- and 2D-bands show increased Raman activity for multilayer regions, and the band intensity ratio provides additional information on the layer thickness for multilayer graphene with a decreased 2D over G ratio for multilayer samples.[16] The use of Raman spectroscopy for detection of single or multiple layers of graphene and analysis of the thickness uniformity and spatial distribution of the flakes is demonstrated in figure 4.9. Correlative SEM - Raman imaging is performed with 5 kV acceleration energy and 98 pA probe current, Raman imaging is performed with and 16 mW excitation power and 1 second spectral integration time. For pretreatment of the data SVD noise reduction is applied that preserves the first 10 S-values of the data. SEM imaging (fig. 4.9A) reveals darker areas for regions with more graphene layers, a typical Raman spectrum measured on the indicated region is presented in figure 4.9B with the D, G, and 2D bands indicated. It should be noted that the 2D band, near 2650 cm^{-1} , [17] is centered just outside the spectral range of the Raman spectrometer with only the tail of the band visible above 2500 cm^{-1} . Analysis of the 2D band intensity is therefore performed only on the visible tail of the full band. A Raman intensity map of each individual band is overlaid with the SEM image and presented in figure 4.9C-E.

Figure 4.9: (A) SEM image of multiple graphene flakes, noticeable by the darker color are multilayers. SEM imaging is performed with SE detection, with 5 kV electron energy, at 2500x magnification. (B) Raman spectrum measured on the position indicated in A averaged over 3x3 pixels, the shown spectra is chosen from a representative location on the graphene flakes. The D, G, and 2D bands are indicated in the spectrum. (C, D, E) integrated Raman intensity maps of the D, G, and 2D graphene bands respectively overlaid onto the SEM image.

A known property of graphene fabrication on nickel is that the layer thickness is not very uniform, individual nickel grains may affect the thickness of the graphene film during CVD deposition.[16, 18] The size of the graphene flakes can be observed with SEM imaging based on their contrast, where thicker graphene layers are darker in SE detection. Correlative Raman imaging is additionally used to measure the graphene uniformity. The signal intensity of the G and 2D bands presented in figures 4.9 D and E shows a strong contribution of regions with multiple layers. Figure 4.9C shows overall a low D-band intensity that is at most at 10% of the G band intensity, from which a low disorder in the sample is concluded. In other works, found in the literature, a more detailed Raman analysis of the graphene CVD fabricated material is performed by transferring the graphene onto an insulating surface, such as SiO₂. [16, 19] It is reported that this transfer recovers the Raman signal from monolayer graphene that is not observed on Nickel substrates.[16, 19] This recovery of the Raman signal of few layer graphene is expected to also improve the layer thickness measurements for multi-layer graphene.

4.4.2 Correlative FIB - SEM - Raman

The combination of Raman with FIB spectroscopy enables the characterization of materials after micromachining. Raman chemical analysis gives insight in the influence of the Ga-ions used in micromachining. Possible changes in the samples are expected at the sample surface such as: material ablation and the formation of defects. Correlative Raman - FIB is demonstrated on two distinct samples, first on polymer beads and second on a silicon waver. For these very different sample materials also different kinds of sample changes are observed.

In figure 4.10 a demonstration of Raman analysis in combination with FIB micromachining is presented with a sample of polystyrene microspheres (1 μm diameter). The polystyrene beads are observed with the SEM before correlative Raman imaging, as presented in figure 4.10A. The bead selected for FIB removal is indicated in a white circle. Raman analysis is performed before (fig. 4.10B,C) and after (fig. 4.10E,F) FIB ablation of one of the beads. Care is taken to prevent an interaction of the ion beam with the surrounding material. Possible re-deposition of removed material can become visible with Raman microscopy. It is observed that after FIB milling the Raman spectrum of the untreated polystyrene beads shows a broadband fluorescent background. This background can originate from material redeposition after FIB milling or from SEM induced EBID. SEM analysis of the sample after FIB milling is shown in figure 4.10D, and with a higher magnification of 25000x in figure 4.10G. From the SEM images a clear residue of the bead is observed.

The Raman spectrum of the residue after FIB ablation is presented in red in figure 4.10H. A broad band around 1400 cm^{-1} is visible indicating the presence of amorphous carbon. Both Raman images were collected with a laser power of 16.5 mW and 1 second integration time. The SEM images were measured with SE detection using the TLD detector. To limit charging of the sample 2 kV acceleration voltage was used with a low probe current of 2.5 pA for figures A and D and 13

pA for figure G, a pixel integration time of 10 μ s for figure A and 30 μ s for figures D and G was used.

Figure 4.10: (A) SEM image before FIB milling, made at magnification 5000x the scale bar denotes 10 μ m. (B) Raman image of the integrated intensity in the 1001 cm^{-1} band. (C) Raman spectrum averaged over multiple polystyrene beads, black area indicates the spectral region presented in B. (D) SEM image after FIB milling, the location of the removed bead is found in the center of the image, the total applied ion dose was $1.1 \cdot 10^{11} \text{ Ga}^+ \mu\text{m}^{-2}$. The image is made at magnification 6500x the scale bar denotes 5 μ m. (E) Raman image of the intensity integrated over the 1001 cm^{-1} band. (F) Raman spectrum of the polystyrene beads after FIB milling of the single bead. A broadband background is observed on the polystyrene spectrum, as can be seen in the comparison with the polystyrene spectrum in C. This background can originate from redeposition of FIB material ablation, or SEM induced EBID. The black area indicates the spectral region presented in E (G) High resolution SEM image of the FIB milled area, the scale bar denotes 2 μ m. (H) Raman spectrum measured of the region where the removed particle was located. A residue of amorphous carbon has remained on the treated location.

Figure 4.10A and B depict Raman analysis after a much lower Ga-ion dose. Figure 4.11A compares the Raman spectra before and after a first dose of $7.6 \cdot 10^6 \text{ Ga}^+ \mu\text{m}^{-2}$. An increase of fluorescence background and of amorphous carbon Raman signal is observed. In figure 4.11B the emission spectrum after a second dose of $6.4 \cdot 10^6 \text{ Ga}^+ \mu\text{m}^{-2}$ ions reveals an intense fluorescence emission, suggesting molecular damage to the polystyrene bead. Additionally, it was noticed that the beads more easily melted in the Raman laser light after FIB treatment. This suggests that molecular changes resulted in an increased absorption of the polystyrene material causing thermal damage under light illumination. These results reveal a problem arising from FIB - Raman experiments performed on carbon based materials. The higher light absorption of the material after FIB irradiation results in increased sample damage during subsequent Raman analysis, this severely limits the capabilities of correlative FIB - Raman analysis for polymers and organic compounds.

Figure 4.11: (A) Spectrum before [black] and after [red] FIB irradiation of a $1 \mu\text{m}$ polystyrene bead, for which a first ion dose of $7.6 \cdot 10^6 \text{ Ga}^+ \mu\text{m}^{-2}$ is used. The 1001 cm^{-1} polystyrene band and an amorphous carbon layer can be distinguished. The Raman spectrum 'before FIB' has been offset for comparison with 160 counts. (B) High fluorescence spectrum from polystyrene after an ion dose of $1.4 \cdot 10^7 \text{ Ga}^+ \mu\text{m}^{-2}$. The signal has significantly increased in intensity and shows a broad fluorescence background. Raman spectra in H and I were obtained with a laser power of 1 mW and a measurement time of 1 s.

The study of ion implantation in Si has attracted interest due to the importance of silicon as a semiconductor material. Ion implantation is generally used as a doping technique, though generally a lower ion energy is used.[20] The use of correlative FIB - SEM - Raman is demonstrated by milling a structure in a silicon substrate. FIB milling will result in removal of material from the substrate surface, however it will also result in defects in the crystal lattice due to ion implantation and disruption of the crystal. The thickness of the damaged layer will be limited due to the low penetration depth of the interacting ions, the penetration depth of gallium on silicon is 30 to 50 nm at 30 kV, with most damage occurring in the

first 30 nm.[21, 22] These defects result in a variation of the stress in the crystal structure, and in a change of the material crystallinity to amorphous silicon.[20, 22] In the Raman spectrum the change in material stress will be observed as a shift in the 1st order silicon band, with a center frequency at 520 cm⁻¹ and a broadening of this band. The occurrence of amorphous silicon is observed as a broad band around 450 cm⁻¹. [20, 22]

To demonstrate the accuracy of Raman analysis after milling on silicon a region has been patterned with an ion dose of 19 pC·μm⁻² or 1.2 · 10⁹ Ga⁺·μm⁻². Raman imaging is performed with 37 mW excitation power, 1 secon pixel integration time, and 700 nm step size. The patterned region is observed with the SEM (figure 4.12A) before correlative Raman analysis. The pattern consists of a set of lines with increasing thickness from top left to bottom right. The lines form a set of squares of 3 μm width. The silicon band is fitted with the imaginary part of the damped harmonic oscillator function (equation 3.2), which describes a single Raman band. The fitted function is compared with the spectrum in figure 4.12B. The parameters for amplitude A, spectral band position ω_0 and band width Γ are extracted from the harmonic oscillator model. This fitting method enables the detection of signal shifts around 0.1 cm⁻¹, which is approximately $\frac{1}{20}$ -th of a pixel in the observed spectrum. This is possible when a high signal to noise ratio spectrum has been obtained. The values for these parameters are obtained for every measured spectrum and the spatial distribution for each parameter is shown in figures 4.12C (signal intensity), figure 4.12D (band width FWHM), and figure 4.12E (central peak position). A high change in signal intensity is observed in figure 4.12C, this signal change should not be attributed to a removal of several micrometers of silicon, it is caused by a surface layer of amorphous or microcrystalline silicon that has a higher absorption coefficient for near infrared light.[23] Stronger signal changes are clearly recognized on the bottom and right hand side of the patterned region, where more FIB milling was performed. To analyze the amorphous silicon band the difference spectrum between treated and untreated silicon has been used. This has revealed the presence of a broad band at 450 cm⁻¹. The integrated band intensity from 390 to 485 cm⁻¹ has been presented in figure 4.12F as a black curve, and the image of the intensity of this band is shown in figure 4.12G.

Figure 4.12: (A): SEM image of the patterned region, the scale bar indicates 10 μm . (B): Raman spectrum of the 1st order Si peak [red] and the damped harmonic oscillator fit to the band [black]. (C): The total Raman intensity in the recorded Si band over the patterned region. (D): The FWHM over the patterned region. (E): The spectral peak position over the patterned region. (F): amorphous silicon band around 450 cm^{-1} , visualized after subtraction of the untreated silicon spectrum. The region of band integration for G is indicated in black. (G): Integrated band intensity map of amorphous silicon 450 cm^{-1} band, as presented in F for a single spectrum.

4.5 SEM - Raman of red and white blood cells

Application of the integrated SEM - Raman microscope is demonstrated for the analysis of cells from blood, erythrocytes and leukocytes. These experiments are performed with a similar HybriScan Raman microscope, integrated on a SEM developed by the company JEOL. Red blood cells are obtained from a blood smear and white blood cells are sorted into neutrophils, eosinophils, and lymphocytes. Correlative analysis of hemocytes provides both a detailed surface morphology and a Raman micro-spectroscopic map of the investigated cells. This facilitates the

development of a library in which both Raman spectral and SEM observed surface morphological features of cells are presented. Furthermore a discussion is made on the effects of the SEM sample preparation protocol on the Raman analysis. Raman spectral libraries of red and white blood cells have been reported in the literature, for example by Wood et al.[24] and Bankapur et al.[25]

It will be discussed how the sample preparation protocol affects the Raman measurements. Samples have to be fixated and dehydrated before they are placed in the SEM vacuum chamber, as such integrated Raman and electron microscopy cannot be applied for living cells. Furthermore because the surrounding medium has changed, by removal of water through dehydration, it can be expected that Raman bands will shift a few wavenumbers compared to cells measured in wet conditions. The full sample preparation protocol is discussed in the next section, a more expansive discussion on sample preparation for iCLEM in general is presented in chapter 2.4.

While this is the first report of integrated Raman - SEM for the analysis of cells, correlative optical - electron microscopy of cells has been reported for fluorescence microscopes integrated with SEM and TEM.[26–28] Sample preparation for these techniques also requires dehydration and fixation of the sample, additionally a staining of the sample with fluorophores is required for such techniques. In this study correlative SEM - and Raman microscopic images will be presented, Raman cluster analysis is used to classify the measured cells, and the surface morphologies of the different investigated cells are reported.

4.5.1 Sample preparation

Red blood cells are prepared by a blood smear on a μ RimTM slide (BioTools) using 1 μ L of blood from a healthy donor. The sample was fixed by submerging it three times in 100% methanol for one second. Then, the sample was left in 100% ethanol for ten minutes for dehydration. This last step was repeated. For drying, the sample was left first in 1:1 hexamethyldisilazane (HMDS)/ethanol and then in 100% HMDS for five minutes each. Finally, it was left overnight at room temperature for the HMDS to evaporate.

White blood cells (WBCs) are prepared by the following procedure. Whole blood from a healthy donor was collected by venepuncture in Ethylenediaminetetraacetic acid (EDTA) treated collection tubes. Ammonium chloride lysing buffer (8.3g NH_4Cl [154.95 mM] (Fisher Scientific), 1g KHCO_3 [9.99mM] (Fisher Scientific), 37mg EDTA [0.099 mM] (VWR Chemicals) in 1L of Milli-Q) with a pH of 7.25 was used to lyse the red blood cells. The blood was diluted 1:14 in the lysing buffer and incubated for five minutes at room temperature. White blood cells were then collected by centrifugation at 300g for five minutes and resuspended in 5 mL cold 1 $\times$ phosphate buffered saline (PBS). The sample was centrifuged at 300g for five minutes, supernatant aspirated and cells were fixed with 1% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were collected by centrifugation at 300g for five minutes and the pellet resuspended in 1X PBS. The sample was sorted using the BDFACSAriaTM cell sorter into four cell populations: neutrophils, eosinophils, lymphocytes and monocytes. Cells were centrifuged at 300g for five

minutes and the supernatant was removed. Dehydration of cells was performed by incubating the cells in increasing concentrations of ethanol: 70% , 80%, and 90% for three, five and five minutes, respectively. After, cells were collected by centrifugation at 400g for five minutes and resuspended in 100% ethanol for five minutes. Cells were then dried by adding hexamethyldisilazane (HMDS) to reach a 1:1 HMDS/ethanol ratio. Cells were collected by centrifugation at 400g for five minutes and resuspended in HMDS for three minutes. Approximately 100 μ L of each sample population was added to μ RimTM (BioTools) slides and samples were left drying overnight.[29]

4.5.2 Experimental methods and data analysis

For experimental analysis the prepared samples are placed in the SEM - Raman vacuum chamber. First regions of interest containing single or multiple cells are localized using SEM imaging. Localization of ROIs is performed with a relatively low magnification (around 200x) and a short integration time. This provides a fast procedure to find regions for correlative Raman analysis, and the low electron dose is used to limit potential damage or EBID contamination of the sample before Raman spectroscopy is performed. The SEM is operated at 2 kV acceleration voltage with secondary electron detection. The low acceleration voltage is advantageous for the investigation of biological samples. These samples consist mostly of compounds with a low atomic weight and limited SEM contrast. A low acceleration voltage increases the electron scattering coefficient, thus improving the signal without the need for higher probe currents. A more comprehensive discussion on the influence of the electron energy on the scattering coefficient is presented in chapter 2.2. The low acceleration energy and probe current furthermore limit sample damage and charging of the specimen.

Raman microscopy is performed on the cells chosen with the SEM. For the measurement the excitation energy is chosen such that no burning or other damage of the cells is observed, as such an excitation power of 10.5 mW is used. Microscopic imaging is performed with a pixel integration time of 1 second, and a scanning step size of 500 nm. A Raman microscopic image is recorded of the selected cells with the focal plane set inat the center height of the cell. After Raman analysis the sample is moved back to the SEM where a high resolution SEM image is recorded of the cell. This image is made at a higher magnification (5000 $\times$ to 10000 $\times$) and with a longer exposure time to obtain a high image quality.[29]

To optimize analysis of the recorded Raman data several pretreatment steps are performed before PCA and HCA is applied. First a constant background is subtracted from all recorded spectra, this background spectrum is measured on the used Raman substrate using the same laser power and integration time. The background consists mostly of contributions from dark current and background light excited on the substrate or in the optical path. To improve the signal to noise ratio singular value decomposition is applied to the data. As explained in section 4.3.1 the number of S components for data reconstruction is chosen such that only noise contributions are rejected.

It has been observed that some contamination occurs during the sample preparation procedure. This contamination is observed in the Raman analysis as two prominent bands at 485 cm^{-1} and 707 cm^{-1} . The spectrum of this contamination is presented in figure 4.13, based on the Raman spectrum it is concluded that the contaminant originates from the HMDS drying agent. A comparison with literature findings shows that the recorded spectrum does deviate where in literature the Raman frequencies for these reported bands are centered at 521 and 664 cm^{-1} ,[30] this indicates that the compound has undergone some molecular changes during the drying process. The HMDS contribution to the Raman spectrum is subtracted using a linear least squares (LLS) fit procedure, in which the band between 440 cm^{-1} and 550 cm^{-1} of the HMDS is fitted to the Raman data.

Figure 4.13: Raman spectrum from a HMDS residue observed after drying of white blood cells. This pure spectrum of the contaminant is used for subtraction of its contribution from the Raman data of the cells.

To ensure that HCA clustering for white blood cells is only performed on actual Raman data, and that no clusters are attributed to variations in background noise, a selection is made of the Raman spectra. In this selection spectra are only included for HCA if a Raman signal is present, this is done by setting a threshold to the total optical intensity per spectrum. This threshold is set such that all cell spectra are included in the analysis. For the presentation of the HCA images all the pixels with spectra excluded from HCA are set in a separate cluster indicated as black pixels, as shown in figure 4.16. Also the average spectrum of these pixels is shown as the black cluster spectrum. Finally a filter is applied to remove fluorescence background contribution from the dataset, this ensures that clustering is performed based on only the molecular specific Raman signal.[11] Before multivariate analysis

a spectral region of 750 to 1750 cm^{-1} is selected. After pretreatment of the data PCA is performed on the dataset, after PCA the spectra are clustered based on the scores of all their PCs in a total of 10 clusters.

4.5.3 Red blood cells

Red blood cells have been prepared using a blood smear, as discussed in the sample preparation section. This sample has also been investigated using blue laser excitation light for resonance Raman spectroscopy which is presented in chapter 7.4. Analysis of red blood cells using the integrated Raman electron microscope is presented in figure 4.14. To prevent potential sample damage Raman experiments are performed with a lower laser power and longer integration time, 5 mW excitation power and 2 second pixel integration time. An overlay of the recorded Raman - HCA image on top of the SEM image is shown in figure 4.14A, and the Raman cluster images are presented in figure 4.14B. The reported Raman cluster spectra in figure 4.14C are averaged over all the pixels within the respective clusters, and presented in the same colors. Clustering of the Raman data is performed on the spectral region of 300 cm^{-1} to 1800 cm^{-1} , the autofluorescence component in the spectra is filtered before HCA. The presented cluster averaged spectra show the signal without fluorescence removal and over the full measured spectral range. Furthermore in figure 4.14C the most prominent Raman bands are indicated with their respective spectral peak positions.

Figure 4.14: Correlative SEM and Raman analysis of erythrocytes. (A) Overlay image showing the Raman cluster image in combination with the SEM image. SEM imaging is performed with SE detection at 2.0 kV electron energy, scale bars denote 10 μm . (B) Raman cluster image as shown in figure A. Raman imaging is performed with 2 second pixel integration time, 5 mW excitation power, and 500 nm scanning step size. (C) Raman cluster spectra, prominent bands are indicated with their spectral peak position.

4.5.4 White blood cells

The correlative analysis of white blood cells with SEM and Raman is presented in figures 4.15 and 4.16. The figures show respectively SEM and Raman images of sorted neutrophils, lymphocytes, and eosinophils. The locations at which the SEM images are placed in figure 4.15 correspond to the locations of correlative Raman images in figure 4.16A. The Raman data is analyzed using hierarchical cluster analysis, all presented Raman images are clustered in a single process and the Raman spectra corresponding to the respective clusters are presented in figure 4.16B. Experiments are performed as stated in the 'Experimental methods and data analysis' section with 10.5 mW laser power, 1 second pixel integration time, 500 nm Raman scanning step size, and for SEM a 2 kV electron energy. A pretreatment of the Raman data is performed as stated in the 'Experimental methods and data analysis' section. First a subtraction of background spectra is applied, a noise reduction procedure with SVD is applied, the spectral component of the HMDS residue (figure 4.13) is removed with LLS fitting, fluorescence background is filtered from the Raman spectra, and a spectral region of 750 to 1750 cm^{-1} is selected, after which PCA and HCA is used for the analysis. Figure 4.16C shows the Raman cluster spectra after pretreatment of the data, including fluorescence filtering, and selection of the spectral region for cluster analysis.

Figure 4.15: SEM images of white blood cells, the first two columns showing neutrophils, the third lymphocytes, and the fourth showing one lymphocyte image and two eosinophil images. From top to bottom in brackets, and from left to right the scale bars denote a distance in μm of: [1, 1, 1, 1, 5], [5, 5, 1, 1], [1, 5, 1, 2, 1], and [10, 5, 5].

It is observed that the white blood cells have distinctly different morphologies, with the neutrophils having a coarser surface compared to the lymphocytes and eosinophils, and the lymphocytes having the smallest size. A measurement of the cell diameters is used to determine the average size of each cell type for this sample preparation protocol. The average diameters are for neutrophils $5.9 \mu\text{m}$, for lymphocytes $4.6 \mu\text{m}$, and for eosinophils $8.5 \mu\text{m}$. The cell morphologies are most clearly presented in figure 4.17 where higher magnification SEM images of single cells are presented, in combination with correlative Raman overlay images. It is noted that the cell morphology, as investigated by SEM, depends on the sample preparation protocol. Sample preparation is performed with the aim to preserve the native cell morphology, however several changes are likely to occur. Dehydration of the cells will result in shrinkage, it is observed that sorting can result in small clusters of cells attaching together, and the eosinophils fixed after sorting have lost surface roughness and become smoother. It is therefore expected that identification of cells is more reliably performed based on Raman analysis.

Figure 4.16: Raman cluster analysis of white blood cells, measured with 10.5 mW excitation power, 1 second integration time, and 500 nm scanning step size. (A) Raman cluster images of cells corresponding with the correlative SEM images presented in figure 4.15. (B) Raman cluster spectra with the same colorcoding as the Raman images. (C) Raman cluster spectra used in multivariate analysis, after data pretreatment.

The Raman cluster images show a good distinction for the different cell types. Color coded clusters can be assigned to specific cell types: pink to eosinophils, red + blue to lymphocytes, and green 1 + green 2 + purple to neutrophils. Example cells are selected from figures 4.15 and 4.16, and presented as overlays in figure 4.17. The overlay images present a larger version of the SEM images showing more details of the selected cells, in combination with the Raman measured image scans. This enables the direct comparison between the three cell types for both SEM observed surface morphology, and the Raman specificity with cluster analysis.

Figure 4.17: Overlays of the correlative SEM - Raman images for (A) neutrophils, (B) lymphocytes, and (C) eosinophils. The scale bars denote 1 μm for A and B, and 5 μm for C

For the actual identification of cell types based on Raman analysis, spectra corresponding to different cell types are presented in figure 4.18. This figure shows the average of all the Raman spectra recorded on the different cell types in figure 4.17. It is observed that while these spectra are similar to each other, they do contain characteristics specific for the respective cell types. These Raman bands are indicated with vertical lines in combination with their Raman frequency. Indicated Raman bands are at 672, 727, and 781 cm^{-1} for lymphocytes,[25] at 831, 1157, and 1605 cm^{-1} for eosinophils,[31] Raman bands from neutrophils are mostly overlapping with bands of the other cell types, however subtle differences in the spectra are observed at for example 849, and 1337 cm^{-1} .[25, 31]

Figure 4.18: Comparison of Raman spectra for white blood cells. Vertical lines are drawn to highlight Raman bands showing specificity for different cell types, their corresponding Raman frequency values are indicated. While the spectra are similar to each other clear differences are seen. For example: the 672, 727, and 781 bands are pronounced for lymphocytes, and the 1157 and 1605 bands are pronounced for eosinophils. The lymphocytes spectrum is offset with 20 counts, and the eosinophils spectrum with 40 counts for clarity.

4.5.5 Raman with ultrastructural FIB - SEM

From the previous section, including figures 4.15 to 4.17, it is concluded that correlative SEM - Raman enables spectral identification of cell types, and provides nanoscale morphological information. There are however remaining questions, specifically to the state of the cell ultrastructure. Considering the sample preparation steps the cells are dried and no embedding is performed, as such a hollow

structure can be expected. The ultrastructural state is investigated using FIB material ablation to visualize the inside of the cell. Correlative SEM - Raman analysis of cells is so far performed on the integrated HybriScan Raman - JEOL SEM microscope, for correlative FIB - SEM - Raman experiments the sample is investigated using the integrated Raman - FEI dualbeam FIB-SEM system.

Figure 4.19: (A) Correlative SEM imaging and Raman cluster analysis presented in an overlay image. The experiment is performed with 1.5 kV SEM acceleration energy, 12 mW Raman excitation power, and 1 second pixel integration time. (B) Raman cluster spectra corresponding with the color coded cluster image in A. (C) SEM images after successive FIB ablation steps. In six FIB ablation steps with intermediate SEM imaging the 3D structure of the cell is investigated, for each ablation step a layer of approximately 700 nm is removed from the sample surface.

3D FIB - SEM block face imaging is a technique in which a FIB is repeatedly used on a sample to remove a surface layer, which is investigated with the SEM.[32, 33] Typically such experiments are performed on cells in embedding material, to preserve the native ultrastructure, and FIB - SEM "slice and view" can be performed on sections several tens of nanometer thick.[32, 33] For this experiment such a rigorous investigation is not required. Correlative SEM - Raman analysis is performed as presented in figure 4.19 A and B. While the cells have not been sorted based on their white blood cell type, the specific cell could be identified based on the Raman spectra of an eosinophil. The incidence angle of the FIB is at 38° and the sample is set at a tilt angle of -10° , as a result FIB slicing is performed for surface elements with a tilt angle of 28° with respect to the substrate. In figure 4.19C multiple SEM images are presented for subsequent FIB ablation steps. The inner structure of the cell shows the cytoskeleton with hollow surroundings, in figures C_{2-4} the two lobes of the eosinophil cell nucleus are observed.

An attempt at performing Raman analysis on intermittent steps during FIB-SEM 3D scanning was unsuccessful. Due to the high light absorption of the organic material after FIB irradiation a high fluorescence emission background that obscures the Raman signal is observed. Correlative Raman microscopy is performed after the FIB-SEM imaging steps C_4 and C_5 . Because a high light absorbance is expected a lower laser power is used so that the sample is not further damaged, a 1 mW excitation power and 1 second spectral integration time is used. Micro-spectroscopic analysis is presented in figure 4.20, it is observed that a strong fluorescence emission is generated at the FIB treated cell surface. Figure 4.20 shows correlative optical and electron microscopic images (A and C) and fluorescence spectra (B and D) observed on locations indicated in the images. The fluorescence emission shows large intensity variations over the cell surface, therefore the optical intensity images are shown in a logarithmic colorscale, to show both high and low intensity emission locations. Figure 4.20B shows averaged spectra measured over the regions indicated in A. The strongest fluorescence contribution is measured on location 1, at the side where the FIB is incident on the cell. This pattern is observed to a lesser extend in fig. C, where the more thinned sample shows a strong fluorescence emission over the entire sample surface. The fluorescence spectral response presented in fig. D, shows similar spectra to observations presented in figure B. Based on the presented data it is concluded that Raman measurements performed after FIB ablation on organic materials will not be feasible with this method due to the high fluorescence background.

Figure 4.20: (A) Overlay of correlative Raman and electron microscopic images, showing the spectrally integrated fluorescence emission in a logarithmic colorscale. Measured with 1 mW excitation power, 1 second integration time, and 500 nm step size, SEM imaging is performed at 1.5 kV, the scale bar denotes 4 μm . (B) Fluorescence spectra averaged over the regions indicated in A, demonstrating the signal variations over the specimen. (C) Overlay of correlative Raman and electron microscopic images, similar to fig. A. Imaging is performed after an additional FIB milling step, the scale bar denotes 3 μm . (D) Fluorescence spectra averaged over the regions indicated in C.

4.6 Discussion

This chapter presents the integration of the HybriScan Raman microscope in a FEI dualbeam FIB-SEM. It has been shown that the integration places no limitations on operation of the FIB-SEM, and enables serial correlative Raman micro-spectroscopic investigation with operation of the FIB-SEM. During operation of the correlative microscope it is observed that the combined irradiation with the electron beam and Raman laser of the sample results in increased risk for sample damage. Therefore a careful choice in both laser power and probe current is required, additionally

the sequence of operation for Raman, SEM and FIB is important since potential sample damage or contamination with FIB or SEM can be observed with Raman spectroscopy. Considering the integrated Raman microscope potential points for improvement are the optical objective numerical aperture. Using a higher NA will result in a stronger laser focus and improved signal collection. However the likely shorter working distance will require a more careful operation of the microscope to prevent a collision between the sample and optical objective. Additionally the spectral range of the microscope is up to approximately 2600 cm^{-1} , as a result Raman bands in for example CH spectral region at 2900 cm^{-1} are not observed. An improvement of the spectral range is however complicated by the lower detection efficiency of common CCDs further into the near infrared.

Correlative SEM - Raman experiments and FIB - SEM - Raman proof of principle experiments have been performed demonstrating applications of the integrated microscope. Advantages of using an integrated microscope, in favor of separate microscopes, become apparent as improvements in the workflow. Switching between analysis systems is much faster which enables sequential FIB - Raman analysis, and facilitates the investigation of additional sample locations based on observations during an experiment. Correlative Raman analysis after FIB micromachining has successfully shown changes in silicon lattice parameters and surface amorphization. However the combination of Raman with FIB for organic or polymer samples results in mostly a high fluorescence signal induced by the FIB irradiation. This complicates the investigation of actual FIB induced molecular changes.

Correlative SEM - Raman analysis of white blood cells has been performed. The sample protocol for dehydration and preservation of the cell morphology preserves the cell's Raman characteristics. One disadvantage of the preparation protocol is that residual components of the HMDS drying agent are observed in the Raman spectra, this contribution to the recorded spectra is subtracted using a LLS fitting procedure. Furthermore a high fluorescence contribution is observed on sorted white blood cells. It appears that this contribution originates from the cell sorting preparation step since it is not observed on unsorted cells. Raman identification of cell types is presented in combination with the specific morphology for the cell types. Finally a correlative FIB - SEM - Raman experiment is performed for observation of the cell ultrastructure. Raman analysis could only be performed before FIB ablation, due to the high fluorescence emission from the sample induced after interaction with the ion beam. Since no embedding materials are used in the sample preparation mostly a hollow ultrastructure and the cytoskeleton are observed with SEM imaging, however the location of the cell nucleus could be seen in several FIB revealed layers.

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CORRELATIVE SEM SERS OF PLASMONIC STRUCTURES

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Abstract

In this chapter correlative Raman - electron microscopy is applied for the analysis of SERS nanostructures. Dimer structures consisting of two coupled plasmonic nanoparticles are chosen as an interesting structure to demonstrate the capabilities of correlative analysis. The effect of the dimer orientation with respect to the polarization state of the laser and the effect of the particle gap size on the Raman signal intensity is observed. Additionally, calculations are performed to simulate the electric near field enhancement. These simulations are based on the morphologies observed with electron microscopy. Using this method experimental observations are compared with the enhancement factor calculated with near field simulations, providing an indication of the SERS enhancement factor. Large differences between experimentally observed and calculated enhancement factors are regularly detected, a phenomenon caused by nanoscale differences between the real and 'simplified' simulated structures. Quantitative SERS experiments are used to reveal the actual structure induced enhancement factor averaged over the full nanostructure surface.

5.1 Introduction

Noble metallic nanoparticles (nps) have attracted general interest due to their specific interaction with light. This interaction excites surface plasmons in the metal - dielectric interface. In nanostructures an intense focusing of light occurs with a high energy density that is far below the optical diffraction limit. Nanoplasmonics has been applied in nanoantennas,[1] waveguides,[2] and in sensing applications.[3–6] Plasmonics is applied in Raman spectroscopy to obtain high signal enhancements of molecules in the vicinity of the particle, resulting in surface enhanced Raman spectroscopy. Low numbers of molecules can thus be detected with the specificity of Raman spectroscopy.

The Raman signal enhancement depends strongly on the geometry of the metallic nanostructure and enhancement factors (EFs) up to 10^9 - 10^{10} have been reported in hot spot locations.[7–9] The EF, averaged over the structure surface, is much lower, namely in the range of 10^4 - 10^6 . [10] With a local EF of 10^7 - 10^8 and higher single molecule Raman spectroscopy has been performed.[9, 11] The most common materials used for SERS experiments are gold and silver. These materials give a signal enhancement that is most prominent in the visible and near infrared spectrum. Plasmonic dimers are of specific interest as a simple composite nanoparticle system. The interest in dimers arises from the high obtainable EF in the gap between the two particles, which we will address as the dimer gap. The field enhancement in the hot spot increases strongly with decreasing gap size and the gap size can be tuned by applying spacer molecules between the metallic nanoparticles.[12–14] Multiple dimer particle systems have been composed, such as 2 spheres with and without separation by spacer molecules, 2 rod-shaped particles,[12, 15, 16] and metallic tips and a gold (or metal) surface.[17, 18] Dimer structures are fabricated by deposition of nanoparticles or through chemical synthesis of coupled particles or through milling structures with a focused ion beam.[19]

The correlative SEM - Raman microscope is used for analysis of plasmonic gold nanoparticles and particularly on particle dimers with a spontaneously formed gap size which results from sparse aggregates deposited on a surface. The orientation of the dimer particle with respect to the linear polarization of the incoming laser field is also investigated. The goal of the investigation is to quantify the effect of the morphology of nanostructures on the local field enhancement and correlate the Raman photon flux with the observed nanoparticle aggregate. The dimer structures are observed with the SEM, and the plasmonic field enhancement is probed by correlative SERS from reporter molecules adsorbed on the gold surface. The SEM - Raman combination enables a high resolution observation of the structure, and an experimental measurement of the signal enhancement. The analysis is further corroborated with a simulation of the near field response of the structures observed with the SEM. A comparison between the recorded Raman intensities and the simulated field enhancements is performed.

5.2 Simulations

5.2.1 Dimer field enhancement

Figure 5.1: (A) Electric near field distribution around a gold monomer of 60 nm diameter. The maximum and structure averaged EF are $1.9 \cdot 10^2$ and 30. (B) Near field distribution around a gold dimer consisting of two 60 nm diameter particles with a gap of 5 nm. The excitation polarization is oriented along the dimer direction. The maximum and structure averaged EF are $5.0 \cdot 10^4$ and $6.9 \cdot 10^2$. (C) Near field distribution with orthogonal excitation polarization, the maximum and structure averaged EF are $1.9 \cdot 10^2$ and 30, as is expected almost identical to case A. To improve the visibility of the local field enhancement distribution a cross section of the XY plane through the center of the nanostructures is made for (D) the monomer, (E) the dimer with on axis excitation polarization, and (F) the dimer with off axis excitation polarization.

The electric near field enhancement from plasmonic structures depends strongly on the structure shape, size, composition, and the excitation wavelength. The field enhancement of a dimer compared to a monomer is presented in figure 5.1. Figure 5.1A shows the near field distribution on the surface of a gold monomer nanoparticle. Figure 5.1B shows the near field for a dimer with excitation light along the dimer axis, figure 5.1C shows the same dimer with excitation light orthogonal to the dimer axis. The simulations are used to obtain the local distribution of the near field enhancement. This information is then used to calculate the EF for a Raman signal of the plasmonic structure. As can be observed, the EF is strongly dependent on the location on the nanostructure. From the calculation the maximum EF, which coincides with the dimer hot spot, and also the average EF over the nanostructure surface are obtained. The structure averaged EF together

with the number of adsorbed molecules determines the measured SERS signal intensity. The gold particles used in this research are 60 nm in diameter, the Raman excitation wavelength is 785 nm, and the gold refractive index is obtained from Johnson and Christy.[20]

Simulations of the electric near field distribution from experimentally observed structures are performed. Typically the enhancement factor for a plasmonic nanostructure is defined as either the maximum EF, in this case in the dimer hot spot, or as the EF averaged over the nanostructure surface. In this research the nanostructure surface is fully covered with Raman reporter molecules, as a result the measured SERS enhancement has to be compared with the surface averaged EF from the simulated structures. Equation 5.1 is used for the calculation of the surface averaged EF, the calculated EFs are presented in the caption of figure 5.1. The equation provides an approximation of the actual EF as it ignores the change in field enhancement at the frequency of the field at the Raman shift, called the E^4 enhancement approximation.

$$EF = \frac{1}{S} \int \frac{|E(\omega_{Laser})|^2 \cdot |E(\omega_{Stokes})|^2}{|E_0(\omega_{Laser})|^4} dS \approx \frac{1}{S} \int \frac{|E(\omega_{Laser})|^4}{|E_0(\omega_{Laser})|^4} dS \quad (5.1)$$

The purpose of the simulations is to calculate the field and signal enhancement of observed dimer structures. The two experimental parameters of interest are the gap size and orientation of the dimer to the excitation polarization. Results of these simulations are shown in figure 5.2. Figure 5.2A shows the EF averaged over the dimer structure in a logarithmic color representation. The signal enhancement has been calculated for different gap sizes (horizontal axis) and excitation polarizations (vertical axis), ranging from 0.5 nm to 15 nm and 0 to $\frac{\pi}{2}$ radians respectively. Figure 5.2B shows, the gap size influence in a logarithmic representation, with the polarization orientation kept at 0° . Figure 5.2C shows the polarization influence with the gap size at 1 nm. The calculations show the expected signal enhancement from dimer structures. The results are compared with the experimentally determined signal enhancement from multiple dimers observed in correlative Raman - electron microscopy. The simulations illustrate that small changes in the nanostructure morphology can strongly affect the enhancement factor, these observations are in good agreement with the literature.[21]

Figure 5.2: (A) Logarithmic representation of the signal enhancement from dimer structures with a gap size between 0.5 nm and 15 nm, the dimers are excited with an excitation polarization between 0° and 90° . (B) Dimer signal enhancement for a 0.5 nm to 15 nm gap size and 0° polarization for two common SERS excitation wavelength 532 nm and 647 nm, and for 785 nm that was used in this study. (C) Dimer signal enhancement for a 1 nm gap size and 0° to 90° polarization. All calculations include the effect of a substrate with $n = 2.9 + 3.3i$.

5.2.2 Substrate effects

For the simulations to accurately represent the experimental situation a substrate has to be included in the calculations. In the experiment the samples are placed on a metallic stainless steel substrate, this substrate is chosen for a low Raman background and high electrical conductivity to prevent SEM charging effects. Due to the high refractive index of steel the substrate is expected to significantly influence the plasmonic field enhancement. For the simulations presented in figure 5.2 a substrate is included in the calculations. Values for the refractive index of the steel substrate are approximated based on its composite materials iron and chromium,[20] at 785 nm wavelength $n = 2.9 + 3.3i$. To further demonstrate the substrate effect on the plasmonic field enhancement figure 5.3 shows the near field enhancement for a dimer structure (A) without substrate, (B) with substrate,

and (C) the ratio of field enhancement with and without substrate. The dimer nanostructure consists of two 60 nm diameter particles with a 3 nm gap. In the dimer gap the field enhancement increases with a factor of 1.14 when the substrate is included in the simulations, this means that in the hot spot inclusion of the substrate increases the calculated SERS signal with over 60% (E^4). It is further observed in figure 5.3C that the strongest increase in field enhancement is at the border near the substrate. However the effect on the SERS signal predominantly originates from the increase in the hot spot field enhancement.

Figure 5.3: (A) Plasmonic field enhancement of a dimer nanostructure consisting of 2 spheres of 60 nm diameter and a 3 nm gap between the particles, excitation is performed with 785nm light. (B) Field enhancement of the same dimer structure with a steel substrate layer included in the simulation, the particles are placed on top of the substrate. (C) Ratio of the field enhancement obtained from the simulation with and without the substrate.

5.2.3 Clusters

Figure 5.4: Near field enhancement simulations on the surface of coupled 60nm diameter spherical gold particles. Performed for (A) monomer, (B) dimer, (C) trimer, and (D) tetramer structures, with a 3 nm gap between the coupled particles.

As is shown in figure 5.1 it is clear that the SERS enhancement increases significantly for small gaps between coupled nanoparticles. As a result the Raman signal predominantly originates from this hot spot location. It is expected that increasing the size of the cluster further increases the enhancement factor. The reason for this is that coupling between the particles additionally drives the free electron oscillation, thus increasing the field enhancement. Figure 5.4 shows the field enhancement on the surface of 1, 2, 3, and 4 coupled nanospheres, the 60 nm diameter particles are separated by a 3 nm gap, and the excitation polarization is directed along the clusters orientation. The maximum enhancement factor from each of the monomer, dimer, trimer, and tetramer structures is $1.9 \cdot 10^2$, $0.25 \cdot 10^6$, $0.66 \cdot 10^6$, $1.7 \cdot 10^6$ respectively. The enhancement factor observed on the monomer structure is naturally much lower because the structure does not provide a hot spot. For increasing cluster sizes an increasing enhancement factor is observed in the hot spot locations, caused by coupling between the multiple particles. This further demonstrates the considerable effect of structure morphology on the enhancement factor. The surface averaged enhancement factor $\langle EF_S \rangle$ is also obtained from the simulations, this property increases for larger cluster sizes not only through the larger maximum enhancement factor but also as a result of the increasing number of hot spots. The calculated $\langle EF_S \rangle$ are: 30, $0.20 \cdot 10^4$, $0.67 \cdot 10^4$, $1.4 \cdot 10^4$

The effect of coupling between increasing numbers of particles is further observed in far field spectra. Figure 5.5 shows the far field scattering (A) and extinction (B) spectra for the discussed structures. In the plasmonic resonance bands a red shift is observed that is also seen for elongated particles such as nanorods or ellipsoids. The coupling of an increasing number of particles increases the interaction cross section with the light field, which results in higher scattering and extinction spectra compared to the sum over respectively 1, 2, 3, or 4 uncoupled particles.

Figure 5.5: Far field (A) scattering and (B) extinction spectra for a monomer, dimer, trimer, and tetramer structure. The extinction spectrum represents the sum of the structure absorption and scattering. The effect of coupled particles is observed through a redshift and increased amplitude of the spectra.

5.3 Experimental methods

5.3.1 Sample preparation

Gold nanoparticles with a diameter of 60 nm prepared by citrate reduction method were obtained from NanopartzTM at 37.7 pM concentration. Rhodamine 6g dye was obtained from Sigma Aldrich (batch: 252433-250MG) in solid form. SERS labeling of nanoparticles was prepared by dissolving R6g in ethanol to a concentration of 8 μM . The mixing ratio of gold nanoparticle solution and R6G solution was 1:7. The dye - and nanoparticle concentrations were verified with UV-Visible absorption spectroscopy before and after mixing of the compounds, the spectra are presented in figure 5.6A. The R6G covered nanoparticles were deposited and dried on a substrate directly before placing them in the correlative light - electron microscope. Raman silent and conductive $\mu\text{Rim}^{\text{TM}}$ slides (BioTools Inc.) were used as substrates. The laser irradiation power is 0.5 mW. The R6g SERS spectrum is spectrally very similar to the spontaneous Raman R6g spectrum indicating little chemical interaction between the gold nanoparticle and the dye molecules, as shown in figure 5.6B. The stability of the SERS signal from nps under laser irradiation was verified by recording the Raman signal of dimer structures during 20 seconds under constant illumination. A constant R6g SERS spectrum is observed for all spectra

during the entire measurement time (figure 5.6 C and D). In imaging experiments it has been observed that larger plasmonic structures become damaged during the Raman experiment. This is observed as sudden change in the SERS signal of the large clusters during Raman microscopy, something which can be seen in figure 5.7A.

Figure 5.6: (A) UV-Vis absorption spectra of Pure R6g and gold nps, and of the mixture used for sample preparation. (B) A comparison of the spontaneous R6g Raman spectrum [black] and the SERS R6g spectrum [red]. Both spectra are normalized to 1, the SERS spectrum is offset with 1 for clarity. The Raman spectrum is measured in an ethanol solution and the SERS spectrum is obtained from one of the investigated dimer nanoparticles. (C and D) Both figures show 20 consecutively measured spectra from a SERS nanoparticle cluster labeled with R6g reporter molecules, measured with 1 second integration time per spectrum and 0.8 mW excitation power. A constant spectrum is observed during the experiment indicating there is no sample damage due to laser irradiation for these structures.

5.3.2 Correlative analysis

In order to acquire data from a sufficiently large number of dimer structures, Raman microscopy images were prepared from an area of more than $2000\text{ }\mu\text{m}^2$ with approximately 25000 spectra. Both SEM and SERS images reveal the locations of particle clusters. Dimer particles can only be selected with certainty from the SEM image. The dimer particles were subsequently analyzed with high resolution electron microscopy to measure the gap size with the highest precision. Application of SEM after the Raman measurements avoids contamination of the nanoparticle clusters by electron beam induced deposition[22] and prevents the potential degradation of Raman reporter molecules adhered to the gold nanoparticles. The electron microscope is operated in immersion imaging mode using SE detection with the TLD, a 15 kV electron energy, and 36.5 pA probe current is used. The SEM resolution for this detection mode is specified at 1.1 nm. Raman experiments are performed with 500 μW excitation power, an integration time per pixel of 1 second and an image step size of 300 nm in x- and y-direction. The Raman microscope has a lateral resolution of 850 nm, and the excitation polarization is oriented along the y-axis as indicated with an arrow in figure 5.7A.

The correlative analysis of the ROI is shown in figure 5.7. Figure 5.7A shows an overlay image of Raman and SEM data. The SEM image was obtained with a magnification of 1500x. The Raman data is represented with a logarithmic color scale, in which the signal intensity corresponds to the fitting parameter of a linear least squares fit of the recorded spectra with the known R6g SERS spectrum. The scale of this printed image is too coarse to observe the nanoparticles in the SEM image. A part of the image, indicated in the white rectangle, is therefore magnified to show the position of the nanoparticles in the SEM recording (figure 5.7B) in blue color scale for increased contrast. The selected dimer is imaged with SEM at a higher magnification of 250.000x, as shown in figure 5.7C. From the high resolution SEM image the dimer orientation, particle diameters, and gap size are obtained. For this dimer the angle, with respect to the light polarization is 40° , the diameters of the nanoparticles are both 60 nm and the gap size is $6 \pm 1.1\text{ nm}$. The measurement of the gap size between the two particles is taken from a line cross section along the dimer axis, as indicated in the SEM image (figure 5.7C). The cross section intensity is presented in figure 5.7D, with the estimated positions of the nanoparticle borders indicated in red. The measured hyperspectral Raman data sets were background corrected and filtered by singular value decomposition.[23] The spectra contain the SERS signal and some fluorescence, which originates from the fluorescence enhanced signal from the R6g molecules and from the gold nanoparticles.[24] The SERS signal was isolated using a filter with a smoother function specifically designed to separate background fluorescence and Raman spectra.[25] A pretreated Raman spectrum, averaged over 9 pixels, selected in figure 5.7B, is presented in figure 5.7E. Figure 5.7 exemplifies the large range in scale during data acquisition. Fig. 5.7A covers an image range of $50 \times 45\text{ }\mu\text{m}^2$, whereas Fig. 5.7C demonstrates nanometer resolution analysis of one of the objects in Fig. 5.7A.

Figure 5.7: (A) Overlay of the Raman intensity image and the SEM image. Regions with dimers are located based on the SEM image and a first estimate of the Raman intensity is made from the overlay intensity map. (B) Magnified region selected from figure A. Gold nanoparticles are visible as white dots in the overlay Raman image. (C) High resolution SEM image recorded on the ROI indicated in B. (D) SEM intensity of a cross section taken along the dimer axis, the observed particle boundaries are indicated in red. (E) SERS spectrum recorded from the nanostructure.

5.3.3 Gapsizes measurement

An accurate measurement of the gapsize in the dimer nanostructure is critical to compare the measured SERS enhancement factor with near field simulations. For this reason the possibility of measuring the gapsize has been investigated with simulations of the secondary electron scattering signal. This is performed in collaboration with J. Sueters and J.P. Hoogenboom of the Technical University of Delft. The simulations are performed with the Geant4 software toolkit that simulates particle (electron) passage and scattering through matter. The simulations are performed with the same settings used during SEM imaging: the electron acceleration energy is 15 kV, a probe current of 36 pA, a dwell time of 5 μs , and a beam width of 1 nm is used. The secondary electron scattering signal is measured in a plane above the sample, the simulated beam position is scanned with 0.5 nm step size across the dimer axis through the center of both nanoparticles. The simulation results are presented in figure 5.8, SE scattering for dimers with a

gapsize of 0.5 nm to 10 nm with steps of 0.5 nm are presented. It is seen that a measureable gap appears for dimers with a gapsize of 2.5 nm or larger, it should therefore be expected that in experiments dimers with a gap smaller than 3 nm will not be resolved in SEM imaging.

Figure 5.8: Simulations of the secondary electron scattering signal from dimer nanostructures with a varying gapsize. Gapsizes of 0 to 10 nm with steps of 0.5 nm are simulated, the locations of the left and right border of the nanoparticles are indicated in red. This is performed in collaboration with J. Sueters and J.P. Hoogenboom of the Technical University of Delft.

Experimental measurement of the dimer gap size is presented in figure 5.9. On the (right) side a line cross section of the SE scattering signal across the dimer SEM image (left) is shown. The positions marked as the edges of the nanoparticles are indicated with a red dot. The SEM images are made with 15 kV acceleration energy with SE detection in immersion mode, a magnification around 250.000x which corresponds to a pixel size of 0.5nm x 0.5nm is used.

Figure 5.9: Experimental measurement of the gapsize from dimer structures. A cross section through the dimer structures, as indicated in the left images, is obtained and the electron scattering signal over the cross section is shown on the right. The observed size of the gap (indicated with red dots) is from top to bottom 4, 6, 6, and 5 nm.

5.4 Microscope and specimen quantification

5.4.1 microscope calibration

A quantitative calibration of the HybriScan Raman microscope is performed to enable quantitative Raman spectroscopy and analysis of the SERS enhancement factor. This calibration is performed using the calibrated 647nm Raman microscope as a reference standard. The quantitative analysis of the 647 nm Raman microscope

is discussed in section 3.2 "Quantitative Raman spectroscopy". Its response function (figure 3.6 black curve) is used to determine the Raman scattering efficiency of two small (sub-diffraction) samples. A polystyrene bead of 200 nm diameter and a 25 nm thick polysulfone layer are analyzed. The measured Raman spectra are presented in figure 5.10. The spectrum of polystyrene figure 5.10A is measured with 70 mW excitation power, and 200 ms pixel integration time. For Polysulfone (figure 5.10B) an excitation power of 75 mW, and pixel integration time of 1 s is used. The spectra show several Raman bands specific for the respective compounds, using the microscope response function the Raman scattering cross section of the polystyrene and polysulfone bands are calculated. Table 5.1 shows the scattering cross sections calculated for a monomer of polystyrene and polysulfone with Raman excitation wavelength at 647 nm. To provide insight in the Raman activity of the respective monomers a schematic of the polystyrene and polysulfone molecules is presented in figure 5.10 C and D respectively. When comparing the scattering cross section between the monomers of polystyrene and polysulfone it should be considered that the sulfone monomer is much larger than the styrene monomer. The sulfone monomer contains 4 benzene rings where styrene contains 1, furthermore the polarizability of sulfone is influenced by the sulfur atom which is heavy in comparison with typical carbon and oxygen atoms.

Figure 5.10: Polystyrene and Polysulfone spectra for quantitative calibration measured with 647 nm excitation light. Polystyrene bead 200 nm diameter (A) measured with 70 mW excitation power, 200 ms integration time, measured dry with coverglass. Polysulfone layer with 25 nm thickness (B) measured with 75 mW excitation power, 1 s integration time, measured in water with coverglass. A schematic of the monomers from (C) polystyrene and (D) polysulfone.

Polystyrene Frequency cm^{-1}	$\sigma \text{ cm}^2$	Polysulfone Frequency cm^{-1}	$\sigma \text{ cm}^2$
620	$0.78 \cdot 10^{-30}$	788	$62 \cdot 10^{-30}$
1000	$9.17 \cdot 10^{-30}$	1067	$32 \cdot 10^{-30}$
1033	$2.20 \cdot 10^{-30}$	1106	$55 \cdot 10^{-30}$
1580 – 1603	$5.74 \cdot 10^{-30}$	1145	$151 \cdot 10^{-30}$
		1582 – 1603	$153 \cdot 10^{-30}$

Table 5.1: Raman scattering cross sections for a polystyrene and polysulfone monomer measured using the 647 nm excitation Raman microscope.

The scattering cross section of these compounds for excitation with 785 nm laser light has to be calculated, this is done using equation 3.6 and 3.7 with a new value for the laser frequency ν_0 . The Raman scattering intensity from the sample is calculated using equation 3.9, of which the parameters 'excitation source intensity' I_0 and 'number of molecules' N are known due to the well-defined sample dimensions. Next a Raman measurement with the 785 nm integrated microscope is performed of the same polysulfone thin layer and of a similar polystyrene bead with 500 nm diameter, the measured spectra are presented in figure 5.11. The spectrum of polystyrene (fig. 5.11A) is measured with 37 mW excitation power, and a 1 s integration time. For Polysulfone (fig. 5.11B) an excitation power of 37 mW, and an integration time of 20 s is used.

Figure 5.11: Polystyrene and Polysulfone spectra for quantitative calibration of the 785 nm Raman microscope. (A) Polystyrene bead 500 nm diameter measured with 37 mW excitation power, 1 s integration time. (B) Polysulfone layer with 25 nm thickness measured with 37 mW excitation power, 20 s integration time.

Using the obtained Raman scattering cross sections and the measured Raman signals from the same compounds the microscope response is calculated as the measured signal in counts divided by the calculated Raman scattering intensity in photons. The microscope response is thus calculated based on the scattering cross sections of the bands in table 5.1 corrected to 785 nm laser excitation. To

obtain the quantitative response function of the microscope for the full spectrum, the response function based on white light calibration as presented in figure 4.5 is scaled to the quantitatively measured signal transmission at the correct spectral position. Figure 5.12 shows the obtained response curve based on the average of the polystyrene and polysulfone microscope response measurements.

Figure 5.12: Quantitative calibration of the spectral response of the 785 nm integrated Raman microscope. The curve showing the relation of detected counts over emitted Raman photons.

It is worthwhile to compare the efficiency of the integrated 785 nm Raman microscope and the home build 647 nm Raman microscope, presented in figure 3.6. For this purpose in table 5.2 the transmission or detection efficiencies of the most important components from both setups are presented. Components with a large spread in efficiency over the investigated spectral region are presented with both the lowest and highest efficiency value. It is observed that the efficiency of most components is comparable, the main differences are in the light transmission through the optical objective, and the CCD electron to count conversion. While the 647 nm Raman objective has a NA of 0.95 and the 785 nm objective an NA of 0.65, almost the same collection angle is reached because the 0.95 NA objective is applied for samples in water. Light transmission through the 0.95 NA objective is lower due to a lower transmission of light collected with the highest part of the NA, transmission for collimated light incident on the center of the objective can be up to 85%. Photons detected with the CCD are converted to electrons, through the photoelectric effect, which are in turn converted to counts during the camera readout. The conversion of electrons to counts depends strongly on the internal gain at which the camera is operated. While the electron to count conversion does

not affect to the quantum efficiency of the camera it does affect the number of recorded counts. The CCD camera used in the 647 nm setup is operated at a higher internal gain, thus providing more counts per detected photon.

Raman 647 Components	Efficiency	Raman 785 Components	Efficiency
Collection angle NA 0.95, in water	15%	Collection angle NA 0.65, in vacuum	12%
Objective	32% - 38%	Objective	80%
Confocal pinhole	90%	Confocal pinhole	60%
Spectrograph	70% - 90%	Spectrograph	70%
CCD camera QE	65% - 95%	CCD camera QE	50% - 95%
CCD electrons to counts	125%	CCD electrons to counts	30%

Table 5.2: Efficiency of the most important optical components in the two Raman setups using 647 nm and 785 nm excitation light.

5.4.2 R6g scattering cross section

Rhodamine 69 (R6g) is used as reporter molecule that is adsorbed onto the gold nanostructure. The spontaneous Raman scatter cross section from R6g is determined using an internal reference standard method.[26, 27] In this experiment a solution of R6g in ethanol is made. A concentration that is comparatively high to samples prepared for SERS experiments is necessary for sufficient signal. This solution is analyzed using UV-Vis spectrometry to determine the exact R6g concentration. Into the solution a small amount of toluene is mixed (5 volume percent). The toluene functions as an internal reference to the R6g signal, its scatter cross section is known and by using it as a reference the R6g cross section is calculated.[28] Absorption spectra of the ethanol R6g solution are shown in figure 5.13. The spectra show a R6g density of 1.7 mM with an absorbance maximum of 150 cm^{-1} . This absorbance is measured with UV-Vis spectroscopy after dilution of the investigated medium with 1 : 10 and 1 : 100 to obtain sufficient light transmission in the spectrometer. The results presented in figure 5.13 are corrected for the dilution factor to correspond with the sample investigated with Raman.

Figure 5.13: (A): The literature R6g absorption spectrum per μM and cm . (B) The measured absorbance spectrum from the R6g in ethanol solution.

Raman spectra of the solution are measured using the HybriScan Raman microscope. Based on the known scattering cross section of toluene and densities of toluene and R6g in the ethanol solution the scattering cross section of R6g is calculated. Research to the toluene cross section performed by "Y. Kato and H. Takuma[28] is performed at an excitation wavelength of 632.8 nm. Therefore a correction is made using formula 3.7 to the fourth power wavelength dependence, and to the wavelength influence on the molecule polarizability. Furthermore the cross section of toluene is changed due to the presence in a different medium ethanol, the refractive index of toluene is 1.49 and of ethanol it is 1.36.[29] As a result there is a different internal field factor L , equation 3.8, which influences the scattering cross section of the toluene reference compound. The measurement provides a quantification of both the R6g and ethanol Raman scattering cross sections. The measured result is the scattering cross section of R6g in the medium ethanol. The desired cross section is the vacuum value which means that the cross section in ethanol value is divided by the internal field factor L with the refractive index of ethanol. The resulting absolute Raman cross section of R6g (left) and ethanol and toluene (right) is shown in figure 5.14.

Figure 5.14: Measured Raman scattering cross section for (A) R6g dye molecules and (B) toluene and ethanol measured simultaneously with R6g.

The Raman scattering cross sections from several of the most prominent R6g bands are presented in table 5.3. These values are obtained through integration over the full spectral width of the specific Raman bands, after a subtraction of the fluorescence background.

Raman bands	Raman frequency (cm ⁻¹)	σ (cm ²)
C-C-C ring in plane bending	610	$2.15 \cdot 10^{-28}$
C-H out of plane bending	774	$2.88 \cdot 10^{-28}$
Aromatic C-C stretching	1308	$6.77 \cdot 10^{-28}$
Aromatic C-C stretching	1360	$8.73 \cdot 10^{-28}$
Aromatic C-C stretching	1509	$10.5 \cdot 10^{-28}$
Aromatic C-C stretching	1650	$1.31 \cdot 10^{-28}$

Table 5.3: The results as shown in figure 5.14A are presented in table format, R6g Raman scattering cross section for multiple prominent bands. The measurement is performed in solution with ethanol, with a small amount of toluene added as internal reference standard.

5.4.3 Molecule adsorption

The number of dye molecules adsorbed on the gold nanoparticles surface in a solution in ethanol is determined. This is performed through the addition and subsequent centrifugation of gold nanoparticles to a solution of R6g in ethanol, as a result the dye concentration in solution will decrease through the adsorption to gold of molecules and next their removal through centrifugation. First a solution of R6g and Gold nps of 80 nm diameter in ethanol is made, 80 nm particle diameter is chosen over 60 nm diameter to aid in the centrifugation step. Figure 5.15A shows the absorption spectra of the solution. The absorption spectrum (blue) is taken of R6g in ethanol solution. The R6g Au80 (black) spectrum is taken after the addition of the gold nanoparticles to the R6g solution. The Au80 (red) spectrum is obtained from a reference solution of gold nanoparticles in ethanol at the same concentration as used in the gold nanoparticles in R6g solution. The concentrations are chosen such that there are $7.0 \cdot 10^7$ nps·m⁻¹ in the solution, when assuming perfectly spherical particles this results in $1.4 \cdot 10^{12}$ nm²·m⁻¹. The dye concentration is at 62 molecules per nm² surface area of gold nanoparticles in solution. The R6g with gold nps solution is centrifuged at 11.000 rpm for 120 minutes, after which absorption spectroscopy is performed on the supernatant to verify the removal of all nanoparticles, figure 5.15B shows the spectrum of the supernatant (red).

Figure 5.15: (A): Absorption spectroscopy of the R6g and Au nps solution before centrifugation. (blue) R6g solution, (black) R6g and gold nps solution, (red) gold nps from a reference solution. (B) Comparison of the absorption spectra from the R6g and Au nps solution before (black) and after (red) centrifugation.

The change in dye concentration between the supernatant and the R6g solution before centrifugation and addition of gold nanoparticles is measured using a confocal fluorescence spectrometer. The spectrometer uses an excitation wavelength of 532 nm which is in the dye absorption band. The percentage of adsorbed molecules is determined using the relative intensity decrease due to centrifugation of the adsorbed molecules. Figure 5.16 shows the fluorescence spectra recorded before addition of the gold nanoparticles (top), and the difference intensity after addition and centrifugation of the gold nps measured in the supernatant.

Figure 5.16: Fluorescence spectroscopy of the R6g solution before addition of gold nps (top) and the difference intensity with the supernatant after addition and centrifugation of the nps (bottom).

Using these measurements the number of molecules adsorbed to gold can be calculated. In the solution with nps and a concentration of C dye molecules a percentage will have been adsorbed to the gold C_{ad} and the rest remains in solution C_{sol} , as described in equation 5.2. The fluorescence intensity is recorded of the supernatant after centrifugation, the only signal contribution is from the remaining molecules C_{sol} . Due to the linear relationship between concentration and fluorescence intensity equation 5.3 can be stated where a reference signal I_{ref} and concentration C_{ref} are introduced. Equation 5.4 is obtained by combining equation 5.2 and 5.3. As reference solution the R6g solution before addition of gold nanoparticles has been chosen, the original number of molecules in solution C is measured using absorption spectroscopy, combined with fluorescence spectra I_{sol} and I_{ref} this enables the calculation of the adsorbed concentration C_{ad} . The concentration of nps added to the solution has also been measured using absorption spectroscopy, which gives the number of dye molecules per np: $5.8 \cdot 10^4$ and the number of molecules per square nanometer: 2.9 (molecules $\cdot$ nm $^{-2}$) . Earlier values have been reported of 0.9 and 1.3 (molecules $\cdot$ nm $^{-2}$) for gold particles and 4.7 (molecules $\cdot$ nm $^{-2}$) for silver particles.[30–32]

$$C_{ad} = C - C_{sol} \quad (5.2)$$

$$\frac{C_{sol}}{I_{sol}} = \frac{C_{ref}}{I_{ref}} \quad (5.3)$$

$$C_{ad} = C - C_{ref} \cdot \frac{I_{sol}}{I_{ref}} \quad (5.4)$$

5.5 Results and discussion

5.5.1 SERS dimers

The SERS signal was measured for many dimers in conjunction with high resolution SEM measurements of the nanoparticle size, orientation and gap size of the dimer structures, the example results are presented in figure 5.17. The electron microscopy images in figure 5.17 (A, D, G, and J) provide the structure morphology parameters of the dimers. The measured gaps in the dimers are 4 nm, 6 nm, 6 nm, and 5 nm respectively, and the dimer - polarization orientation angles are 50°, 45°, 37°, and 23°. This gap size was determined by the procedure presented in figure 5.9. Figure 5.17 further shows the SERS spectra recorded from the same structures figure 5.17 (B, E, H, and K) and the corresponding simulations of the near field enhancement (C, F, I, and L).

Figure 5.17: Recorded nanoparticle dimers, with the recorded corresponding Raman spectra. (A, D, G, and J) SEM images are used for the analysis of the gap size, orientation, and np diameter. (B, E, H, and K) Raman spectra measured from the dye labeled dimers. (C, F, I, and L) Simulation of the electric near field enhancement, the simulated morphology corresponds to the np morphology recorded with the SEM. The structure averaged EF obtained from the simulations is respectively $9.8 \cdot 10^2$, $6.1 \cdot 10^2$, $6.9 \cdot 10^2$, and $1.8 \cdot 10^3$. The EF obtained from the measured SERS intensities are $7.5 \cdot 10^2$, $8.4 \cdot 10^2$, $6.3 \cdot 10^2$, and $1.6 \cdot 10^3$ respectively.

The combined investigation of the nanostructures with SERS, SEM, and near field simulations based on the SEM observed morphology enables a comparison of the SERS enhancement factor between measurements and simulations. Surface

averaged enhancement factors are calculated based on the measured signal intensity divided by the unenhanced Raman signal intensity. The unenhanced signal is calculated using the number of molecules per surface area gold, the R6g scattering cross section (table 5.3), and the detection efficiency of the microscope (figure 5.12). For the dimers presented in (A, D, G, and J) the measured surface averaged enhancement factors are respectively $7.5 \cdot 10^2$, $8.4 \cdot 10^2$, $6.3 \cdot 10^2$, and $1.6 \cdot 10^3$. Using the near field simulations the enhancement factors are calculated as the E^4 enhancement averaged over the entire structure surface (equation 5.1). The structure averaged EF obtained from the simulations is respectively $9.8 \cdot 10^2$, $6.1 \cdot 10^2$, $6.9 \cdot 10^2$, and $1.8 \cdot 10^3$. The experimental SEM-Raman analysis and simulated SERS enhancements confirm the general notion of the electromagnetic enhancement in Raman light scattering.

A comparison of the amplitude of the SERS signals with the simulated EF was made for all observed dimers. The simulation predicts a prominent decrease in signal when the gap size increases. The gap size is a well-known property of np aggregates that has a dominant influence on the signal strength.[15] A comparison between the measured and simulated SERS enhancement as a function of gap size is shown in figure 5.18. The presentation of the enhancement factor as a function of the gap size requires a correction of the measured signal to the variation in dimer orientation with respect to the polarization direction of the incident field. The amplitude of the SERS enhancements in figure 5.18 corresponds to a dimer orientation parallel to the light polarization for nps with a diameter of 60 nm. A comparison of the simulated and measured enhancement factor, shown in figure 5.18, reveals only an approximate agreement between the gap size and the measured signal strength. Although the general trend matches reasonably well a precise agreement is absent. The error bars in figure 5.18 indicate an uncertainty in the measurement of the gap size, originating from the finite SEM resolution. It can be noted that the experimentally measured EF is on average lower compared to the EF determined from near field calculations. The logarithmic average for all measured structures equals a ratio of 1.5. The logarithmic average is used to give the same weight to a ratio above as to a ratio below the simulated EF.

Figure 5.18: Measured (black stars) and simulated (bold black line) enhancement factor from dimers with varying gapsizes. EF values are averaged for the signal from all molecules present on the dimer structure as represented in equation 5.1, EF values are corrected to correspond to polarization excitation parallel to the dimer axis.

The precise knowledge of the structure could help in future research of the interpretation of the chemical and electromagnetic contribution to the SERS EF. We conclude that the integrated SEM - Raman has enabled correlative analysis of a large number of nanoparticle structures, providing a quantification of the plasmonic enhancement in conjunction with the structure morphology. The electromagnetic contribution to the EF can be simulated for an ideal particle dimer and compared with the experimental results. More precise simulations can be obtained by inclusion of a more detailed description of the morphology of the nanoparticles. Such knowledge can be obtained for example through higher resolution TEM imaging.

5.5.2 Single nanoparticles

Because of the large variation in SERS signal strength observed from dimers there is a desire to investigate single nanoparticle SERS signals. It is investigated whether the signal variation is as strong for monomer structures as it is for dimer structures. This could give insight into whether the SERS signal variation originates from small variations in the nanoparticle hot spot morphology. From the previous experiments on dimer nanoparticles of 60 nm diameter it was noticed that the Raman signal from the 60 nm monomer was too weak for detection. Therefore larger spheres are chosen to obtain a higher plasmonic enhancement, additionally the larger surface area provides more reporter molecules thus increasing the signal strength. Figure

5.19 shows the plasmonic field enhancement for a 60 nm and a 150 nm diameter gold nanoparticle. The simulations are performed for 785 nm excitation light and the presence of the substrate is not included. A higher local field enhancement is observed for the larger nanoparticle. As a result the surface averaged SERS enhancement factor in the 4th power approximation increases with a factor 2.1 for the 150 nm vs. the 60 nm sphere, with the surface averaged EFs: $\langle \text{EF}_S^{150\text{nm}} \rangle = 59$ and $\langle \text{EF}_S^{60\text{nm}} \rangle = 28$. Aside of the increased local enhancement the 150 nm sphere provides a larger surface area, in comparison with the 60 nm sphere the area is increased with a factor of 6.25. This makes the total signal 13.1 times higher for the 150 nm compared to the 60 nm sphere.

Figure 5.19: Electric field enhancement simulated for (A) a 60 nm diameter particle and (B) a 150 nm diameter particle. The maximum field enhancement for both spheres is respectively $3.4\times$ and $4.2\times$.

When the substrate is included in the near field simulations a significant increase in the near field enhancement is observed. This is shown in figure 5.20 where the field enhancement for a structure without substrate (fig. 5.20A) and a structure with substrate (fig. 5.20B) is shown. The ratio of the near field enhancement between figures A and B is shown in figure 5.20C, as can be expected it is observed that the field enhancement increases strongest closer to the substrate. Including a substrate in the calculations increases the enhancement factor stronger for the monomer particles in comparison with dimer particles as presented in figure 5.3. The reason for this is that for the dimer the signal originates mostly from a single hot spot that is farther away from the substrate, and is therefore less influenced by the substrate. The final surface averaged EF for a 150 nm gold sphere with the substrate included in the simulation is calculated to be 290.

Figure 5.20: (A) Plasmonic field enhancement of a 150 nm monomer nanoparticle for excitation with 785nm light. (B) Plasmonic field enhancement of the same structure and excitation wavelength, but with the inclusion of a steel substrate layer in the simulation. (C) Ratio of the field enhancement obtained from the simulation with and without the substrate.

The simulations suggest that the observation of single nanoparticles is possible using the 150 nm spheres. In figure 5.21A a SEM Raman overlay image recorded on a sample of 150 nm spheres is shown. The red colormap represents the scores of the linear least squares fit performed with a normalized spontaneous R6g Raman spectrum, the intensity map is given in a logarithmic representation. The Raman experiment is performed with 0.3 mW excitation power, 1 second exposure time per pixel, and 300 nm scanning step size. It is observed that monomer particles are well visible in the Raman image. Figure 5.21B shows the SERS spectrum measured from a single monomer particle averaged over 3x3 pixels in the Raman image.

Figure 5.21: (A) Overlay of the Raman and SEM images measured on a sample of 150 nm diameter labeled gold nanoparticles. A logarithmic intensity scale is used, showing that monomer nanoparticles are observed. (B) SERS spectrum observed of a monomer particle indicated in A.

Quantitative analysis of the observed monomer nanoparticles shows a large spread in enhancement factors. The observed EFs are presented in figure 5.22, because of the large spread in intensities the data is presented with a boxplot, the measured EFS vary between 250 and 3300. The average enhancement factor is $1.6 \cdot 10^3$. Multiple monomers are observed to have much higher intensity in comparison with simulations. This higher EF potentially originates from the interaction of the nanoparticle with the metallic substrates, this could result in a hot spot at the particle substrate interface. It can be concluded that a spread in enhancement factor is not necessarily limited to cluster structures with a clearly present hot spot.

Figure 5.22: Measured enhancement factors of 150 nm gold nanoparticles.

A comparison between the 150 nm monomer particles and dimer structures of 60 nm particles is difficult to make. From monomer structures regularly a higher signal is measured in comparison with dimers of smaller nanoparticles, this is partly a result of the 3 times larger surface area for the 150 nm monomers in comparison with 2 particles of 60 nm diameter. The monomer structures surface contains $2.0 \cdot 10^5$ molecules, where the dimer structure contains $6.6 \cdot 10^4$ molecules and the dimer SERS signal originates from just a fraction of its total surface area. The surface averaged enhancement factor of the monomer particles is expected to be smaller (290) in comparison with many dimers, the dimer enhancement depends on the structure parameters but is typically above 1000 as shown in figure 5.2A.

5.5.3 Discussion

Correlative electron Raman microscopy is a powerful tool for the analysis of the plasmonic properties of nanostructures, providing a quantification of the plasmonic enhancement in conjunction with the structure morphology and near field simulations based on the observed morphology. However, several observations have been

made that limit the comparison between the measured and simulated enhancement factors for individual nanostructures.

One of the observations made with electron microscopy is the faceted shape of the nanocrystals.[33] The nanoparticle surface is observed to consist of facets and ridges, instead of the commonly assumed perfectly spherical shape. The presence of facets, their orientation inside a dimer hot spot, and the sharpness of ridges all influence the plasmonic enhancement. This may contribute to the difference in signal enhancement observed for the four dimers presented in figure 5.17, where the nanoparticle morphology is presented in conjunction with their SERS signal. The effect of the gap shape has been investigated in other work,[34] where dark field spectroscopy is applied for the analysis of gaps with a parallel or anti-parallel alignment of the facets in the dimer gap. In this work[34] dark field spectroscopy has shown for dimers with the same gap size a red shift for structures with anti-parallel alignment in comparison to structures with parallel aligned facets. For dimers with the same gap distance but different orientation of facets a higher local field enhancement is observed in simulations for gaps with anti-parallel alignment of facets,[34] however a larger gap volume is achieved for parallel alignment increasing the number of molecules in the hot spot.

Additionally a decrease in penetration of the electron beam in small gaps in between particles that compose a dimer places an uncertainty on the accuracy of the gap size measurements, especially for gap sizes of 3 nm and smaller. The strong influence on the local field enhancement by the gap size therefore results in an uncertainty in the expected enhancement factor. The signal enhancement varies strongly with nanometer small gap sizes and even small errors in the measured gap will strongly affect the simulated EF,[21] which is reflected in the data. Finally it is observed that the experimentally measured EF is on average lower compared to the EF determined from near field calculations, with a ratio of 1.5. Such a difference in observed enhancement can potentially be attributed to morphology variations of the nanocrystals (vide supra), but potentially also to a chemical contribution to the SERS enhancement. By inclusion of a more detailed description of the morphology of the nanoparticles in the near field calculations an approximation can be made of this chemical contribution to the enhancement factor.

It is expected that analysis of monomer nanoparticles lowers the variation in the measured EFs since there is no local hot spot that is strongly influenced by the nanoscale morphology of the particles. However still a large variation in measured EFs is observed. Furthermore the observation is made that the experimentally observed enhancement factor for monomer structures is higher than expected based on near field simulations. On inspection of the near field simulations this variation could result from the nanoscale morphology variations at the particle - substrate interface. As is seen in figure 5.20 the field enhancement increases strongly at the particle boundary interface, which potentially results in a small local hot spot. Such a hot spot could also be strongly affected by nanoscale variations in the particle morphology.

5.6 Conclusions

This chapter presents the application of the integrated Raman - electron microscope for correlative analysis of plasmonic nanostructures. Nanostructures with varying morphologies are observed with the SEM, and their plasmonic Raman enhancement is measured with the correlative Raman microscope. This analysis is performed first on dimer structures, consisting of two 60 nm diameter gold nanospheres with a small gap, and second on monomers of 150 nm diameter gold spheres. The gold nanoparticles are labeled with R6g dye molecules of which the SERS response is measured to probe the plasmonic field enhancement from the nanostructure with Raman spectroscopy. With the SEM observed structure morphology it is possible to model the observed structure in simulations to calculate the near field enhancement. The simulations provide insight into the near field distribution of the plasmonic enhancement and are compared with the SERS intensities measured from the structures. For a comparison with measured SERS intensities the simulated EF is averaged over the full nanostructure surface, which is comparable with the Raman measured EF that originates from dye molecules over the full surface area. Simulations are performed for monomers and for dimer structures with a gapsize varying from 0 to 15 nm using an excitation field polarization from 0° to 90° .

To directly compare the measured Raman response from plasmonic nanoparticles with near field simulations a quantitative measurement of the SERS enhancement factor is required. For this purpose a quantitative calibration of the Raman microscope is performed. This calibration enables the system to be compared with other Raman microscopes and enables a measurement of the Raman scattering cross section or densities of investigated compounds. Additionally the Raman scattering cross section of the R6g dye molecules and the surface coverage density of the dye molecules is measured. The quantitative measurement of the EFs from dimer structures shows a large variation even for structures with similar parameters such as gapsize and orientation. This variation is expected to originate from the limited SEM resolution in the measurement of the gapsize and nanoscale variations of the particle morphology which could have a large effect on the enhancement in the structure hot spot. However an approximate agreement with the simulations is observed showing on average a slightly lower enhancement than expected from simulations with a ratio of 1.5. For 150 nm diameter monomer particles the comparison between simulations and experiment has shown a similar variation in the experimentally observed enhancement factor. The result is at first unexpected but could originate from interaction of the nanoparticle with the substrate. Monomer nanoparticles have shown a stronger SERS signal than expected from initial simulations, the 150 nm diameter spheres have shown similar signal strengths in comparison with dimer structures partly due to the larger surface area containing more reporter molecules. We conclude that quantitative SERS is demonstrated for many nanostructures, enabling a direct comparison between the experimentally observed signal strength and SEM observed morphologies. Additionally the comparison of the measured enhancement factor with near field simulations of the enhancement can provide new insight into the nanostructure properties.

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WHISPERING GALLERY MODES OBSERVED THROUGH EBID

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Abstract

Surprisingly intense spectra of whispering gallery modes (WGMs) were observed in polymer microbeads during Raman spectroscopic analysis after SEM electron irradiation. In this chapter this phenomenon is investigated, it will be proposed that whispering gallery mode resonances became visible after local deposition of hydrocarbon material through electron beam induced deposition. Illumination of deposited material with the near infrared Raman excitation laser generates a broad light spectrum, acting as a local "white light" source that couples matching wavelengths with the WGMs sustained by the sphere. This facilitates spectroscopic analysis of the sustained WGMs and provides the Q-factor and free spectral range for investigated particles. Analysis by an integrated SEM and Raman micro-spectrometer offers a direct approach to the analysis of WGM resonators as they are, for instance, used in sensing applications. Integration of multiple techniques (FIB, SEM, and optical spectroscopy) enables control over the optical resonator. Through EBID patterning on specific locations on the sphere and through FIB ablation modification of the sphere morphology a controlled treatment of the resonator is achieved. This is achieved by applying specific patterns by SEM treatment, and FIB micromachining to the optical resonator morphology.

6.1 Introduction

Optical microresonators confine light within a micro-cavity, which results in a significant increase of the energy density for resonator modes. In particular, WGM resonances are observed on microspheres that confine light propagating along the sphere surface by internal reflection. Such WGMs are for example employed as sensors for the detection of molecules that attach on the sphere surface. A change at the surface influences the WGM spectrum.[1–6] In other applications, the WGM has been used to achieve an increased light density, which enables Raman and spectroscopic analysis with a higher excitation energy density thus achieving an enhanced signal.[7, 8] Cavity enhanced Raman spectroscopy has also been demonstrated for microscopic applications.[9] Optical ring resonators are furthermore applied for high quality optical filters in combination with coupled optical waveguides.[3]

We report on the use of the integrated FIB-SEM-Raman microscope for analysis of WGM resonators. The electron beam is used to locally deposit hydro-carbon material that upon laser illumination acts as a white light source with emission also in the near infrared.[10, 11] Subsequent spectroscopic analysis reveals the WGM spectrum due to coupling of the excited white light with available modes. Analysis reveals the WGM spectrum, the mode free spectral range, Q-factors of observed modes, and relative mode intensities. WGM resonances become visible in spectral analysis with the Raman spectrometer after treatment of the resonator surface with the SEM. We propose that the ring resonator signal originates from photoluminescence light excited with the Raman laser from EBID material, the broadband photoluminescence light couples into the resonator for favorable wavelengths which are subsequently detected with the confocal spectrometer. Electron beam induced deposition is commonly used in for example: fabrication of nanostructures,[12, 13] fabrication of electronic interconnects,[12, 14] or deposition of a carbon protective layer for TEM analysis.[15]

Potential applications of integrated light and electron microscopy investigation of optical microresonators are as a non-destructive analysis method for fabricated optical microresonators. This can be especially useful for waveguide structures prepared in combination with FIB milling,[16] as these are directly in-situ analyzed. Polymer beads with diameters between 2 and 10 μm were used as microresonator. These beads are regularly used in Raman imaging as test samples due to their strong signal and broad range of available diameters. It should be noted that no WGMs are observed from untreated polymer beads in normal Raman imaging. However detection of optical resonances during Raman analysis of microspheres has been used for morphology specific analysis, in those works the WGM resonances are observed from a fluorescent component in the material.[17–19]

6.2 Theoretical description

6.2.1 Electron beam induced deposition

Deposition of hydrocarbon material occurs mostly through interaction of secondary electrons scattering from the sample surface, with trace gasses near the sample surface. This is because the lower energy of the SE electron, typically up to 50 eV, gives an increased interaction compared to the e-beam electrons.[13, 20] EBID is performed on the top half of the bead as indicated in figure 6.1A. After SEM treatment and after incremental steps of the treatment process, micro-spectroscopic analysis of the WGM spectrum is performed. The 785 nm laser excitation light is coupled into the sphere with a 0.65 NA optical objective. Due to the excitation direction light propagation occurs along the vertical cross section of the bead, as schematically represented in figure 6.1B. A SEM image of the treated polymer bead is shown in figure 6.1C, the area on which EBID is performed is visible as a dark square. Furthermore charging effects are visible on the center of the bead as a white ring. The outer circumference of the bead doesn't undergo charging effects due to charge neutralization of higher SE scattering from the surface.[21]

Figure 6.1: (A) Schematic presentation of EBID with the electron beam. Deposition occurs mostly through interaction of secondary electrons with trace gasses. Deposition occurs only the top surface of the bead as this part is irradiated with the e-beam. (B) Schematic presentation of laser excitation and propagation inside the microbead. (C) SEM image of EBID material on a 10 μm diameter sphere, visible as a dark square on the treated region.

Spectra observed from the EBID hydrocarbon material deposited on a flat surface show a high photoluminescence contribution over the full measured spectral range. In other work Raman analysis of amorphous hydrocarbon material shows similar results with a fluorescence background observed for EBID on an organic material (resin), and with the typical amorphous carbon Raman spectrum observed for EBID on an inorganic (cinnabar) substrate.[10, 11] The literature suggests that the EBID signal is substrate dependent and confirms that EBID provides a broadband spectrum, which we found to be suitable for coupling with the optical resonator modes. The photoluminescence spectrum from EBID is measured in the integrated SEM - Raman microscope for material deposited on a metallic substrate and on a polymer layer. The spectrum observed for deposition on a metal substrate is presented in figure 6.2A showing both the broadband photoluminescence and a Raman band of amorphous carbon between 1400 cm^{-1} and 1600 cm^{-1} . [22] For deposition on a polymer substrate the amorphous carbon Raman band is typically not observed, however a broadband photoluminescence contribution is still visible, figure 6.2B. Recording of the spectra is performed with 1 mW excitation power and 1 second integration time. It should be noted that the intensity of these spectra are not comparable to spectra recorded from polymer beads. The reason for this is a higher SE scattering and EBID deposition on the curved bead surface compared to a flat substrate, and that the deposition rate varies with the vacuum pressure near the sample which is not controlled in the experiment.

Figure 6.2: Spectra of EBID are measured with 1 mW excitation power and 1 second integration time. Spectroscopy of deposited material is performed on (A) a metallic substrate and (B) a polymer (polysulfone) substrate.

6.2.2 Optical resonators

Optical resonators typically exist as an arrangement of mirrors that confine light in the optical cavity. The light confined in the cavity reflects multiple times between the mirrors. This results in constructive or destructive interference of

light inside the resonator, depending on the wavelength and optical length of the cavity. Constructive interference exists for wavelengths that fit an integer number of times in the optical path length OPL as presented in equation 6.1, where m and λ_m are the mode number and corresponding wavelength. For these favorable wavelengths an increased optical density and optical feedback is achieved inside the resonator. This enables multiple applications the most common of which is the laser where the optical feedback and a gain medium in the resonator are used to achieve coherent light emission. An important parameter of the cavity resonator is the Q factor, a resonator with a high Q factor has a low energy dissipation per cycle. High Q factors are achieved by a high reflection coefficient for the mirrors and low losses in the optical path, they result in narrowband spectral modes. The Q factor is determined from the width of the modes $\delta\nu$ and spacing between the modes ν_{fsr} called the free spectral range, equation 6.2

$$OPL = m \cdot \lambda_m \quad (6.1)$$

$$Q = \frac{\nu_0}{\delta\nu} \quad (6.2)$$

Ring resonators are closed loop ring waveguides that confine light through internal reflection. In and out coupling is generally achieved through optical waveguides. Constructive interference is achieved for wavelength modes that fit an integer number of times in the ring resonator optical pathlength. For a perfectly spherical resonator the optical path length is calculated with the ring radius r and the effective group refractive index of the ring resonator medium $n_{g,eff}$, equation 6.3. Waveguide optical ring resonators have numerous applications in integrated optics, for example as high quality optical filters with coupled optical waveguides, and for on chip sensing applications.

$$OPL = 2\pi \cdot r \cdot n_{g,eff} = m \cdot \lambda_m \quad (6.3)$$

Of particular interest are whispering gallery mode resonators. These are dielectric microspheres that confine light propagating along the sphere surface by internal reflection. Observation of WGM resonances is presented in figure 6.3. WGMs are excited in a 10 μm diameter polystyrene bead through laser excitation of EBID material deposited on the bead surface. Figure 6.3A shows the intensity map of the observed WGM signal. During the experiment the laser is scanned over the sphere and the combined Raman and WGM signal is collected with the confocal spectrometer. It is observed in figure 6.3A that the spatial dependence of the measured WGM signal follows approximately a cosine squared profile along the resonator circumference. From the observation it is concluded that an optimized resonator signal is measured for light polarized along the bead surface. Such locations are indicated in blue squares in figure 6.3A. This profile is investigated in more detail as presented in figure 6.7. In figure 6.3B the Raman spectrum from an untreated polystyrene bead is presented as reference to the WGM signal. The spectrum is obtained from the areas indicated in figure A before EBID treatment of the sphere and is identical to the standard Raman spectrum of polystyrene. Figure 6.3C shows the measured spectrum (black) after EBID. The Raman spectrum of

polystyrene is accompanied by additional fringes with a typical spacing in agreement with what is expected from WGM resonances. The spectrum (red) of the WGM resonances in figure 6.3C is obtained after subtraction of the polystyrene Raman scattering spectrum. In the inset the same red spectrum is repeated on an expanded amplitude scale to bring out fine details in the WGM response. Two resonator modes can be distinguished, indicated in the figure as the transverse electric (TE) and transverse magnetic (TM) mode. Assignment of the modes is based on the TM mode having a shorter resonance wavelength $\lambda_{TM} < \lambda_{TE}$, and that the TM modes have a lower amplitude.[1, 23] The WGM spectrum is used to extract specifications from the resonator such as the mode free spectral range $\Delta\lambda_{FSR}$ and spectral width $\delta\lambda$, used to calculate the Q factor. A measurement of the Q-factor is limited to the resolution of the optical spectrometer, which is 5 cm^{-1} . This sets an upper limit on the resolvable Q-factor magnitude of 2300. For the resonator presented in figure 6.3 the Q-factor varies between 350 and 650 for TE modes.

Figure 6.3: (A) Optical intensity map showing the WGM signal intensity image. The laser polarization orientation is indicated with the red arrow. Two regions are indicated with blue squares, the spectra presented in B and C are obtained as the average from these pixels. (B) Polystyrene Raman spectrum observed for the untreated bead measured on the same region as the indicated positions in A. (C) Observation of whispering gallery modes including the polystyrene Raman signal (black) and with the polystyrene Raman signal subtracted (red). The inset shows the same red spectrum, enlarged to visualize both the TM and TE modes.

Characterization of the WGMs is performed on spheres of a suitable dielectric compound. It is expected that most beads of a transparent compound are suitable to sustain WGMs. This is verified with the analysis of treated glass (SiO_2) and poly(methyl acrylate) (PMA) which is compared with measurements on polystyrene as presented in figure 6.3. In figure 6.4 the observation of WGM resonances on 8

μm diameter polymethylacrylate (A) and $7.75\ \mu\text{m}$ diameter glass (B) microbeads is presented. It is observed that mainly glass microbeads have a lower Q-factor compared to the polymer beads, this indicates higher losses in light propagation which could result from irregularities in the glass bead surface. It is furthermore observed that spectra of the glass microsphere show optical resonances also before SEM treatment. This is a direct result of the broadband Raman contribution of glass between 1500 and $2000\ \text{cm}^{-1}$, the Raman scattered light couples into the resonator modes and observed as WGM resonances. SEM treatment of glass increases the WGM resonator signal, mainly on spectral regions with a low Raman contribution from the glass. It can be concluded that the polymer beads are more suitable for characterization of the WGMs, mainly due to the higher Q-factor and resonator signal.

Figure 6.4: (A) WGM resonances observed on a polymethylacrylate bead of $8\ \mu\text{m}$ diameter. Optical resonances are observed only after SEM treatment and show spectra similar to observations made on polystyrene. (B) WGM resonances observed on a glass bead of $7.75\ \mu\text{m}$ diameter. Observed optical resonances have a lower Q-factor compared to polymer beads, which could result from higher losses due to irregularities on the surface. WGM resonances can be observed before SEM treatment due to the strong broadband glass Raman spectrum, after treatment the resonances become stronger over the full spectral range.

6.3 Methods and materials

The integrated optical - electron microscope configuration enables fast analysis of WGM resonators on intermittent steps of SEM irradiation. The optical microscope laser is used for excitation of the white light spectrum from the EBID hydrocarbons, the subsequently excited WGMs are detected with the confocal spectrometer. Spectroscopic analysis of the beads is performed before and after EBID. Treatment of the polymer beads is performed by irradiation with the SEM e-beam at a beam energy of $2\ \text{kV}$, and probe currents between $13 - 52\ \text{pA}$. This low acceleration voltage is chosen because it provides a high secondary electron scattering coefficient, which results in an efficient EBID process. Excitation of the WGM resonances is performed with 1 to $3\ \text{mW}$ of $785\ \text{nm}$ laser light, which is a sufficiently low irradiation to prevent bleaching or sample damage.

The spheres are polystyrene beads with diameters ranging from 2 to 10 μm obtained from Polysciences, inc. The precise diameter specifications are reported together with the results and are obtained with SEM imaging. WGM resonances are furthermore observed on 8 μm PMA and 6 - 13 μm glass beads both obtained from Sigma-Aldrich. The beads are deposited on conducting metallic substrates designed to have a low light background and which are suitable for correlative light and electron microscopy. The $\mu\text{Rim}^{\text{TM}}$ substrates are obtained from BioTools, inc.

To obtain an optimum representation of the resonator signal in the data analysis several pretreatment steps are performed before fast Fourier transformation (FFT) is applied to the measured spectra. For FFT the resonator signal has to be isolated from other contributions in the measured spectra. If this is not done these signal contributions will act as a background with a difficult to recognize FFT spectrum. First to improve the signal to noise ratio a singular value decomposition is applied and the first S-values are used for data reconstruction. The number of S-values is manually selected and typically around 30. Second a filter is applied to remove "smooth" contributions from the spectra. This filter mainly removes the broadband photoluminescence contribution to the signal and preserves the Raman and WGM signal.[24] Third the polystyrene Raman spectrum is removed from all the recorded spectra, this is performed by subtraction of a pure polystyrene Raman spectrum scaled to the 1001 cm^{-1} Raman band intensity. As a result the WGM signal is isolated from all other contributions. For Fourier transformation the WGM spectrum is interpolated to a linear frequency axis, and the transformed spectra are presented with a X-axis equal to the optical pathlength of propagation in the resonator. This data analysis method is applied for all discussed experiments.

6.4 Results

6.4.1 Successive EBID treatment

Analysis of WGM resonators reveals an increasing resonance signal as a result of increased electron irradiation. This is demonstrated through incremental SEM irradiation steps with intermediate spectroscopic analysis of the treated resonator. The results are presented in figure 6.5. Polystyrene beads of 6 μm diameter were used for this experiment. Figure 6.5A shows the observed spectra after irradiation of a polystyrene beads top surface with a cumulative electron dose of [14.6, 58.6, 132, 176, 249] $\text{e}^- \cdot \text{nm}^{-2}$, figure 6.5B shows spectra on a 2nd polystyrene bead for an electron dose of [310, 621, 932, 1242] $\text{e}^- \cdot \text{nm}^{-2}$, the spectra are vertically offset for clarity in figure 6.5A with incremental steps of 50 counts and in figure 6.5B with steps of 100 counts. The polystyrene Raman signal is subtracted from all the observed spectra to present only the WGM resonances. It is observed that the WGM Q-factor undergoes little change upon an increase of the irradiation dose. A shift of the WGM spectrum is however observed, this shift is a result of a change in the refractive index of the surrounding medium caused by material deposition.[6, 25] This is shown with more detail in figure 6.5C where a single TM and TE mode of the normalized spectra shown in figure A [black] and figure B [red] are presented. Figure 6.5D shows the FFT spectra of the same WGM signals,

A [black] and B [red]. The FFT spectra show clearly the optical pathlength of the resonance modes. The first order resonance is found at the optical pathlength 27.2 μm , which corresponds with a 6 μm diameter ring and an effective group refractive index of 1.44, using equation 6.3. To determine the Q-factor the free spectral range ($8.0 \cdot 10^2 \text{ cm}^{-1}$) and $\delta\nu$ (30 cm^{-1}) for TE and (50 cm^{-1}) for TM) are obtained, which translates to a Q-factor of 420 for TE and 235 for TM modes, with mode number 32 and 33.

Figure 6.5: (A and B) Observation of WGMs on 2 polystyrene beads, both of 6 μm diameter, with increasing SEM irradiation steps. An offset between the consecutive spectra of 50 counts is given for bead A and 100 counts for bead B, with each consecutive step represents the spectrum measured after a new SEM EBID treatment. (C) Magnification of a single TM - TE mode combination of the spectra presented in A (black) and B (red). Only a small change in the WGM spectra is observed for higher carbon deposition concentrations. The spectra are normalized to 1 and given an offset to improve a direct comparison of their spectral peak position. (D) FFT spectra of WGM shown in A (black) and B (red). The optical pathlength of the resonators is identified from the first order maximum 27.2 μm . (E) The ratio of the detected WGM signal intensity vs the observed white light emission intensity from EBID material. The white light emission intensity is measured on the center of the treated polystyrene sphere as the smooth baseline under the Raman and WGM peaks. The arrow indicates the direction of increased EBID.

The resonator signal intensity increases close to linear with the increasing white light intensity, which increases for increasing EBID. Figure 6.5E presents the linear relationship between the resonator signal strength and the generated white light

intensity. Observations are in agreement with the proposition that SEM treatment of the beads visualizes WGM resonances through EBID deposition. Successive SEM treatment steps increases the EBID deposition and therefore the photoluminescence emission that is coupled to the resonator modes. The WGM signal is proportional to the EBID white light emission. Figure 6.5C shows that there is little change in the resonator Q-factor for increasing EBID, as is expected when there is only an increase in white light intensity coupled to the resonator.

6.4.2 Resonator diameters

Figure 6.6: (A) Observation of WGM resonances on polystyrene beads with diameters 1.9, 3.05, 4.45, 6.0, and 10.2 μm . (B) FFT of the spectra presented in A. The optical pathlengths of the resonators are indicated as blue stars in the figure on the corresponding FFT bands.

Further analysis of polystyrene beads with different diameters reveals more properties of WGMs. Figure 6.6A and B respectively show WGMs and their FFT spectra for polystyrene beads of 1.9, 3.05, 4.45, 6.0, 10.2 μm diameter. The diameters have been measured with SEM. The electron dose used for analysis of the beads is respectively $[1.1, 1.0, 0.8, 1.2, 0.8] \times 10^3 \text{ e}^- \cdot \text{nm}^{-2}$. A significant increase in Q-factor for increasing resonator diameters is observed, the 3, 4.5, 6 and 10 μm diameter resonators have TE mode Q-factors up to 118, 163, 473, and 1155 respectively. The optical pathlength from the 3, 4.5, 6 and 10 μm beads is obtained from the first order FFT peak: 12.5, 20.1, 27.2, and 45.7 μm respectively, when dividing with the ring circumference the effective group refractive index $n_{g,\text{eff}}$ is obtained: 1.3, 1.44, 1.44, and 1.43. It is observed that an increasing diameter results in higher Q factors, which is presumably due to smaller bending losses. The small frequency oscillations in the FFT spectra are due to window effects and are most prominent in the 0 to 20 μm length scale, especially on the 2 μm diameter sphere. The mode pattern for most of the beads is regular and can be interpreted in a straightforward manner

considering TE and TM mode propagation. For the largest investigated beads (10 μm diameter) higher order modes and therefore more complicated resonator spectra are observed, as presented in figure 6.6A.

6.4.3 Excitation polarization

As was observed in figure 6.3A the spatial dependence of the measured WGM signal follows approximately a cosine squared profile along the resonator circumference. It is proposed that this effect originates from the laser polarization orientation, this is experimentally verified by imaging the bead for multiple rotations of the polarization orientation with 0° , 45° , 90° , and 135° . The measurements show that the observed resonator signal rotates with the laser polarization. It can be concluded that an optimized profile rotates with the laser polarization. This situation provides an optimized propagation of excitation light in the resonator through lower bending losses in internal reflection with TE polarized light. This results in a higher excitation light density in the resonator, thus achieving an increased WGM signal. Additionally out-coupling of propagating WGM light occurs in the direction of propagation, therefore this position of the confocal detection spot is ideal to observe light that is coupled out of the resonator.

Figure 6.7: (A) FFT spectrum recorded on the border of the polystyrene sphere with the laser polarization parallel to the bead surface. The first order Fourier band from the WGM signal is indicated with red dots, the intensity of this region is presented in the intensity maps. (B-E) Intensity maps for an excitation polarization of 0° , 45° , 90° , and 135° . The polarization direction is indicated with red arrows.

The beads are treated with a $8.2 \times 10^2 \text{ e}^- \cdot \text{nm}^{-2}$ electron dose and analyzed with 2.24 mW laser power, and 1 second per pixel integration time. In the experiment the entire sample and Raman stage are rotated using the SEM rotation stage, which is effectively the same as a rotation of the light polarization. Figure 6.7A

shows the FFT spectrum recorded on the side of the bead with parallel polarization orientation. The intensity of the first order Fourier band, indicated in fig. 6.7A with red dots, is presented for each recorded spectrum in the intensity maps figure 6.7B-E. The polarization orientation used for the four recorded images is indicated with a red arrow.

6.4.4 Patterned resonator treatment and micromachining

The combination of FIB and SEM with spectroscopic characterization enables control over the resonator treatment. This is first demonstrated by patterning only specific locations of a bead with the SEM. In figure 6.8A a 10 μm diameter polystyrene bead is presented on which EBID is performed on 4 areas of 1.5 $\mu\text{m} \times 1.5 \mu\text{m}$. The initial SEM image is made with a very low electron dose ($8 \times 10^{-3} \text{ e}^- \cdot \text{nm}^{-2}$), which results in an image with a low signal to noise ratio. The 4 treated locations are visible in the image with a better signal quality. The EBID step is performed with an electron dose of $1.8 \times 10^3 \text{ e}^- \cdot \text{nm}^{-2}$. Because EBID material is present only on these specific locations WGM resonances will be observed exclusively for locations where the excitation light path travels through these areas. This effect is observed in figure 6.8B where the measured WGM intensity map is presented, it is clear that WGM resonances are only recorded on the treated locations and on the 180° opposite side of the sphere. The intensity map is obtained from the first order FFT peak of the WGM resonator signal. WGM spectra are similar to the earlier observed spectra as presented in figures 6.8 C and D. These spectra are obtained from the highest intensity pixel in figure B. The spectra are measured with 0.56 mW laser excitation power. Both the measured and FFT spectrum are presented. An area in the FFT spectrum in figure D is indicated, the indicated band intensity image is presented in figure B. An overlay of the Raman and SEM figures is created to show the correlation between EBID treatment and resonator intensity, figure 6.8E.

Figure 6.8: (A) SEM image of a 10 μm diameter polystyrene bead. A very low electron dose is used for the initial image to limit overall EBID deposition. On 4 locations, visible in the image, an EBID treatment step is performed with a $1.8 \times 10^3 \text{ e}^- \cdot \text{nm}^{-2}$ electron dose. (B) Intensity map of the WGM resonances measured with 0.56 mW laser excitation power, the intensity of the integrated first order FFT peak is shown. (C-D) WGM spectrum and corresponding FFT spectrum recorded on the patterned sphere on the position with maximum resonator intensity. An area indicated in red in figure D shows the used FFT peak for the intensity map of figure B. (E) Overlay of the optical and electron microscopic images.

Control of the WGM resonator is further facilitated with FIB micromachining. This is demonstrated through ion beam milling of specific locations on a treated polystyrene microsphere. The sphere is presented in figure 6.9A with an overlay of the SEM image with the optical micro-spectroscopic image. SEM imaging is performed with 2kV acceleration voltage and 13 pA probe current. The optical image is created with 0.56 mW excitation power, 1 μm scanning step size, and 1 second pixel integration time. An intensity map of the first order FFT peak from the WGM spectrum is presented. In subsequent steps FIB material ablation is performed on 3 locations indicated in figure 6.9B, first on the top part of the sphere, second on the bottom part, and third on the left part. Spectroscopic analysis of the bead is performed after each of the 3 FIB treatment steps, the measured intensity maps of the 1st FFT band are presented in figures 6.9C-E. It has previously been observed that laser excitation of polystyrene beads after FIB treatment can result in damage to the bead. This has been discussed in connection with figure 4.10. For this reason spectroscopic analysis is not performed directly on the treated locations. Figure C presents a scan over the bottom 7 μm of the sphere, figure D presents a scan over the center 3 to 7 μm of the sphere, and figure E presents a scan over the center 3 to 7 μm band on the 7 μm area on the right side of the sphere.

Figure 6.9: (A) Overlay of the SEM image and optical micro-spectroscopic image, showing the SEM image of the bead and WGM intensity optical image. (B) SEM image of the polystyrene bead after three FIB ablation steps, material ablation is performed on the top, bottom, and left side of the sphere. (C-D) WGM intensity maps measured after each of the three FIB ablation steps. The presented intensity map is obtained from the integrated first order FFT band. Imaging is performed on regions of the sphere where no FIB interaction has taken place, first on the bottom 7 μm , second on the 3 to 7 μm band through the center of the sphere, and third over the 3 to 7 μm band excluding the left side of the bead. (F-G) WGM spectra and the corresponding FFT spectra, measured before FIB ablation [black], after the first FIB ablation step [red], after the second FIB ablation step [blue], and after the fourth FIB ablation step [green]. The spectra are measured on the maximum intensity position on the bead.

For the first two FIB treatment steps the WGM resonator signal does not decrease for a measurement performed on the left or right side of the bead. This is in good agreement with our description of light propagation in the microsphere, because both the excitation and observed WGM light travel along the XZ plane between the FIB treated locations. A significant change is observed in figure 4.10E after the final FIB step, this is a direct result of the breaking of the resonator. The WGM spectra measured after each of the FIB treatment steps are presented in figure 6.9F. The spectra are measured with excitation on the right side edge of the bead at the maximum intensity position. The corresponding FFT spectra are presented in figure 6.9G. The spectra are presented in the order they are measured, before FIB treatment [black], after first FIB machining [red], after second FIB machining [blue], after third FIB machining [green]. It is observed that after the first FIB treatment the resonator signal intensity increases. This is possibly an effect of increased photoluminescence of regions on which a low ion dose interaction occurred. It is further observed that the optical pathlength and Q-factor only change significantly when the resonator is intentionally broken in the final (third) FIB milling step.

6.5 Conclusions

In summary, a surprising observation is reported of whispering gallery mode optical resonances after electron beam treatment of polymer micro-spheres. A proposed explanation for this observation originates from EBID material of trace gases in the vacuum chamber of the SEM. The WGM resonances were observed in optical spectroscopic analysis of the photoluminescence from EBID material after narrow-band laser illumination. The WGMs exist on the spheres before SEM treatment and are made visible by EBID of hydrocarbon material. Broadband photoluminescence emission from EBID is excited with the Raman laser and couples to the local field of the WGM resonator and is observed with the optical spectrometer. Spectroscopic analysis with a Raman spectrometer integrated with the SEM provides direct access to the measurement of the free spectral range and Q-factors for modes observed in particles with different sizes and with different materials, namely polystyrene polymethylacrylate and glass. It has been observed that the resonator signal increases linearly with the electron dose which has been used to deposit hydrocarbon material. The photoluminescence spectral emission from deposited hydrocarbon material enables a direct observation of the resonator optical path length after Fourier transformation of the luminescence spectra.

Microscopic imaging of the resonator signal reveals areas of more efficient coupling of the local field to the WGM resonator versus areas with less efficient coupling. It is concluded that this spatial variation on signal intensity originates from the propagation of excitation light inside the resonator for different orientations of the polarization direction with respect to the resonator boundary. The excited WGM signal intensity can thus be controlled with the laser polarization state. Control over the excitation of WGM is further enabled through specific EBID patterning with the SEM, and through micromachining of the resonator with the

FIB. The effects of these treatment procedures have shown controlled treatment over the WGM resonator.

We conclude that an observed, surprising effect of intense WGM resonances in spheres with a combined SEM-Raman microscope is in full agreement with existing knowledge of cavity resonances. The combination of EBID and optical spectral analysis as a new method for analysis of WGM resonators has thus been presented and may provide opportunities for the characterization of WGM resonators that are applied in for example sensing applications and integrated optics.

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BLUE LIGHT EXCITATION RAMAN

Abstract

In this chapter a high resolution Raman microscope, using 405 nm excitation light, is developed to help bridge the gap in resolution between electron and optical microscopy. The Raman microscope is a standalone system and correlative SEM microscopy is performed by transferring samples between the separate SEM and Raman systems, and relocating the ROI using sample markers. Applications of the Raman microscope for resonance Raman spectroscopy and for high resolution analysis of SERS nanostructures are presented. Resonance Raman spectroscopy is performed on red blood cells, for which the excitation wavelength is on resonance with the hemoglobin Soret band. The results demonstrate the system capabilities for resonance experiments showing a strong signal and enabling fast microscopic imaging. The strong optical resolution enables a more accurate analysis of SERS nanostructures. Experiments are performed on clusters of silver nanoparticles, for which the blue excitation wavelength is close to the plasmon resonance. SERS spectra are analyzed in combination with correlative high resolution SEM imaging, and simulations to the local field enhancement for the SEM observed structure morphologies. A qualitative analysis to the SERS signals in combination with near field simulations is presented.

7.1 Introduction

In the integrated Raman - electron microscope discussed in chapter 4 there is a large resolution difference between the optical and electron microscope. This resolution gap complicates correlative analysis for structures smaller than the diffraction limit. While the resolution gap will not be fully bridged using conventional confocal microscopes it is expected that higher resolution optical microscopes will improve the correlation of Raman - SEM images. In this chapter a high resolution Raman microscope is demonstrated. Using blue excitation light and a high numerical aperture objective the optical resolution is significantly improved compared to our integrated Raman microscope. While the blue light excitation Raman microscope is not integrated in a SEM correlative electron microscopy images have been obtained. This is done with the use of SEM grids and markers to find the ROI in the separate microscope.

Use of blue laser excitation light changes the experimental aspects compared to Raman microscopy with red or NIR excitation. As has been stated, one of the advantages is the increased resolution obtained from a shorter excitation wavelength. Experiments can however become more complicated, the main reason for this is the higher photon energy and a stronger light - matter interaction. Most materials have a higher absorption coefficient for light of a shorter wavelength, this potentially results in a higher autofluorescence emission that acts as a background on the Raman signal. Additionally a high light absorption increases the chance of sample damage under laser irradiation. To compensate for this, a lower excitation power is used. One significant advantage arising from the increased light - matter interaction is the increased Raman scattering cross section. As was stated in equation 3.7 the Raman scattering cross section is proportional to $(\tilde{\nu}_0 - \tilde{\nu}_R)^4$, or approximately λ_{Laser}^{-4} . Compared to 785 nm light the use of 405 nm excitation increases the scattering cross section approximately 14 $\times$.

Another useful application becomes possible for blue light excitation. Resonance Raman spectroscopy was briefly discussed in chapter 3.1.2 and equation 3.6. When the Raman excitation wavelength is close to the molecule absorption band a resonance scattering process occurs increasing the Raman scattering signal up to 10³ times.[1] Figure 7.1 shows the Jablonski diagrams for normal Raman scattering (left) and resonance Raman scattering (right). The closer the excitation photon energy is to the first excited state the higher the resonance signal enhancement. This effect is prominent for blue excitation Raman spectroscopy since the excitation energy is much closer to the first excited state of many molecules.

Figure 7.1: The Jablonski diagram for (A) normal Raman scattering and (B) resonance Raman scattering. The indicated energy states are the ground state E_g , the vibrational states E_{vib} , and the first excited state E_{e1} .

7.2 Setup

The Raman setup uses a diode laser that produces 405 nm light. The laser (Omicron-Laserage Laserprodukte GmbH) is a narrowband light source with a stable wavelength and intensity. The laser is coupled to a single mode optical fiber. The fiber collimator produces a collimated beam with 8 mm diameter that fills the full aperture of the optical objective. The optical objective, obtained from Nikon Instruments (CF Plan Apo 50x EPI), is a 0.95 NA apochromat chosen for a high light transmission and low chromatic aberrations, it is suitable for working in dry conditions and is therefore compatible with samples that are also investigated in the SEM vacuum chamber. The excitation power incident on the sample is at maximum 4 mW. The incident power is tunable using the laser control down to 0.4 mW. To further control the light intensity neutral density (ND) filters are placed on a flip mount, depending on the used ND filters an arbitrarily low incident irradiation can be achieved. Further in the optical path from the laser to the objective a half wave plate (HWP) is placed to control the excitation polarization light.

Raman scattered light is collected in the backscattered direction using the same objective, using an optical edgefilter the laser wavelength is separated from the Raman light. The used filter is a 407nm razoredge filter, obtained from Semrock inc. (partnumber: LP02-407RU-25). For confocal detection a 15 μm pinhole is placed at the entrance of the spectrograph. The homebuilt spectrograph provides a spectral detection range from -50 to 2950 cm^{-1} . An Andor - Newton EMCCD (model number DU970P-BVF) is used for Raman detection, providing in full vertical binning 1600 pixel spectra. To calibrate the spectrometer a krypton lamp was coupled into the beam path using a flip mirror, the calibration procedure is covered in more detail further in this chapter.

To observe the sample and determine the ROI for Raman microscopy a video camera (Allied Vision Technologies Stingray F-125) and LED illumination is used. The optical path of the LED and CCD camera is aligned onto the sample using two 50/50 pellicles, as presented in figure 7.2. This setup enables the observation of specimens mounted on top of non-transparent substrates and shows the laser focus spot in the video image. A lens with focal distance 100 mm is used to project the image onto the camera, providing a magnification of $25\times$ and a field of view of $130\times 130\text{ }\mu\text{m}$.

Figure 7.2: Schematic representation of the blue light excitation Raman microscope. The following abbreviations are used: M = Mirror, FM = Flip Mirror, Coll = Fiber collimator, HWP = Half Wave Plate, ND-F = Neutral Density Filter, OBJ = Objective, S = Sample, CP = Confocal Pinhole

The sample table, shown in figure 7.3 (right), is placed on a XY- piezo stage to enable scanning of the sample during confocal imaging. The stage has a travel range of $50 \times 50 \mu\text{m}$, for longer range sample movement a manual translation stage is used. The sample table is designed to be compatible with specimen holders from the FEI - SEM microscopes and standard calcium fluoride 20 mm diameter substrates. For axial scanning the optical objective is placed on a piezo pifoc stage, as shown in figure 7.3 (left). The Z-stage has a travel range of $400 \mu\text{m}$, which combined with the XY-stage enables 3D microscopic imaging.

Figure 7.3: Photographs of the microscope mount for the optical objective (left) and of the sample table placed onto the XY-piezo stage (right). The sample table has 4 locations for specimen mounts, on which the FEI - SEM sample stubs can directly be placed. The piezo stage has a travel range of $50 \times 50 \mu\text{m}$, it is placed onto a manual XY stage for sample movements over a larger area.

7.2.1 Calibration procedure

The calibration routine for this Raman microscope is similar to the procedure presented in chapter 3.2.1 for the 647 nm excitation Raman microscope. First a wavelength calibration for the Raman spectrometer is performed. For this procedure the spectrum from a krypton gas lamp (figure 7.4A-B) and a toluene Raman spectrum (figure 7.4C) are measured. The krypton spectrum contains a set of high intensity bands in the spectral region above $1000\text{-}2800 \text{ cm}^{-1}$ and a set of low intensity bands for $0\text{-}1000 \text{ cm}^{-1}$, both spectral regions are presented in figures 7.4A and 7.4B respectively. In table 7.1 the peak positions are given for the krypton spectrum in (nm) and for the toluene Raman spectrum in (cm^{-1}).

Figure 7.4: Wavenumber calibration procedure of the spectrometer. (A) Spectrum of the krypton lamp. (B) Magnification of the first 450 pixels of the krypton spectrum. (C) Toluene Raman spectrum. The peaks used for curve fitting of the spectrometer wavelength axis are indicated with blue dots. (D) A 4th order polynomial curve fit through the krypton and toluene spectral peak positions vs. pixels of the CCD camera. (E) Difference values between the 4th order polynomial curve fit and the respective krypton and toluene peak positions. The peak with the maximum difference value is indicated with an asterisk in the toluene spectrum.

Krypton (nm)	Toluene (cm^{-1})
405.704	522.0
406.513	786.8
408.834	1003.8
409.873	1030.7
414.512	1211.7
427.397	1605.4
428.296	2920.4
431.958	
436.264	
437.612	
439.997	
445.392	
446.369	
450.235	

Table 7.1: Peak positions for the krypton lamp and toluene Raman spectrum. [2, 3]

For the spectrometer calibration a 4th order polynomial fit is performed through the peak positions over the 1600 CCD pixels. The obtained curve is shown in figure 7.4D, the positions of the used spectral lines are indicated in red for toluene and black for krypton. To verify the quality of the fitted polynomial curve the spectral position difference of the fit with respect to the krypton and toluene peak locations is shown in figure 7.4E. During the fitting procedure the exact emission wavelength of the excitation laser is also determined to be 404.90 nm. This value is obtained as the wavelength for which there is a minimum difference between the spectra peak positions and the curve fit, as measured by the fixed wavelength krypton peaks and the toluene peaks whose wavelength depends on the excitation laser.

The calibration of the relative detection efficiency of the Raman microscope is performed with a measurement of the spectrum from a calibrated white light source. The lamp calibration spectrum obtained from the supplier (Ocean Optics) has been verified with a measurement on the blue Raman setup using a second calibrated light source (Avantes Avalight-Hal). The measured signal from the white light source is compared with the known emission spectrum from the calibrated source, the ratio of the measured and calibration spectrum gives the microscopes response function for the full spectral range. For this first the readout background of the CCD camera is measured, the background spectrum is presented in figure 7.5A. The CCD is operated at full vertical binning and cooled to -70°. The white light spectrum and all subsequent measured Raman spectra will be corrected for this background. As white light source the deuterium lamp from Ocean Optics (D2000) is used, the measured spectrum is shown in figure 7.5B [black]. The deuterium lamp is chosen for its high power in the blue spectral range. It is observed that the deuterium lamp contains a large number of narrowband peaks, these peaks can have a negative influence on the response calibration curve as small spectral shifts in the spectrometer can result in large deviations between the measured and calibration spectrum. To prevent this a Whittaker smoother function is applied to the measured and calibration spectra,[4] the smoothed spectrum is shown in red. The smoothed measured lamp spectrum [black] and calibration spectrum [red] are shown in figure 7.5C. The microscope spectral response function is shown in figure 7.5D, a stable response of the microscope over the full spectral region is observed with the exception of the first 250 cm⁻¹ where the edgefilter is suppressing the signal.

Figure 7.5: (A) CCD background signal measured with 250 ms integration time and averaged over 500 spectra. (B) White light spectrum from a deuterium gas discharge lamp [black] the background spectrum in figure A is subtracted from this spectrum. The lamp is placed below the optical objective and is measured with 250 ms integration time and averaged over 1000 spectra. Multiple high intensity peaks are observed in the spectrum, in combination with even very small errors in the wavenumber calibration this could lead to undesirable peaks in the calibration response spectrum. Therefore a smoothing filter is applied to remove such peaks resulting in a smoothed spectrum [red]. (C) The measured smoothed spectrum [black] and calibration reference spectrum [red]. (D) Microscope spectral response function, obtained as the ratio of the measured white light spectrum over the reference white light spectrum.

7.2.2 Setup characterization

For characterization of the Raman microscope first the optical resolution is determined. The lateral resolution is measured by imaging of a sample of sub-diffraction size with a low scanning step size. As sub-diffraction size sample a sparse distribution of quantum dots (CdSeS/ZnS alloyed quantum dots, 6 nm diameter, 450 nm fluorescence emission) is made, quantum dots have been chosen in favor of polystyrene beads because polymers degrade under blue laser excitation. To prevent fluorescence bleaching a low incident power of 5 μW is used, further an integration time of 50 ms and scanning step size of 40 nm are used. The observed Q-dot cluster is analyzed with PCA and the first principal component scores map

is shown in figure 7.6A, it can be seen that there is a small difference in resolution over the X and Y axis. The microscope resolution is determined as the FWHM of the X and Y cross section through the intensity image. The X and Y cross sections are shown in figure 7.6 C and D respectively, showing a FWHM resolution of 0.27 μm and 0.36 μm . The measured emission spectrum from the Q-dots is presented in figure 7.6B. The axial cross section is determined with a Z-scan of a silicon wafer through the laser focus. Due to the high absorption coefficient of silicon for 405 nm light, $8.1 \cdot 10^4 \text{ cm}^{-1}$, [5] the Raman signal drops to 26% for material at a depth of 100 nm and to 7% for 200 nm, sufficient for an axial resolution measurement. The normalized Z-scan intensity is shown in figure 7.6E, the measured FWHM indicated with red dots is 0.84 μm . The Raman measurement on silicon is performed with 10 nm step size, 100 ms integration time, and 3.5 mW excitation power, the spectrum measured in the laser focus is presented in figure 7.6F.

Figure 7.6: Lateral and Axial resolution characterization. (A) PCA scores image of a quantum dot showing the resolution spot size. Some astigmatism is observed as an elongation of the resolution voxel over the y-direction. (B) Fluorescence emission spectrum from the Qdots cluster. (C) X cross section through the intensity map shown in A, the half maximum points are indicated (red) giving a FWHM resolution of 0.27 μm . (D) Y cross section with a FWHM of 0.36 μm . (E) The axial resolution is measured by a Z scan of a silicon substrate through the laser focus, due to the high light absorption of silicon it acts as a thin layer sample less than 100 nm thick. The figure shows the normalized intensity with indicated half maximum points, the FWHM is 0.84 μm . (F) The silicon Raman spectrum as measured in focus.

Additionally the spectral resolution of the microscope is characterized as the FWHM of the narrowband peaks observed from the Krypton gas lamp spectrum shown in figure 7.4A. The spectral bands of the gaslamp are much narrower than the

spectral resolution of the Raman spectrometer, it provides therefore a good source of narrowband lines for a measurement of the spectral resolution. By interpolating the measured spectrum of the krypton peaks the FWHM of each peak is accurately determined, the average FWHM of all observed krypton bands is 5.2 cm^{-1} .

7.2.3 Quantitative characterization

Calibration of the microscope detection efficiency is performed similarly to the calibration of the 647 nm excitation Raman microscope. The calibration is performed with a quantitative measurement of the toluene Raman signal in comparison with the known scattering coefficient. Additionally a measurement of the transmission and detection efficiencies of all components in the setup is performed. The two results are compared to determine the validity of the detection efficiency calibration.

A calculation of the signal detection efficiency starts at the sample and microscope objective. The fraction of light emitted in the objective collection angle $\frac{\Omega}{4\pi}$ is calculated using equation 3.16 to be 11% with an NA of 0.95 and a toluene refractive index of 1.52. The measurement of light transmission through the optical objective is performed using the 405 nm laser, measuring the optical intensity before and after the objective. The objective transmission curve provided by the supplier is used and scaled at the measured signal transmission at 405 nm, the result is presented in figure 7.7A. Other optical components in the detection path are maxmirrors obtained from Ocean Optics and an edgefilter from Semrock, the light transmission from these components is obtained through the suppliers.

The transmission efficiency of light through the spectrograph is polarization dependent. This is a result of polarization dependent light reflection of the incident light on the prism surfaces. For light incident on the prism surface a polarization in plane with the surface will result in higher reflection losses. Transmission through the spectrograph is measured using the 405 nm laser for both S and P polarized light, as adjusted with the half wave plate. Because the quantitative calibration will be performed using toluene as standard sample the transmission efficiency of toluene through the spectrometer is measured for both S and P polarization excitation light. With the measured ratio for these signals, and the measured ratio of transmission for S and P polarized laser light in the spectrometer, the depolarization ratio of the toluene 1003.8 cm^{-1} band is measured to be 0.0913. The transmission of Raman scattered light through the spectrometer for this depolarization ratio is calculated using measured S and P polarization transmission values. Next a white light calibration is performed using the deuterium lamp presented in figure 7.5B and scaling the white light calibration to the determined quantitative transmission value. The spectrograph calibration is presented in figure 7.7B, the transmission value presented here includes truncation of the beam at the pinhole entrance. Finally Raman spectra are detected on a Newton - Andor CCD camera over an array of 1600 pixels, the camera spectral detection efficiency is obtained from the supplier. The camera is operated at 0.8 electron per count at gain setting $4\times$, providing 1.25 counts per electron. Figure 7.8 (black curve) shows the resulting detection efficiency of the combined optical components.

Figure 7.7: Transmission spectrum from (A) the optical objective and (B) the homebuild spectrograph. The objective transmission spectrum is obtained from the supplier provided transmission spectrum which has been scaled to light transmission for scattered light as measured using the 405 nm excitation laser. The spectrograph relative transmission spectrum is measured using a calibrated lamp as white light source, this spectrum has been scaled to absolute transmission measured with the 405 nm laser at P-polarization.

The quantitative calibration is also performed using a Raman measurement from toluene and using its known Raman scattering cross section. The number of toluene molecules in the microscope voxel is determined using the resolution measurements presented in figure 7.6 and the density of toluene. The axial focal length is corrected for light refraction at the toluene surface, this is done by a multiplication with the refractive index. Resulting in $5.3 \cdot 10^8$ molecules within the FWHM of the lateral and axial resolution. The Raman scattering cross section of toluene is known from literature for 632.8 nm excitation for the 1003.8 cm^{-1} band. Calculating the cross section for 405 nm excitation is done using equation 3.7, resulting in $2.94 \cdot 10^{-29} \text{ cm}^2 \cdot \text{sr}^{-1}$. The detection efficiency for the microscope is calculated as the measured signal over the calculated number of emitted Raman photons for the toluene 1003.8 cm^{-1} band. This detection efficiency is used in combination with the white light calibration curve presented in figure 7.5C to obtain the quantitative calibration curve, as presented in figure 7.8 (red).

The two microscope detection efficiency curves are not in precise agreement, there is a 10% to 15% difference between the two curves. The efficiency measured using the toluene Raman scattering cross section provides a higher value compared to the value determined based on the individual microscope components. A possible explanation of these differences is the effect of aberrations in the Raman excitation and detection volume, the optical objective used in the experiments is mostly suitable for dry samples. This might result in a longer axial focal length which would lead to some changes in the Raman intensity.

Figure 7.8: Detection efficiency obtained as the multiplied transmission and detection curves of all the individual setup components [black], and measured using toluene as a quantitative compound [red]. The presented values represent the efficiency of detected counts divided over the total number of scattered Raman photons in the center of the Raman voxel.

7.3 Sample preparation

Red blood cells are prepared by a blood smear on a μ RimTM slide (BioTools) using 1 μ L of blood from a healthy donor, with the same method as discussed in chapter 4. The sample was fixed by submerging it three times in 100% methanol for one second. The methanol was replaced for 100% ethanol for ten minutes for dehydration. This last step was repeated. To dry the sample, the ethanol was replaced for 1:1 hexamethyldisilazane(HMDS)/ethanol for five minutes. Finally, the sample was left for five minutes in 100% HMDS and left to dry overnight.[6]

Preparation of SERS nanoparticles is performed similar to the method presented in chapter 5.3.1 for gold nanoparticles. Silver nanoparticles with a diameter of 50 nm prepared by citrate reduction method were obtained from NanoComposix at 0.02 $\text{mg}\cdot\text{ml}^{-1}$ concentration. Rhodamine 6g dye was obtained from Sigma Aldrich (batch: 252433-250MG) in solid form. SERS labeling of nanoparticles was prepared by dissolving R6g in ethanol, and adding the silver nanoparticle solution. The dye - and nanoparticle concentrations were measured with UV-Visible absorption spectroscopy before and after mixing of the compounds, the spectra are presented in figure 7.13A. The presented spectra are measured from R6g in ethanol solution [black], 50 nm diameter silver nanoparticles added to the R6g ethanol solution [red], and 50 nm diameter silver nanoparticles in only ethanol as reference [blue]. A comparison between the measured and simulated absorption spectra for the silver nanoparticles in ethanol is presented in figure 7.13B. It is observed that the measured nanoparticles show a broader and more red shifted extinction spectrum in comparison with simulations. The concentration of nanoparticles in solution is estimated using the calculated extinction spectrum to be $3.15 \cdot 10^9 \text{ nps}\cdot\text{ml}^{-1}$, the rhodamine 6G concentration is measured from figure A to be 5 μM . The R6G

covered nanoparticles were deposited and dried on a substrate directly before the SERS experiments. Raman silent and conductive $\mu\text{Rim}^{\text{TM}}$ slides (BioTools Inc.) were used as substrates. A comparison between the SERS spectrum and the spontaneous Raman spectrum from R6g is shown in figure 7.13C. The R6g SERS spectrum is spectrally very similar to the spontaneous Raman R6g spectrum indicating little chemical interaction between the silver nanoparticle and the dye molecules. It is observed that the R6g Raman spectrum measured with 405 nm excitation differs from the 785 nm excitation spectrum as presented in figure 5.6B. This is an effect of the excitation of the Raman spectrum at a different resonance condition, the measured spectrum therefore shows more correspondence with the literature for 488 nm excitation.[7]

Figure 7.9: (A) UVVis absorption spectroscopy for SERS sample preparation showing: R6g in ethanol solution [black], 50 nm diameter silver nanoparticles added to the R6g ethanol solution [red], and 50 nm diameter silver nanoparticles in only ethanol as reference [blue]. (B) The silver nanoparticles in ethanol reference spectrum (A blue) is presented [black] with the simulated far field scattering spectrum of silver nanoparticles in ethanol for a density of $3.15 \cdot 10^9 \text{ nps} \cdot \text{ml}^{-1}$. (C) Normalized spontaneous Raman spectrum [black] of R6g powder measured with 11 μW excitation power and 50 ms integration time, and Normalized SERS spectrum [red] of R6g adsorbed on silver nanoparticles measured with 2 μW excitation power and 50 ms integration time. The SERS spectrum is offset with 0.5 for clarity.

7.4 Results and discussion

The blue Raman microscope was tested in applications for resonance Raman microspectroscopy of red blood cells and for high resolution SERS analysis of silver nanoparticle clusters. These experiments demonstrate first the resonance Raman capabilities for the 405 nm Raman microscope, and second demonstrate analysis of silver SERS samples with high resolution microscopy. Correlative SEM images are made of the samples investigated with Raman. Because the Raman microscope is a standalone system, sample markers and finder grids were used to relocate the ROI in the SEM.

The theory for resonance Raman scattering has been discussed in chapter 3.1.2, equation 3.6, and figure 7.1. Resonance Raman scattering increases the scattering cross section potentially up to 10^3 times, depending on the details in excitation wavelength and the specific molecule. For Raman spectroscopy with blue light

excitation resonance Raman is of specific interest, this is because for many molecules the excitation frequency is much closer to the frequency of the first vibronic modes. Porphyrin-like molecules typically have absorption bands around 400 nm, which makes them suitable compounds to demonstrate resonance Raman imaging with this microscope. Red blood cells have been chosen to investigate the resonance Raman capabilities, because these contain a large amount of the porphyrin complex hemoglobin.

Silver nanostructures have been shown to provide a significantly higher Raman enhancement in comparison with gold.[8] Furthermore the plasmon resonance wavelength for silver is in the range of 400 nm, depending on the structure morphology and the surrounding medium refractive index.[9] This makes the blue excitation Raman microscope a useful system for analysis of these plasmonic structures. The excitation wavelength is close to the plasmonic resonance, and the high resolution is advantageous for the investigation of such small nanostructures.

7.4.1 Resonance Raman of red blood cells

The absorption spectrum of red blood cells shows a strong absorption band around 400 nm corresponding to the Soret band of hemoglobin.[10, 11] A schematic representation of the Heme b group of hemoglobin is presented in figure 7.10F. It consists of an iron atom inside a porphyrin ring, the iron is covalently bound to four nitrogen atoms of the porphyrin molecule. Oxygenated and deoxygenated hemoglobin can be distinguished. In oxygenated hemoglobin an oxygen molecule is bound to the Fe-atom and in deoxygenated hemoglobin no oxygen is bound. The two states can be distinguished in Raman spectroscopy due to a shift of a few wavenumbers of the 1357 cm^{-1} band (for deoxygenated hemoglobin) to 1375 cm^{-1} (for oxygenated hemoglobin).[12, 13] In the experiments always a mixture of oxygenated and deoxygenated hemoglobin is observed.

A resonance Raman experiment of dried red blood cells is presented in figure 7.10. A Raman image of $35\times 35\text{ }\mu\text{m}$ with 120×120 pixels is made with an integration time of 50 ms per pixel and a laser power of 0.257 mW. The high Raman scattering cross section allows the use of a short integration time with low laser power, which enables the collection of 14.400 spectra in 12 minutes. Figure 7.10 A and B show the first principal component scores image and loading plot, the first PC has captured 93% of the variance in the Raman data. To perform correlative electron microscopy the sample is moved to a separate JEOL SEM, the ROI investigated in Raman microscopy is located in the SEM with the use of carbon grids. The SEM image, presented in figure 7.10C, is recorded with SE detection, 2 kV acceleration energy, and $22000\times$ magnification. An overlay figure of the correlative SEM-Raman data is presented in 7.10D. Finally for Raman analysis the spectrum of a measured cell is presented in figure 7.10E, the spectrum is averaged over the location indicated in a white square in figure A. Prominent peaks in the Raman spectrum are observed and the corresponding wavenumber peak positions are indicated. In figure E both the 1357 cm^{-1} and 1375 cm^{-1} bands specific for deoxygenated and oxygenated hemoglobin are observed, demonstrating the presence of both states.

Figure 7.10: Raman experiment of multiple red blood cells measured with 50 ms integration time and 0.257 mW excitation power. The first principal component scores image (A) and loading (B) show the first 93% of the data variance. (C) SEM image measured on the same sample location, the scale bar denotes 10 μm . (D) Overlay image of the Raman scores image and the high resolution SEM image. (E) Raman spectrum measured on the location indicated in figure A, naturally this spectrum is very similar to the first PCA loading presented in B. Prominent Raman peaks are indicated with their spectral peak positions. (F) Schematic of the Heme b complex of hemoglobin showing the porphyrin ring with an iron atom at its center.

The resonance Raman spectra show a strong signal considering the short spectral integration time and the low laser power that have been used. Furthermore the fluorescence background contribution to the spectra is low. High quality Raman images can therefore be obtained at a fast imaging speed of 12 minutes for a 120×120 pixels scanning area, or 20 spectra per second. Resonance Raman spectroscopy of red blood cells is of general interest and their spectral response has been observed for many different excitation wavelengths,[14] showing strong spectral differences for different wavelengths. To our knowledge no resonance Raman results on RBCs with 405 nm laser excitation have been reported. However, multiple references for excitation with a 406.7 nm krypton ion laser are available.[11, 12, 15] The measured spectra compare well with these reported results. Red blood cells have also been investigated using the integrated SEM - Raman microscope for which 785 nm excitation light was employed, as presented in chapter 4.5.3 figure 4.14. A comparison between excitation with 405 nm and 785 nm light is presented in figure 7.11.

A comparison between the Raman spectra for 405 nm and 785 nm excitation of RBCs shows large spectral differences as a result of the different excitation conditions. Such findings have also been reported in other works.[14] A comparison with these studies has shown that the 405 nm excitation and also the 785 nm excitation Raman spectra are in agreement with the literature.[12, 14, 16] Although for NIR excitation a stronger fluorescence background contribution and broader Raman bands are observed (figure 7.11B). Using these spectra a comparison between the Raman scattering cross sections for the two excitation wavelengths can be made. Such a comparison will only be a rough estimate, first because the two spectra show different Raman bands and are therefore difficult to compare, and second because the quantification of the 405 nm microscope detection efficiency is still subject to some uncertainty. The Raman peak intensity for NIR excitation shows a higher signal (approximately 100 counts) in comparison with 405 nm Raman (approximately 30 counts). However the blue light excitation experiments are performed with a much shorter integration time T (405nm: 50 ms vs. 785nm: 2000 ms) and lower laser power P (405nm: 0.257 mW vs. 785nm: 5 mW). For this comparison the microscope detection efficiencies E have to be taken into consideration, these efficiencies are 2% for the blue light excitation microscope (figure 7.8) and 0.7% for the NIR excitation microscope (figure 5.12). The calculation of the measured signal intensity ratios I^{meas} corrected for spectral integration time, laser power, and microscope efficiency shows a ratio of approximately $80 \times$ higher Raman scattering for blue light excitation in comparison with NIR excitation.

$$\frac{I_{405}^{meas}}{I_{785}^{meas}} \cdot \frac{T_{785}}{T_{405}} \cdot \frac{P_{785}}{P_{405}} \cdot \frac{E_{785}}{E_{405}} \approx 80$$

Figure 7.11: Comparison of Raman spectra measured on red blood cells (A) with 405 nm excitation wavelength, and (B) with 785 nm excitation. The presented spectra are obtained from the figures 7.10 and 4.14. The peak position of the most prominent Raman bands are indicated, demonstrating the differences in Raman response for different excitation conditions.

7.4.2 Silver SERS

Silver has several different plasmonic properties in comparison with gold. First the plasmonic resonance wavelength is around 400 nm, depending on the nanostructure morphology and the medium, which is significantly blue shifted in comparison with gold. Second, it has been shown that silver provides a significantly higher plasmonic enhancement than gold or other metallic compounds.[8] Since silver plasmonic nanostructures are resonant for blue light, they will show a significant SERS enhancement factor for Raman experiments with the blue Raman system. A comparison between silver and gold SERS enhancement is provided based on near field simulations. Next correlative Raman and SEM analysis is performed on a sample containing many dried silver nanoparticles with R6g adsorbed reporter molecules. A qualitative analysis of the signal enhancement from different clusters is performed, and compared with near field simulations.

The SERS theory has been described in chapter 3.3, specifically the Clausius-Mossotti equation 3.17 is presented.[17] The equation describes the relation between the relative permittivity and polarizability of a material. It is therefore useful to compare the dielectric permittivity of gold and silver. Figure 7.12 shows both the real (top) and imaginary (middle) part of the relative permittivity from gold and silver, the values are obtained from Johnson and Christy.[18] The g factor as presented in equation 3.17, and repeated here: $g = \frac{\epsilon(\lambda)-1}{\epsilon(\lambda)+2}$ is shown in the figure (bottom). Clear resonances are observed at 370 nm for silver and 550 nm for gold.

Figure 7.12: (Top) Real part of the relative permittivity for silver and gold. (Middle) Imaginary part of the relative permittivity. (Bottom) Clausius - Mossotti g factor, relating the material polarizability to the relative permittivity. For gold and silver resonances are observed at respectively 550 nm and 370 nm.

A comparison between silver and gold nanospheres for SERS experiments is made using near field simulations, the results are presented in figure 7.13. The simulations are performed using 405 nm and 785 nm as excitation wavelengths for silver and gold nanoparticles of 50 nm diameter. The local field enhancement for the different structures is presented in figure 7.13, (A) shows Ag with 405 nm excitation, (B) Ag with 785 nm, (C) Au with 405 nm, and (D) Au with 785 nm, all figures are presented with the same intensity colorscale. The simulations are performed with the excitation polarization oriented along the x-direction and the incident light coming from the positive z-direction. The black ring around the spheres is a result of the discretized mesh structure where the E-field cannot be calculated on mesh facets that intersect with the particle boundary.

Figure 7.13: A comparison of the simulated field enhancement for 50 nm diameter nanospheres, of the compositions gold and silver, and with excitation wavelength 405 nm and 785 nm. Near field simulation for silver nanospheres excited with (A) 405 nm light, and (B) 785 nm light show a surface averaged EF of 410x and 24x respectively. Gold nanospheres excited with (C) 405 nm light, and (D) 785 nm light show a surface averaged EF of 27x and 26x respectively. (E and F) Far field scattering [black] and extinction [red] spectra for nanospheres in air [solid lines] and in ethanol [dashed lines], for silver and gold respectively.

It is clear that the field enhancement is significantly higher for silver nanostructures excited near resonance with 405 nm light. The surface averaged SERS enhancement factors are calculated in the fourth power approximation, using equation 5.1: $\langle \text{EF}^{\text{Ag } 405} \rangle = 410\times$, $\langle \text{EF}^{\text{Ag } 785} \rangle = 24\times$, $\langle \text{EF}^{\text{Au } 405} \rangle = 27\times$, $\langle \text{EF}^{\text{Au } 785} \rangle = 26\times$. Furthermore the far field scattering and extinction spectra are presented in figures (E) for a silver nanosphere and (F) for a gold nanosphere. The far field simulations have been performed for particles in vacuum and in ethanol (dashed line). It is observed that the light interaction at the plasmon resonance wavelength is stronger for silver in comparison with gold, as can be seen from the higher scattering cross section.

Further simulations are performed to the hot spot effect in dimer nanostructures. Simulations are performed for dimers consisting of 50 nm diameter silver spheres with varying gapsizes. The local field enhancement for a structure with an example gap of 5 nm is presented in figure 7.14A. The field enhancement in the hot spot is approximately $1.3 \cdot 10^8\times$ and a surface averaged enhancement of $1.4 \cdot 10^6\times$ is calculated. For varying gapsizes the surface averaged enhancement is presented in figure 7.14B, a logarithmic plot is used to show the high variation in enhancements for the different gapsizes. In comparison the simulations for gold nanostructures presented in figure 5.2B show a maximum surface averaged enhancement of 10^5 to 10^6 for similar structures for the excitation wavelengths 532 nm, 647 nm, and 785 nm. A local maximum for a gap of roughly 4 nm is caused by the red shift of the plasmon resonances for structures with a smaller gap. Far field scattering (figure C) and extinction (figure D) spectra show that the red shifted plasmon resonance frequency coincides with the excitation field wavelength for a structure with a gap of approximately 5 nm. It is noted that higher order modes are observed in the scattering and extinction spectra for dimers with a gap of 5 nm or smaller. These higher order modes are observed as small secondary peaks at 350 nm to 370 nm spectral region. This is in correspondence with experiments and simulations reported in the literature.[19, 20]

It is noted that the simulations are not able to properly describe the response for interparticle gaps smaller than 2 nm. The reason for this is that quantum mechanical effects, such as electron tunneling and nonlocal screening, are not included in these simulations. Nonlocal screening refers to electron - electron interactions that reduce a sharp charge localization at interfaces, it is therefore mostly relevant for structures with narrow gaps (smaller than 2 nm) with a strong charge localization at the particle boundary.[20, 21] For gaps smaller than 1 nm electron tunneling starts to produce a charge transfer over the dimer junction, this results in a decrease in the surface charge and a smaller electric field in the dimer hot spot.[20, 21] The calculated enhancement factors with a gap larger than 2 nm are in agreement with values presented in the literature. The enhancement for silver nanosphere dimers have reported E^4 values in the hot spot of up to $10^9 - 10^8$ for structures with a 3 to 5 nm gap.[22]

Figure 7.14: (A) Simulated near field enhancement for a dimer nanostructure consisting of two silver nps of 50 nm diameter separated by a 5 nm gap, and excited with 405 nm light. The calculated hot spot SERS enhancement is $1.3 \cdot 10^8 \times$, and the surface averaged EF is $1.4 \cdot 10^6 \times$. (B) Simulated surface averaged enhancement factor for dimer nanostructures with a gap size varying from 1.5 to 20 nm, in a logarithmic representation. (C) Far field scattering spectra simulated for dimer structures with a 2, 5, 10, 15, and 20 nm gap size, and (D) the corresponding extinction spectra.

Correlative SEM and Raman analysis of clusters of silver nanoparticles is presented in figure 7.15. First Raman analysis is performed on the stand alone 405 nm Raman system, next the sample is transferred to the electron microscope (FEI dualbeam FIB-SEM system) where the ROI is located using markers. Raman analysis is performed using a low excitation power (2.1 μ W) and short integration time per pixel (50 ms), a sample area of $20 \times 20 \mu$ m is measured with 80 nm scanning step size ($250 \times 250 = 62500$ pixels). SEM imaging is performed using SE detection with the TLD, an electron acceleration voltage of 15 kV, probe current of 36 pA, and pixel dwell time of 30 μ s are used. Analysis of the Raman measurement is performed after pretreatment of the data. As pretreatment singular value decomposition and reconstruction with the first 20 S-values is used to suppress noise in the dataset, in the next step fluorescence background is filtered from the dataset.

Figure 7.15: (A) Overlay image of Raman LLS fit scores with SEM. Raman imaging is performed with 2.1 μW excitation power and 50 ms spectrum integration time. SEM imaging is performed with SE detection and 15 kV acceleration energy, the scale bar denotes 10 μm . The excitation polarization orientation is along the vertical axis, similar to experiments performed with the integrated 785 nm Raman microscope. (B) higher magnification image of the region indicated in A, the scale bar denotes 1 μm . (C) Spectrum used for LLS fitting of the Raman data.

The SERS signal intensity per pixel is calculated using a LLS fit of a normalized R6g Raman spectrum to the measured spectra. Fluorescence background is subtracted from the R6g spectrum, and a spectral region of 1100 to 1800 cm^{-1} is selected for fitting of the spectrum to the SERS measured data. The final LLS fitting spectrum is presented in figure 7.15C. The LLS coefficients are presented in an overlay figure onto the SEM image, as presented in figure 7.15A, and in figure 7.15B at a higher magnification of the region selected in A. The Raman images are presented with the same colorscale, since the fitting spectrum is normalized at 1 the presented coefficients directly relate to the peak intensity of the R6g SERS spectrum.

The investigated area shows a large variety of cluster sizes, which enables an analysis on the SERS intensity from different clusters. The high Raman resolution greatly improves the collection of the SERS signal from specific clusters, specifically in an environment of many more nanoparticles at a sub micrometer distance. This has been demonstrated in figure 7.15 A and B, where the signal from separate clusters at a distance of 500 nm is distinguished. It has however been observed that the SEM secondary electron scattering shows a lower contrast for silver in comparison with gold nanoparticles. Added to this is that the size of the silver nanoparticles is more polydisperse and the nps have a more faceted morphology. As a result the gap size is not accurately resolved in SEM analysis.[23] Therefore the SERS signal strength is investigated for different cluster sizes without a complete analysis of the interparticle gaps.

The SERS signal for monomers is naturally much weaker in comparison with clusters. However by averaging over 3x3 pixels in the Raman image and the use of SVD for noise reduction on the dataset has enabled the detection of the monomer signal. Figure 7.16 shows the SERS response for three monomers, selected from the measurement in figure 7.15. The SEM images of the nanoparticles are shown in figure 7.16A, and the corresponding SERS signals in figure 7.16B. Although the recorded SERS signal has a peak intensity of only 1 count, still a discernable signal is clearly observed.

Figure 7.16: (A) Secondary electron SEM image of multiple 50 nm silver nanoparticle monomers, recorded at 15 kV acceleration energy at 100.000x magnification, the scale bar denotes 400 μm . (B) R6g SERS spectra of the silver monomers indicated in figure A, measured with 50 ms integration time and 2.1 μW excitation power. Spectra are averaged over 3×3 pixels in the Raman image and location 2 and 3 are offset with 2 and 4 counts respectively.

The SERS signal from multiple clusters is observed in the measurement, several examples are presented in detail in figure 7.17. High resolution SEM images of selected structures are presented in figure 7.17 A, D, G, and J. The images show the clusters indicated in a circle of 300 nm diameter, this circle represents approximately the FWHM resolution of the Raman microscope. This gives an indication of how small nanoparticle clusters are observed inside a region with multiple other particles during Raman imaging. Naturally some background will still be observed from particles just outside the circle boundary, especially since the presented Raman spectra are selected as an average of 3×3 pixels with 80 nm pixel step size. The SERS spectra of the reporter molecules on the silver nanoparticles are shown in figure 7.17 B, E, H, and K. These spectra are obtained as an average of 3×3 pixels, selected from the dataset after the SVD noise suppression step, and before filtering of the R6g fluorescence contribution to the signal. Furthermore a background is subtracted from the spectra, this background is obtained from spectra measured on an empty region on the substrate. The spectra show clearly a stronger signal for larger clusters, especially in comparison with the signal from monomers as presented in figure 7.16. However a precise correlation of the signal strength in comparison with near field simulations is at this moment not provided, because the nanoscale morphology is not measured with sufficient accuracy. An approximate solution to the field enhancement is obtained with near field simulations based on the corresponding structure morphologies. Figure 7.17 C, F, I, and L present the local field enhancements for observed nanostructures, the simulations provide some insight in the hot spot locations and their relative intensity.

Figure 7.17: (A, D, G, J) Secondary electron SEM images of 50 nm silver nanoparticle clusters, recorded at 15 kV acceleration energy and 36 pA probe current. A circle of 300 nm diameter, that represents the Raman FWHM resolution, around the structures is shown. Naturally some background will still be observed from particles just outside the circle boundary. (B, E, H, K) R6g SERS spectra corresponding to the nanostructures presented in the corresponding SEM images. The measurement is performed with 50 ms integration time and 2.1 μW excitation power, the spectra are averaged over 3×3 pixels in the Raman image. (C, F, I, L) Near field simulations showing the local field enhancement around the silver nanoparticle clusters, visualizing the locations of hot spots and their relative intensity. The simulations show only an approximate comparison with the corresponding SEM images, because the interparticle gaps could not be accurately observed and the particles are more polydisperse in comparison with gold.

7.4.3 Discussion

The use of a short excitation wavelength for Raman microscopy has, as discussed, important advantages considering resolution and applications for resonance Raman spectroscopy. However it is important to consider the increased risk of sample damage when using blue excitation light. Experiments for red blood cells and SERS are performed with a low laser power, which limits the risk of thermal damage to the sample. Potentially there is still a higher light absorption that can result in photo-degradation or in the extreme case in thermal degradation. As an example polymer samples have shown a strong degradation due to absorption of the blue light. Additionally in figure 7.10 the SEM image shows a hole in the middle of the center red blood cell, this is caused by a long term illumination with the Raman laser. After the initial Raman measurement was finished the stage moves to its center position which in this case resulted in the laser being centered on the cell for a duration of several minutes. It is noted that the experiments are performed on dry samples, this is required for correlative SEM images. For certain samples, especially resonance Raman on cells, it is expected to be advantageous to use an immersion objective for investigation of the specimen in water.

A comparison between silver and gold nanoparticles for SERS reveals several important aspects that need to be considered. It is generally known that the fabrication of silver nps results in more disperse particles, for both shape and size, in comparison with gold.[23] This limits a direct comparison between SERS experiments and near field simulations based on SEM observed morphologies. SEM observations have furthermore shown a lower contrast for silver in comparison with gold. Although the particles could be easily observed the interparticle gapsize could not be resolved with the nanoscale resolution required for accurate near field simulations. For this reason the choice is made to present only approximate near field simulations with the corresponding SEM-SERS data, and to focus first on the analysis of cluster sizes instead of the nanoscale morphology. The same reporter molecule R6g is applied as was used for SERS experiments on gold nanoparticles, as presented in chapter 5. While this choice helps in the comparison between these different experiments it is noted that R6g is not an ideal reporter molecule when using a 405 nm excitation wavelength. The R6g absorption resonance is around 500 - 550 nm, which means that for 405 nm the excitation is performed in between vibronic resonance modes.

The comparison between silver nanoparticle clusters of varying sizes shows naturally a stronger SERS signal for larger clusters, especially in comparison with the signal from monomers. However this signal difference is lower than initially expected based on field enhancement simulations as presented in figure 7.14. This signal difference is difficult to quantify since the near field simulations are only used to approximate the local field enhancement, but overall a stronger variation in signal intensity is expected. Potential causes to this are additional damping effects to the field enhancement that are now not considered. Considering electromagnetic damping effects, electron screening is a potential factor although it is expected to only have an effect for gaps smaller than 2 nm. Another potential effect is the oxidation of silver in air, which would effectively create a thin isolating shell around

the metallic nps. SERS experiments have been performed directly after drying of the nanoparticles but oxidation could still affect the sample.[24] Further research to this is recommended for the investigation of these effects.

Further research to plasmonic structures can provide more insight into the differences between silver and gold nps. For example SERS experiments to gold nanoparticles with 405 nm excitation, or conversely to silver nps with 785 nm excitation, would provide an interesting comparison between the different samples. The use of correlative electron microscopy is expected to further aid in this comparison by facilitating the localization of specific or similar silver or gold clusters, and SEM naturally provides the visualization of morphologies required for correlative near field simulations.

Finally the high resolution of the blue Raman microscope might be further improved by imaging with smaller scanning step sizes in combination with an image deconvolution algorithm. This is a known image processing technique that is more commonly employed with fluorescence imaging.[25] However in combination with the chemical specificity of Raman, and for the many sub-diffraction SERS structures, it might improve the localization of specific particles also in the proximity of other nearby sample features.

7.5 Conclusion

In this chapter the development and utilization of a high resolution Raman microscope employing a 405 nm excitation source is presented. The microscope is developed to help bridge the resolution gap between electron and Raman microscopy, and so to improve correlative SEM - Raman imaging. The system development, Raman calibration procedure, and setup characterization are presented. A strong lateral and axial resolution of 0.3 - 0.35 μm lateral and 0.84 μm axial FWHM is obtained using a high NA objective (0.95) suitable for samples in dry conditions. The system is furthermore suitable for resonance Raman spectroscopy since the blue excitation wavelength is close to vibronic resonances for multiple molecules, specifically for porphyrin complexes.

Experiments for resonance Raman spectroscopy on red blood cells, and for SERS analysis of silver nanoparticles, are presented. Resonance Raman spectra measured for red blood cells show a strong signal that is in good agreement with the literature. The strong signal enables fast imaging with only 50 ms integration time per spectrum and a low excitation power of 0.257 mW. Results are compared with Raman spectra measured with 785 nm excitation (reported in chapter 4) which results in a very different resonance excitation condition. Correlative SEM images are obtained by translating the sample to a separate SEM (JEOL system). The microscope is furthermore utilized for SERS analysis of silver nanoparticles. A comparison of the plasmonic enhancement for gold and silver with 405 nm and 785 nm excitation is performed based on near field simulations. The strong enhancement from the silver nanostructures enables the use of a very low excitation power (2.1 μW) and short integration time (50 ms), with this short integration time an image of 250×250 (62500) pixels is recorded in less than an hour. The high Raman

resolution enables identification of SERS signals from clusters and monomers also in regions on the sample with many other nearby clusters. Correlative SEM images are obtained by translating the sample to a separate SEM (FEI system). Finally a qualitative comparison of the SERS signal with near field simulations based on the SEM observed morphologies is presented.

We conclude that the strong Raman resolution greatly improves the correlation of Raman and SEM images. This is of specific interest for SERS nanostructures, due to their small size. It would therefore be very useful to integrate the Raman microscope with SEM, thus further utilizing the high resolution for correlative SEM - Raman microscopy.

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CONCLUSIONS AND OUTLOOK

In this thesis we have presented the integration of a confocal Raman microscope in a dualbeam FIB-SEM. The combination of Raman and electron microscopy in a single system enables correlative chemical specific and nanoscale resolution analysis of diverse samples. Applications of the integrated system have been demonstrated with proof of principle experiments on diverse samples such as: organic polymers and cells, micron-sized crystals and graphene flakes, and FIB treated silicon and organic materials. Using an integrated system in favor of two separate microscopes results in a workflow improvement. Upon translating the sample between the electron and optical microscope the ROI is preserved thus enabling faster analysis, and since the sample does not leave the vacuum chamber there is no risk of contamination during translation. Possibly more important is that switching back and forth between microscopes is much easier. This enables to perform sequential Raman - SEM - Raman - SEM imaging, which can be desired when new structures are observed during later stages in the experiment.

The combination of nanoscale imaging and chemical specific analysis is especially important for the characterization of plasmonic nanostructures. Nanoscale structure morphologies are correlated to their effect on the Raman signal. In this thesis we have shown a thorough analysis for dimer structures to the effect of the gapsize and dimer orientation on the SERS activity. Simulations to the near field enhancement from the structure are compared with a quantitative experimental analysis of the SERS enhancement. An overall agreement between simulations and experiments is observed, however large variations in the experimental SERS response remain. These variations are attributed to variations in the nanoscale morphologies for the different dimer structures that are not included in the simulations.

During Raman measurements on polymer microbeads an observation is made of surprisingly intense whispering gallery mode resonances. WGM resonances are observed in the Raman spectra after the microbeads are imaged by SEM. It is noted that optical WGM resonators have diverse applications, like plasmonic nanostructures a common application is as sensor for biomolecules. An investi-

gation of the observed phenomenon shows that the WGM signal originates from photoluminescence that is excited at the bead surface from amorphous carbon material that is deposited with the electron beam (EBID). Characterization of the signal reveals the mode propagation pattern and the resonator Q-factor, the results are in agreement with the existing knowledge of WGM resonators. With knowledge on the WGM mode propagation and on the origin of the signal, it becomes possible to use a controlled SEM or FIB treatment of the microbeads to excite WGM resonances on controlled locations.

With the previously reported results the integrated Raman - FIB - SEM has been applied for correlative analysis of structures with sizes varying from about 100 nm to about 50 μm . The analysis of nanoscale structures, e.g. plasmonic nanoparticles, has shown that the resolution of the Raman microscope is in fact lower than would be desired for an accurate correlation with SEM imaging. It is observed that occasionally the signal from small structures is obscured by comparatively much brighter structures that are located in their vicinity, at distances of approximately 1 μm . These different structures are naturally well distinguished with the SEM. To improve the correlation of SEM and Raman images a separate high resolution Raman microscope is developed. The stand-alone Raman microscope uses a short excitation wavelength of 405 nm and a high NA objective of 0.95 to achieve an optimum resolution. Applications of the system are demonstrated for experiments on erythrocytes, and for SERS experiments on silver plasmonic nanoparticles. For these samples the blue excitation light offers significant advantages, namely through a resonance Raman excitation with the erythrocytes hemoglobin Soret band. Furthermore the excitation wavelength is close to the plasmonic resonance wavelength of the silver nanoparticles and therefore results in a strong SERS enhancement factor. It is noted that only a qualitative analysis is performed, as such no exact signal enhancements are calculated. All Raman measurements are examined in combination with correlative SEM images measured on the separate SEM systems, and with near field simulations to the observed structures

At this point a discussion on the outlook and current developments for integrated correlative Raman and electron microscopy will be provided. In our opinion the development and later the integration of the high resolution Raman microscope, reported in Chapter 7, fits with a direction that the iCLEM field is taking. In this direction new applications are achieved through the use of more sophisticated microscopy techniques, preferably integrated with the electron microscope. This trend is already observed for integrated fluorescence and electron microscopes, where super resolution optical microscopes are integrated with TEM systems. The equivalent for Raman would be a correlative TERS system, in practice however such a system is more complex and might not be easily integrated in typical electron microscopes. Another combination of a new integrated microscopy technique is presented in this thesis, namely correlative FIB and Raman. Several applications of the FIB - Raman combination are described in chapter 4, however other interesting applications are likely feasible. For example the fabrication with FIB of specific structures in nanocarbon materials, e.g. graphene and carbon nanotubes, can

be directly analyzed with correlative Raman microscopy. Additionally 3D SEM-Raman imaging, in which the FIB is used to reveal deeper sample layers, is possible. Examples of samples for which this would be an interesting analysis method are photonic crystals, waveguide structures, and pharmaceutical tablets. Sequential FIB - Raman operation will likely require a long operation time potentially up to several days when many large Raman image stacks are obtained. The development of new software tools can aid in a semi-automated experimental characterization. Such a system would enable an automated experiment to run for potentially several days. Similarly software that enables automated Raman imaging on selected locations in a large field of view SEM image will significantly improve the data collection by having the system perform continuous automated acquisition.

With the application of integrated Raman - electron microscopy becoming more common an increase in experience on the effect of combined sample interactions is expected. The combination of high energy electron irradiation with laser beam irradiation can lead to chemical changes in the investigated sample. Such chemical alterations could have a dramatic effect on the Raman spectral analysis, for example through breaking of chemical bonds and amorphization of the material. These effects are occasionally observed during correlative analysis, however a more thorough understanding of the interactions that take place and on how to prevent them would greatly benefit the iCLEM field. Naturally the ion beam interactions result in stronger specimen alterations. Due to a strong fluorescence induced background this has proven to complicate correlative Raman analysis after FIB treatment on organic polymers and cells. Correlative Raman analysis might remain possible using different FIB treatment techniques or using certain sample treatment procedures. It is recommended that the effect of a lower ion energy on the fluorescence background is investigated. Another suggestion is that the injection of some gas to the FIB treated sample might neutralize free radicals created during the ion irradiation.

In chapter 4 it is observed that for ultrastructural analysis of cells a new sample preparation protocol might be desired. While correlative SEM - Raman analysis works well for the characterization of different cell types, the use of FIB - SEM for 3D ultrastructural analysis reveals only the cell cytoskeleton. There is already a lot of information on ultrastructural analysis of such samples using 3D block face scanning FIB-SEM, and TEM. However the sample preparation protocols for 3D block face imaging are expected to be incompatible with Raman spectroscopy. To preserve the Raman signal a dedicated sample treatment will be necessary. A suggestion is the use of cryo-prepared specimens. Cryo sample preparation has the advantage that it does not alter the chemical characteristics of the specimen, while it preserves the sample morphology for SEM imaging. For this reason it is a technique with proven results for correlative fluorescence and electron microscopy. However a few challenges have to be considered. First a cryo sample stage is required on which sample treatment is performed and that preserves the specimen temperature in the vacuum chamber. Second there is the potential of ice layer formation during sample preparation, this might obscure both the Raman and electron scattering signal. It is interesting to note that during Raman analysis, the material state of the vitreous ice can be obtained from the Raman spectra. Initial experiments on cryo Raman analysis of organic samples and monitoring

of the vitreous ice state have been performed. Results are not presented in this thesis, but show the possibility for cryogenic sample preparation in combination with Raman characterization.

With the rapid progression taking place in the field of iCLEM we can look forward to numerous new developments and applications. While some challenges remain in preventing sample damage and using suitable preparation protocols, it is expected that novel methods and protocols will be developed. We see initial applications for the integrated Raman electron microscope mainly in the materials science field. In this thesis initial experimental analysis is performed for nanocarbon materials, it is expected that in the future the system can function as a great tool for the analysis of graphene and carbon nanotube structures and products. As a product analysis tool the integrated system might find opportunities for the analysis of for example food products or pharmaceuticals. Raman and SEM are frequently used as separate techniques for the analysis of such specimens, and the integrated system will add a reliable method to characterize with chemical specificity specific SEM observed sample features. Of specific interest is correlative analysis of plasmonic structures and monitoring of catalytic processes. Plasmonics has been thoroughly investigated in this thesis, however numerous different plasmonic structures and materials exist and new insights for these specimens could be obtained using correlative SEM - SERS analysis. Catalysis is another field in which nanoparticle structures are of great importance and that benefits from chemical specific monitoring of processes and products. As such monitoring of processes in an environmental SEM - Raman system, and the analysis of used nanoparticles promises an interesting application. It is expected that a more widespread use of integrated SEM - Raman systems will further improve on the development of the available commercial microscopes, thereby expanding the field of iCLEM.

SUMMARY

In this thesis we have described the integration of a Raman microscope in a focused ion beam - scanning electron microscope (FIB-SEM). Raman micro-spectroscopy enables chemical specific characterization, while electron microscopy enables high resolution imaging. The Raman - SEM combination thus enables the correlation of chemical specific micro-spectroscopy with nanometer resolution morphological imaging. FIB materials ablation adds to this a micromachining capability. The Raman - FIB combination enables spectroscopic analysis of FIB treated samples. Conversely FIB ablation of surface material can be used to reveal deeper layers in a specimen for subsequent Raman analysis. The integration into a single system facilitates fast sequential analysis and treatment on any single location on the sample.

Capabilities of the integrated microscope are demonstrated on a wide variety of sample materials. Chemical specific identification of micron sized materials, combined with SEM analysis of surface morphological features is performed for graphene flakes and crystal particles. Raman analysis to the effects of FIB treatment for different materials shows large differences depending on the investigated material. Raman characterization with FIB milling on silicon substrates shows changes in the crystal lattice bond strength and amorphization of the material surface layer. After FIB treatment of carbon based materials, namely polymers and cells, a strong light absorption is observed in subsequent Raman analysis of the amorphized materials. This absorption results in a strong fluorescence emission that obscures the Raman signal and can lead to thermal damage. In another application analysis of cells is demonstrated for the first time with integrated correlative Raman - SEM. This has shown the capability of cell type identification based on Raman micro-spectroscopy and combined SEM characterization of the surface morphology for different cell types.

Studies are performed to surface enhanced Raman spectroscopy (SERS) from plasmonic nanoparticles and to optical whispering gallery mode (WGM) resonances observed on polymer beads. Both have applications as sensors to for example targeted biomolecules. Sensitivity is obtained for plasmonic nanostructures through a strong enhancement of the Raman signal from molecules in their vicinity, and for WGM resonators through spectral changes in the response as a result of molecules adhering to the bead surface. A quantitative analysis is performed to the Raman

signal enhancement for different plasmonic nanostructure morphologies. In so called hot spots a strong ($10^8\times$) Raman enhancement is achieved, the enhancement is strongly dependent on the material nanoscale morphology. The critical factor in this study is the SEM resolution with which nanoscale variations in morphology are observed. The study to the optical response from WGM resonators started with a sudden observation of optical resonances during Raman analysis of 10 1 μm polystyrene beads after SEM irradiation of these beads. Characterization has revealed that the beads function as WGM resonators, the source of the propagating light originates from photoluminescent material deposited during SEM irradiation of the bead surface. The experimental observation is in agreement with the literature on WGM resonators. With this knowledge a controlled FIB and SEM treatment is demonstrated, it enables the excitation and detection of optical resonances on specifically chosen locations on the bead.

This thesis is concluded with a chapter that reports on the development of a stand-alone high resolution Raman microscope. This system uses a short wavelength (405 nm) excitation laser and high NA (0.95) objective to achieve a strong optical resolution. While this system is not integrated with SEM, correlative imaging is performed by translating the sample between the independent microscopes and relocating the ROI based on sample features and potential markers. Applications of the microscope are demonstrated for resonance Raman measurements on erythrocytes and for SERS experiments on silver nanoparticles. Especially for studies on small structures such as plasmonic nanoparticles the high Raman resolution is a strong advantage. A more detailed microscopic analysis is obtained and the correlation with SEM images is improved. The developed high resolution system paves the way for integration with SEM and bridging the resolution gap between optical and electron microscopy.

SAMENVATTING

In dit proefschrift is de integratie beschreven van een optische Raman microscoop in een focused ion beam (FIB) - rasterelektronenmicroscoop (SEM: scanning electron microscope). De Raman microscoop/spectroscoop maakt het mogelijk om een chemisch specifieke analyse uit te voeren voor materiaal karakterisatie, terwijl de rasterelektronenmicroscoop de mogelijkheid geeft om materiaal te observeren met hogere (nanometer) resolutie. Met de combinatie van deze technieken is een systeem gerealiseerd waarmee chemisch specifieke analyse snel gecorreleerd kan worden aan nanometer resolutie observaties van dezelfde onderzochte structuren. De FIB wordt onder andere gebruikt voor ablatie van oppervlakte materiaal waarmee het mogelijk is op microschaal structuren te fabriceren. In combinatie met Raman microscopie worden materialen geanalyseerd nadat ze met de FIB zijn behandeld, waardoor bijvoorbeeld defecten in kristalstructuren worden herkend. Deze integratie van technieken maakt een snelle opeenvolgende materiaal analyse en behandeling mogelijk op elk gekozen locatie.

Applicaties van de geïntegreerde microscoop zijn aangetoond voor diverse materialen. Als voorbeeld is voor lagen grafen en micron grote kristallen materiaal identificatie met Raman spectroscopie gecombineerd met SEM analyse van de structuur morfologie. Het toepassen van Raman analyse voor karakterisatie na FIB behandeling van materiaal laat zien dat er sterke verschillen zijn afhankelijk van de onderzochte materialen. Bij FIB ablatie op silicium wafers zijn veranderingen waargenomen van bindingssterktes in het kristalrooster ook heeft de Raman analyse laten zien dat de FIB behandeling een laag amorf silicium achterlaat. Deze observaties tonen een degradatie van de kristalstructuur aan. Daarentegen is Raman karakterisatie na een FIB behandeling van organische componenten, zoals polymeren en biologische cellen, moeilijker. Bij deze materialen is een sterkere degradatie van de moleculaire structuur waargenomen, dit resulteert experimenteel in een absorptie van het Raman laser licht. Deze licht absorptie resulteert in een sterke fluorescentie achtergrond emissie en in een groter risico op thermische schade aan het materiaal. Ook is voor het eerst een gecombineerde Raman - SEM analyse uitgevoerd op biologische cellen. De identificatie van cel types met Raman spectroscopie en gecombineerde SEM analyse van de oppervlakte morfologie is toegepast voor rode en witte bloedcellen.

Met behulp van de geïntegreerde microscoop zijn voor dit proefschrift twee studies uitgevoerd. De eerste is een studie voor oppervlak versterkte Raman spectroscopie (SERS: surface enhanced Raman spectroscopy), en de tweede is een studie naar optische resonanties (WGM: whispering gallery modes) die geobserveerd zijn in polymeer microbollen. Zowel de SERS structuren als de sferische resonatoren hebben applicaties als sensors voor bijvoorbeeld specifieke biologische moleculen. Met plasmonische SERS nanosensoren is de detectie van moleculen mogelijk door middel van de enorme versterking van Raman verstrooiing voor moleculen in de nabijheid van de nanostructuur. De plasmonische signaalversterking is sterk afhankelijk van de vorm van de nanostructuur, voornamelijk in zogenaamde hot spots kan de Raman versterking oplopen tot $10^8 \times$. Door de combinatie van SEM en Raman karakterisatie is dit effect onderzocht, de nanostructuur wordt zichtbaar met de SEM en de signaalsterkte wordt gekarakteriseerd met de Raman microscoop. Bij WGM resonatoren ontstaan veranderingen in het resonantie spectrum wanneer moleculen zich hechten aan het oppervlak van de resonator. Het onderzoek naar WGM resonatoren is gestart na een verrassende observatie van optische resonanties tijdens Raman analyse van polystyreen microbollen. Karakterisatie van dit fenomeen laat zien dat de resonanties ontstaan na elektron irradiatie van het microbol oppervlak met de SEM. Deze irradiatie zorgt voor een depositie van amorf koolstof dat een fotoluminescente emissie geeft tijdens Raman analyse, deze licht emissie koppelt met de microbol WGM modes en wordt geobserveerd in het Raman spectrum. De experimentele observaties, hoewel in eerste instantie verrassend, zijn in overeenstemming met de WGM literatuur. Met kennis van de literatuur en de experimentele mogelijkheden van de FIB-SEM is een gecontroleerde behandeling van de microbollen mogelijk, waardoor excitatie van resonanties uitgevoerd wordt op specifiek geselecteerde locaties.

In het laatste hoofdstuk van dit proefschrift is beschreven hoe een hogere resolutie Raman microscoop is ontwikkelt. Deze microscoop is niet geïntegreerd met een SEM, het gebruikt een kortere (405 nm) excitatie golflengte en een sterk optisch objectief (0.95 NA). Deze eigenschappen resulteren in een hoge optische resolutie. De hoge resolutie optische microscoop dient ter overbrugging van het resolutie verschil tussen elektron en licht microscopie, dit vergemakkelijkt een correlatieve analyse met beide systemen van sub-micron objecten. Applicaties van de hoge resolutie en korte golflengte Raman microscoop zijn aangetoond voor resonantie Raman spectroscopie op rode bloedcellen en voor de analyse van zilver plasmonische nanostructuren. De zilver nanostructuren zijn resonant voor blauw excitatie licht, en de hogere resolutie Raman analyse maakt een meer gedetailleerde analyse van het preparaat mogelijk. Alle Raman experimenten zijn gecombineerd met SEM analyse, met de SEM zijn dezelfde locaties terug gevonden door middel van markerings op het substraat.

ABBREVIATIONS

CLEM	Correlative light and electron microscopy
iCLEM	Integrated correlative light and electron microscopy
SEM	Scanning electron microscope
ASEM	Atmospheric scanning electron microscope
STEM	Scanning transmission electron microscope
FIB	Focused ion beam
TEM	Transmission electron microscope
SE	Secondary electrons
BSE	Backscattered electrons
ETD	Everhart-Thornley detector
TLD	Through the lens detectors
EM	Electron microscopy
OM	Optical microscopy
RM	Raman microscopy
FM	Fluorescence microscopy
NIR	Near infrared
CVD	Chemical vapor deposition
SERS	Surface enhanced Raman spectroscopy
TERS	tip enhanced Raman spectroscopy
EF	Enhancement factor

NPS	nanoparticles
WGM	Whispering gallery mode
EBID	Electron beam induced deposition
TE	Transverse electric
TM	Transverse magnetic
FFT	Fast Fourier transform
NA	Numerical aperture
ROI	Region of interest
FWHM	Full width at half maximum
SNR	Signal to noise ratio
NIST	National institute of standards and technology
AFM	Atomic force microscope
STM	Scanner tunneling microscope
STED	stimulated emission depletion
STORM	stochastic optical reconstruction microscopy
PALM	photoactivated localization microscopy
XRF	X-ray fluorescence
EDX	Energy-dispersive X-ray spectroscopy
DDA	discrete dipole approximation
DDSCAT	Discrete dipole scattering
MNPBEM	Metallic nanoparticle boundary element method
BEM	Boundary element method
SVD	Singular value decomposition
PCA	Principal component analysis
HCA	Hierarchical cluster analysis
LLS	Linear least squares

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* Authors contributed equally

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