UNIVERSITÀ DEGLI STUDI DI TRIESTE

XIX CICLO DEL
DOTTORATO DI RICERCA IN NANOTECNOLOGIE

DESIGN AND DEVELOPMENT OF MICROARRAYS FOR FUNCTIONAL GENOMICS

(Settore scientifico-disciplinare MED/08)

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1 INTRODUCTION

1.1 Biosensors in the post-genomic era

With the pre-genomic era that was characterized by the effort to sequence the human genome just being completed, we are entering the post-genomic era that concentrates on harvesting the fruits hidden in the genomic text. In contrast to the pre-genomic era which, from the announcement of the quest to sequence the human genome to its completion, has lasted less than 15 years, the post-genomic era can be expected to last much longer, probably extending over several generations. Indeed, with the completion of the Human Genome Project, it has been shown that there are only about 20'000 genes in the human DNA\(^1\) with an estimated number of proteins between 500'000 and 1'000'000. Such complexity, which wasn't predicted by the Central Dogma\(^2\), arises countless unknown factors about how the molecular interaction systems work, such as the relationships of the mRNAs found in a cell and its reactivity to the stimuli it undergoes. In such context, functional genomics studies undertake a great relevance to lead toward the understanding of the mechanisms involved in genetic expression and its regulation in connection with the metabolic pathways.

The relevance of these studies can also be laid considering that those studies go beyond a simple survey of the genome but are able to discriminate between genes population of different tissues of the same organism and even between stages of cells of the same tissue. For the first time, an observation method of the metabolic processes and their regulation at cell level I given. Therefore these data are considered fundamental as an effective base for the development of new diagnostic and therapeutic techniques. For the above reason, the study of the parameters involved in such interactions, is a topical subject but, due to the complexity of the biological processes and the impossibility to simulate them, it is fundamental to collect real data. Those data will be then evaluated with the tools given by the Computational Biology\(^3\).

Unfortunately, while doing functional genomics studies, collecting the data with conventional techniques may require a long time. In this context, various biosensors\(^4\)
were developed and have been an increasingly technology of impact for biomolecular interaction analysis (BIA). These technologies have greatly simplified the task of BIA with their easy access to high-quality kinetic and thermodynamic data. More exciting progresses are being made to allow large-scale interaction studies for helping the development of a complete set of proteomic interaction maps in an age where the full genomes have been sequenced.

Biosensors are devices that combine the specificity of biological molecules with physical detection strategy, e.g., using electronic or optical systems. Biosensors use specific bio-recognition elements immobilized on the surface to transduce the biological signal. The transducer detects the specific molecular interaction and converts the signal for electrochemical, optical, mass, thermal or other measurements.

The majority of the biosensor techniques can be grouped into two categories, based on labeling and label-free interrogation, respectively. The earliest types of sensors require radio active, enzymatic, fluorescent or electrochemical labels to report the binding event. However, there is an increasing awareness of novel techniques that do not require labeling of the ligand or the receptor, such as optical sensors, electrochemical sensors, acoustic sensors, mass spectrometry, calorimetry, etc.

The label-free sensors are obviously advantageous in avoiding the additional cost/time. In particular, the concept eliminates undue detrimental effects from the labels that may interfere with the fundamental interaction. There are an increasing number of commercially available instruments that enable label-free screening for virtually any receptor–analyte complex. Calorimetry sensors are even providing the “gold standard” in the measurement of interaction thermodynamics. However, although the sensitivity of label-free biosensors has been greatly boosted by enormous engineering efforts, it can never compete with that of the labeling biosensors. For example, single-molecule spectroscopy has been widely realized at room temperature by laser-induced fluorescence. Also, the labeling by gold nano-particles has also gained great reputation in ultra-trace detection of biomolecules. Since both labeling and label-free technique have their advantage and limitation (Figure 1.1), both techniques are driven forward in parallel in order to accelerate the acceptance of biosensors in a larger abundance of areas.
1.2 Requirements for the next genomics sensors

With the fall of the Central Dogma, the complex system of interactions that can now be observed must be studied with newer techniques which require a new kind of technology that has to be developed.

In fact the most common methods for the mRNA analysis are Reversed-Transcriptase Polymerase Chain Reaction (RT-PCR) and oligonucleotide arrays (so called microarray). Both have good qualities, in fact PCR based methods have a good dynamic range and the Microarray technique allows to analyze thousands of genes at the same time, but both have major problems. RT-PCR is the technique with the highest sensitivity for the identification, analysis and quantification of mRNA\textsuperscript{13}, but with method is only possible to study a gene at the time. It is not easy compare different mRNA expression levels with RT-PCR because of the variations in PCR efficiency (only relative quantification can be obtained for example while extracting mRNA from paraffin embedded tissues, PET, due to the impossibility to use any competitor or internal standard). On the other hand, with microarrays is only possible to compare two different sample populations, therefore you have a relative quantification. In addition, a huge amount of mRNA must be used, unlikely to obtain from extremely degraded samples (as for paraffin embedded tissues, PET), fact that keeps at stake the RT-PCR,
which allows to study samples where the nucleic acids are scarce and partly degraded, provided that the target sequence is intact\textsuperscript{13}. Furthermore, reliable and reproducible measurements are essential: to acquire high quality data, it is important that all steps involved in this technology are optimized and standardized to reduce experimental error. Such need for standardization has been recently reviewed\textsuperscript{14}. Currently, there are no generally accepted standards for performing and analyzing a Microarray study\textsuperscript{15}.

Since nucleic acids have been identified as important ‘fingerprints’ for individuals, species and even diseases, development of detection strategies has become a challenging task in ongoing research. The purpose of the studies is to conquer the inescapable need of the time consuming and error prone PCR reaction and establish new array DNA platforms, according to a direct nucleic acids measurement protocol that will avoid the time consuming and error prone Reversed-Transcriptase Polymerase Chain Reaction where genes can only be analyzed individually and the measurements are affected by the polymerase activity and selectivity. In order to maintain a good dynamic range, to correlate in a precise way the expression levels for thousands of different mRNAs, work with tiny amount of mRNA, eventually on raw samples to reduce the loss of material during purification steps, and therefore

\begin{figure}
\centering
\includegraphics[width=\textwidth]{microarray_figure.png}
\caption{A microarray experiment. The top part of the figure shows steps in the sample preparation process while the bottom part shows steps in the fabrication of the microarray. The samples are hybridized to the microarrays (centre).}
\end{figure}
perform an accurate functional genomic study, new device which will integrate all this characteristics are required.
Therefore there is a claim for new detection systems that could be developed taking advantage of the newest nanotechnology fabrication techniques at the nanometre scale.
This new technology will be required to work even with degraded nucleic acid obtained for example from paraffin embedded tissues (PET).

1.2.1 Molecular Analysis in archive tissues

The last decades have led to revolutionary developments in the field of molecular biology and genetics, both from the methodology and scientific point of view. This new knowledge have been and important base for medical practise and, in particular, in the development of molecular medicine.
The new technologies and knowledge in molecular medicine are nowadays applied to develop a new personalized approach to medicine, allowing to evaluate the progression of a pathology combining information gathered from multiple sources, considering risks and specific elements for single medical cases. To integrate the information coming from the clinical practise with those from the molecular analysis studies, the major problem is to define the more significant biomolecular targets for each disease.
The recent development of biology and molecular medicine had a scarce practical application, up to now. Indeed, cell culture and animal models, before being applied, have to be tested and validated at human level. The very same analysis, if performed on human tissues, has the advantage to be able to validate the basic researches and to lead to the direct application into clinical practise of the results obtained. It has been seen in fact, that after the histomorphological typifying it is necessary to evaluate the expression level of the molecular target of the therapy.
The creation of biobanks of human tissues covers a fundamental role for the development of an integrated approach for the diagnosis and therapy, especially in the oncological field.
Introduction

The possibility to have to hand whole collection of human tissues as a tool for molecular research, opens the way to countless possible applications and may lead to the real development of personalized molecular medicine.

The best choice would be to collect fresh cryopreserved tissues, which is clearly an unlikely scenario, mainly to the costs and complications connected to such project and to the difficulties related to the transportation of frozen materials if shared in multicentric projects across Europe. The thawing out of the samples would indeed lead to the irreparable degradation of the sample.

The best alternative to fresh tissues is represented by the paraffin embedded tissues (PET), an extraordinary resource for clinic molecular studies. All together, they constitute the hugest collection of pathological samples, available for retrospective studies, useful to understand for example the mechanisms behind pathologies or the different expression levels of a gene.\(^6\)

Human tissues have been collected for decades in the Departments of Pathological Anatomy's archives, in every hospital around the world. This allows to perform morphological analysis every time is needed but it is important to state that this has led to an incredible development of the knowledge and ability to classify the pathologies, laying the foundations for modern clinical practice.\(^17\)

Using these huge archives, it is possible to run through all the stages of a pathology, both common or rare. The paraffin embedded technique is a quite cheap preservation method that doesn't affect the morphology of the tissue and it is widely compatible with the antibodies used in immunohistochemistry.\(^18\)

In the past the tissues were preserved this way to be able to study the morphology later on, without even thinking that this solution is suitable for molecular biology studies. Nowadays is possible to extract nucleic acids from PET and then perform molecular analysis.\(^19\)

The problem connected with PET is the degradation level of the nucleic acids which allows only the study of short sequences.

Unfortunately, the mRNA quantity that can be obtained from PET is not enough to use them with the Microarray technology unless resorting to the retro-transcription of the mRNA into cDNA, which brings in all the problems related to the PCR methods like the non-proportional amplification of the sequences.
1.3 Why Surface plasmon based (sensing) techniques can be a possible approach

A surface plasmon resonance (SPR) is a collective electron resonance, pinned at the interface between two optically dissimilar materials, one of which is a metal (e.g. Au, Ag, Cu). SPR is being explored for its potential in optics, magneto-optic data storage, microscopy and solar cells, as well as being used to construct sensors for detecting biologically interesting molecules. Theoretically and experimentally, the following distinctive features of SPR have been discovered and proven to be valuable:

a) It is a short range phenomenon, with the electric field decaying with 1/e distance of hundreds of nanometres in the adjacent media.

b) It provides an enhanced electromagnetic field at the interface;

c) It propagates with high attenuation owing to the high loss in the metal.

d) It is mostly p-polarized, giving spatial orientation information about the dipoles with which the SP interacts.

Based on feature (a), SPR becomes an optical technique that uses evanescence waves to measure changes in refractive index and/or thickness very close to the sensor surface. A great deal of work has been done in the exploitation of SPR for optical biosensing, since its first thin-film sensing was conducted in the late seventies.

In 1990, the first commercial SPR biosensing instrument was introduced by Biacore. Subsequently, a number of commercial SPR biosensor instruments are available.

It is becoming a mature biophysical method and a leading technology for characterizing biomolecular interactions.

Feature (b), the enhancement in the localized field has long been recognized and used for different spectroscopies of adsorbing species, including Raman scattering, fluorescence, nonlinear optical response (e.g. second order nonlinear polarizability), infrared-absorption and diffraction (scattering), etc. Correspondingly, the techniques are
named to be surface plasmon enhanced Raman scattering (SE-SERS)\textsuperscript{22}, surface plasmon enhanced fluorescence spectroscopy (SPFS)\textsuperscript{23}, surface-enhanced second harmonic generation (SHG)\textsuperscript{24}, surface-enhanced infrared absorption (SEIRA)\textsuperscript{25} and surface plasmon enhanced diffraction (SPD)\textsuperscript{26}. Feature (c) offers a lateral resolution of SPR at the micrometer scale for thin-film imaging purposes. Following the early work by Knoll et al\textsuperscript{27}, surface plasmon microscopy (SPM) has been well-engineered\textsuperscript{28} and even commercialized\textsuperscript{29}. SPM is also showing its compatibility to the biochip technology. Feature (d) has not gained as much attention as that of (a), (b) and (c). However, there are still a few efforts\textsuperscript{30} reported, using SPFS or SEIRA to take full advantage of the p-polarized excitation source. Provided that SPR is an in situ technique that can be performed both in liquid and air, it is particularly well suited as a technique to develop new diagnostic biological tools for nucleic acids detection.

1.4 Aim of the study

Several studies explore the possibilities offered by the Surface Plasmon Resonance (SPR) as a possible technique to develop a biosensor devoted to DNA analysis. The aim of this study is to explore the possibility to improve the performances of the SPR using nanofabrication of plasmonic crystals, in order to develop a new tool for functional genomics studies with new biological architecture. This could be a possible base for a new type of array DNA platforms according to a direct nucleic acids measurement protocol that will avoid the time consuming and error prone reversed-transcriptase Polymerase Chain Reaction, where genes can only be analyzed individually and the measurements are affected by the polymerase activity and selectivity. The SPR technique was not yet established in our group, therefore the aim of this study is to introduce from scratch this method in our group in order to establish a new platform that could be used also for other applications. Consequently, an in dept understanding of the theory behind the technique is required and the study starts with a
thorough investigation of DNA hybridization based on functional self-assembled monolayer systems.
In particular, the Surface Plasmon Fluorescence Spectroscopy (SPFS) will be explored as the main detection method. The SPFS technique was firstly introduced by the Knoll’s group at the Max Planck Institute for Polymer research in 20031. Extensive DNA hybridization studies have been carried out since then32. The need of the fluorescence to detect the DNA hybridization is due to the fact that currently the SPR itself cannot detect such small molecules. This is not true for example for proteins since, possessing a larger size than oligonucleotides, the protein binding can be visible in both the SPR. This dual-channel sensing ability of SPFS can be fully used for uncovering more interfacial information which could be of great interest if possible also for the DNA. Therefore this study tries to improve this aspect of the technique taking into account the possibilities offered by the nanofabrication with the goal in mind to realize, in the future, a completely label-free system, in order to be able to perform DNA studies directly on the raw samples, reducing thus also the time needed to prepare the samples. Based on the understanding that fluorescence suffers severe quenching if the fluorophores are too close to the metal, it has been designed an architecture to extend the interaction platform out of the ‘quenching’ region that is simpler than those previously adopted33.

1.5 Overview of the project

The present study is made by two separated parts converging upon the same aim that is to exploit new analytical methodologies for molecular biomedicine applications. The power of these new technologies comes from the potentiality that nanofabrication gives to molecular biology once the two fields are joint and that may allot to overcome the present issues related to the traditional molecular biology’s techniques. Therefore, a nanofabrication approach has been applied to a nucleic acids sequence analysis.
Table 1.1. Schematic overview of the project. The fabrication part is divided into two categories. One is related to the lithographic work, which will be coupled with the second category related to the biological aspects of the project. Jointly, these two parts will be characterized in order to realize a new DNA analysis platform.

There are different ways to analyze nucleic acids:

- Electrochemical methods\cite{34};
- \(^{32}\)P radiolabeling;
- XPS (x-ray photoelectron spectroscopy)\cite{35};
- Secondary ion mass spectrometry\cite{36};
- Neutron reflectivity\cite{37};
- Grazing angle FT-IR\cite{38};
- Surface-enhanced Raman spectroscopy\cite{39};
- AFM\cite{40};
- SNOM\cite{41};
- SPR\cite{42}. 


Introduction

For several reasons, SPR has been the choice we decided to choose. It is an \textit{in-situ} optical method that allow to work both in air and in water. The possibility to work in aqueous environment and being a non destructive method, make SPR a particularly well suited method for biological applications. In addition, the possibility in the future to develop label-free technologies may allow, once the proper substrates are realized, to perform direct analysis on biological samples without the need to process them in order to label them or purify them, step which reduce the amount of the analyte since the yield it is never 100%. Furthermore, there is the possibility to improve the SPR methods using appropriately patterned substrates that can be realized with x-ray lithographic techniques.
2 THEORY

2.1 Surface Plasmon optics

Surface plasmon resonance spectroscopy (SPR, SPS or SPRS) is a century-old technique dating back to the finding of Wood’s anomaly seen in for the reflected light from diffraction gratings\textsuperscript{43}. It was later investigated in 1957 by the pioneering work of Ritchie\textsuperscript{44}. Since then, there has been a considerable amount of work done on the fundamental properties of plasmons and the SPR theory has been well established\textsuperscript{12}. There are also numerous examples of their applications, which mainly focus on developing a wide variety of optical sensors.

Surface plasmons (SPs) are electromagnetic waves that propagate along the surface of a conductor, usually a noble metal (such as Au, Ag, Pt, etc.). They are essentially light waves that are bound to the surface by the interaction with the free electrons of the conductor. As a result, the free electrons respond collectively by oscillating in resonance with the light wave. The resonant interaction between the surface charge oscillation and the electromagnetic field of the light constitutes the SPs and gives rise to its unique properties.

2.1.1 Maxwell equations – a starting point

The Maxwell equations are given by:

\[
\nabla \cdot \vec{D} = 0 \quad \nabla \times \vec{E} = -\frac{\partial \vec{B}}{\partial t} \\
\nabla \cdot \vec{B} = 0 \quad \nabla \times \vec{H} = \frac{\partial \vec{D}}{\partial t}
\]  --- 2.1
Here, \( \vec{E} \) is the electric field (V m\(^{-1}\)) and \( \vec{H} \) is the magnetic field (Am\(^{-1}\)). They are related to the electric displacement (or the dielectric flux density or electric flux density) \( \vec{D} \) (C m\(^{-2}\)) and magnetic flux density (or magnetic induction) \( \vec{B} \) (T; tesla = NA\(^{-1}\)m\(^{-1}\))

\[
\vec{D} = \varepsilon \varepsilon_0 \vec{E} \\
\vec{B} = \mu \mu_0 \vec{H}
\]

where \( \varepsilon \) and \( \varepsilon_0 \) are the dielectric constant (without dimension) and the electric permittivity of free space, respectively. \( \mu \) and \( \mu_0 \) are the magnetic permeability (without dimension) and the magnetic permeability of free space, respectively.

![Figure 2.1. The charges and the electromagnetic field of SPs propagating on a surface in the x-direction are shown schematically in the left panel. The exponential dependence of the field \( \vec{E}_z \) on both +z and −z sides is seen on the right panel.](image)

2.1.2 Wave equations and dispersion relation of surface plasmons

The solution of Maxwell equations for the electric field \( \vec{E} \), in the case of plane waves, is presented by the equation:

\[
\vec{E} = \vec{E}_0 e^{i(k \cdot r - \omega t)}
\]
Where $E_0$ is the electric field amplitude, $\vec{r}$ is the position vector, $\omega$ is the angular frequency ($\omega=2\pi f$, $f$ frequency), $t$ is the time, and $\vec{k}$ is the wave vector which is in the direction of the propagation. The magnitude of $\vec{k}$ is given by:

$$|k| = \sqrt{\mu_0\varepsilon_0 \omega^2}$$  \hspace{1cm} (2.4)

in vacuum (or air as a first approximation), $\varepsilon=1$, $\mu=1$ and $\lambda=2\pi/\omega$, $k=2\pi/\lambda=\omega/c$, then:

$$c = 1/\sqrt{\mu_0\varepsilon_0}$$  \hspace{1cm} (2.5)

The electron charges on a metal boundary can perform coherent oscillations, which are called surface plasmon polaritons (SPPs or SPs). These charge oscillations can be localized in the $z$-direction, and accompanied by a mixed transversal and longitudinal electromagnetic field that propagates along the $x$-axis and vanishes at $|z| \to \infty$ on both sides of the metal/dielectric interface, and has its maximum at $z=0$ (cf. Figure 2.1). The plasmon waves have $p$-character because the surface charges induce the discontinuity of the electromagnetic field in the $z$-direction, which does not apply for $s$-waves (no $E_z$ component). In other words, only transverse magnetic plane waves (i.e. TM waves) can be applied to excite SPPs. Considering the dielectric ($\varepsilon_1>0$, medium 1)/metal ($\varepsilon_2=\varepsilon_2'+i\varepsilon_2''$, medium 2) interface, the electromagnetic fields on both sides are expressed as:

$$H_1 = (0, H_{y1}, 0)e^{i(k_{x1}x+k_{z1}z-\omega t)}$$  \hspace{1cm} (z > 0)

$$E_1 = (E_{x1}, 0, E_{z1})e^{i(k_{x1}x+k_{z1}z-\omega t)}$$

$$H_2 = (0, H_{y2}, 0)e^{i(k_{x2}x-k_{z2}z-\omega t)}$$  \hspace{1cm} (z < 0)

$$E_2 = (E_{x2}, 0, E_{z2})e^{i(k_{x2}x-k_{z2}z-\omega t)}$$  \hspace{1cm} (2.6)

$k_{x1}$ and $k_{x2}$ are the wave vectors in $x$-directions and $k_{z1}$ and $k_{z2}$ the ones along the $z$-axis. Considering the continuity relations of the in-plane components:

$$E_{x1} = E_{x2}, H_{y1} = H_{y2}$$  \hspace{1cm} (2.7)

and inserting (2.7) into (2.6) yields:
\[ k_{x1} = k_{x2} = k_x, \]  

inserting (2.6) into (2.1), together with (2.5) one obtains:

\[ + k_{x1} H_{y1} = - \frac{\omega}{c} \varepsilon_1 E_{x1} \]  
\[ + k_{x2} H_{y2} = + \frac{\omega}{c} \varepsilon_2 E_{x2} \]  

Together with the continuity relations (2.7), one obtains the dispersion relation of SPs:

\[ \frac{k_{x1}}{\varepsilon_1} + \frac{k_{x2}}{\varepsilon_2} = 0 \]  

This reveals that SPs can only exist at the interface between two materials that have dielectric constants of opposite sign, e.g., a metal/dielectric interface.

The wave vector \( k_i \) can be decomposed into \( k_{xi} \) and \( k_{zi} \), together with (2.8), one obtains:

\[ k_x^2 + k_{zi}^2 = \varepsilon_i \left( \frac{\omega}{c} \right)^2 \]  

From (2.10) together with (2.11), one yields the dispersion relation of SPs in another format:

\[ k_x = \frac{\omega}{c} \left( \frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2} \right)^{1/2} \]  

In the case of the dielectric (\( \varepsilon_1 > 0 \), medium 1)/metal (\( \varepsilon_2 = \varepsilon_2' + i\varepsilon_2'' \), medium 2) interface, and assuming \( \varepsilon_2'' < |\varepsilon_2'| \), the complex \( k_x \) is expressed by:

\[ k_x = k_x' + ik_x'' \]  

with:

\[ k_x' = \frac{\omega}{c} \left( \frac{\varepsilon_1 \varepsilon_2'}{\varepsilon_1 + \varepsilon_2'} \right)^{1/2} \]  
\[ k_x'' = \frac{\omega}{c} \left( \frac{\varepsilon_1 \varepsilon_2'}{\varepsilon_1 + \varepsilon_2'} \right)^{1/2} \frac{\varepsilon_2''}{2\varepsilon_2'^{1/2}} \]
2.1.3 Spatial extensions of SP fields and propagation of SPs

From (2.11) and (2.12) and assuming $|\varepsilon_2| > \varepsilon_1$, one obtains:

$$k_{z1}^2 \approx \left( \frac{\omega}{c} \right)^2 \left( \frac{\varepsilon_1^2}{\varepsilon_1 + \varepsilon_2^i} \right)$$

--- 2.16

$$k_{z2}^2 \approx \left( \frac{\omega}{c} \right)^2 \left( \frac{\varepsilon_2^2}{\varepsilon_1 + \varepsilon_2^i} \right)$$

--- 2.17

Since $\varepsilon_1^2 + \varepsilon_1 < 0$, the wave vectors $k_{z1}$ is purely imaginary. The optical fields decay exponentially as $E_z \propto e^{-|k_{z1}|z}$ into their respect medium.

The distance (depth) where the field falls to 1/e, becomes:

$$\tilde{z} = \frac{1}{|k_{z1}|}$$

--- 2.18

The intensity of SPs propagating along the metal/dielectric interface (x-axis) decreases as $E_x \propto e^{-2|k_x|l}$. Therefore, the propagation length $L_x$:

$$L_x = \frac{1}{2|k_x|^2}$$

--- 2.19

can be defined, which influences the lateral resolution for surface plasmon microscopy applications. Also for 633 nm light on the gold/air interface, $L_x = 10 \mu m$. For the same light on the gold/water interface, $L_x = 4 \mu m$. The damping of the electromagnetic field causes heat and dissipated into the metal and the dielectric.

2.1.4 Excitation of SPs by light

The dispersion relation of a free photon in a homogenous dielectric ($\varepsilon_1$) is

$$k_{ph} = \frac{\omega}{c} \sqrt{\varepsilon_1}$$

--- 2.20
which is always smaller than the wave vector of SPs at the dielectric/metal interface, $k_{sp}$ (cf. $k_x$ in (2.12)). This is shown graphically as $\omega(k_x)$ plots in Figure 2.2 (cf. curve (a) and curve SP-I). The $\omega(k_x)$ curve of SPs approaches asymptotically the dispersion curve of the free photon ($|\varepsilon_2| >> \varepsilon_1$ for $\omega -> 0$) with no intersection of the curves. Thus, SP cannot directly transform into light on a smooth surface, i.e., it is a non-radiative wave. Another consequence is that, the p-polarized light cannot be used to excite SPs directly, due to its insufficient $k_{ph}$. Experimentally, there are two coupling techniques to enhance $k_{ph}$, namely, the prism coupling and the grating coupling, in order to match the optical momentum at the interface.

2.1.4.1 Prism coupling

The light travels through a half-cylindrical prism ($\tilde{\varepsilon}_p$), with its $k_{ph}$ being enhanced to
\[ \frac{\omega}{c} \sqrt{\varepsilon_p} \] by a factor of \(\sqrt{\varepsilon_p / \varepsilon_1}\). The dispersion curves before and after the enhancement by the prism are shown as curve (a) and (b) in Figure 2.2, with a slope of \(\frac{c}{\sqrt{\varepsilon_1}}\) and \(\frac{c}{\sqrt{\varepsilon_p}}\), respectively. At a certain wavelength of the laser (\(\omega_{\text{laser}}\)), the dispersion curve of SP-I determines a laser incident angle \(\theta\) for the excitation of SPs (cf. curve (d)). This occurs if the projection of the wave vector \(\frac{\omega}{c} \sqrt{\varepsilon_p}\) on the x-axis matches the x-component of \(k_{\text{SPP}}\).

\[ \frac{\omega_{\text{laser}} \sin \theta}{c} \sqrt{\varepsilon_p} = k_{\text{SPP},x} \]  

As is shown in Figure 2.2, the enhanced wave vector \(\Delta k_x\) from point 1 to 2 is the contribution from the prism. The excitation of SPs is recognized as a minimum in the totally reflected intensity, which can be quantitatively described by Fresnel’s equations for multi-layer system\(^45\), with the aid of the transfer matrix algorithm\(^46\).

Two prism coupling geometries are possible, which are called Otto configuration\(^47\) and Kretschmann-Raether configuration\(^48\), respectively. Various shapes of prisms can be used for exciting SPs, such as triangular, half cylinder prisms, hemisphere prisms. For simplicity, in the following discussions we will use half cylinder, since the light incident angle will not be changed by the refraction at the air/prism interface. For the triangle prism, the corresponding angle conversion should be considered.

For the Otto configuration (panel (A) in Figure 2.3), the metal surface is separated by an air or dielectric gap at a distance of sub-micrometer from the prism. The resonant excitation of SPPs is achieved on the metal surface through the gap where the evanescent fields from both sides of the gap overlap. With the experimental parameters listed below the schematic, a Fresnel simulation yields a deep and sharp minimum in the angular-dependent reflectivity curve due to the occurring of the surface plasmon resonance.
The Kretschmann-Raether configuration is more versatile because it is experimentally less difficult to realize. As shown in panel (B) of Figure 2.3, it requires a finite thickness $d_m$ of the metal layer, which is directly attached to the base of the prism. The thickness $d_m$ influences the coupling angle $\theta$, as well as the coupling efficiency, e.g., the minimum reflectivity. This dependence can be simulated by Fresnel's equations using the transfer matrix methods. As a general understanding, it is a result of the destructive interference between the partially reflected light wave from the prism/metal interface and the re-radiated light wave from the metal/dielectric interface. A Fresnel simulation of the usual experimental conditions is also shown.

For a more convenient experimental handling, the metal layer is usually deposited on a glass substrate with the same refractive index as that of the prism. Then an index-matching fluid couples the prism and the substrate optically. For practical reasons,
substrates with lower refractive indices are sometimes coupled to the high index prism for surface plasmon excitation.

The refractive index mismatch between the prism and the substrate brings extra features in the angular reflectivity curve, as shown in panel (C) of Figure 2.3. For example, the reflectivity curve is superimposed by an angular-dependent interference pattern, which is caused by the extra reflection beam at the prism/substrate interface interfering with the beam from the substrate/metal interface. Another point is that, at an angle slightly higher than the SPR minimum angle, a second total reflection occurs at the prism/substrate interface and the SPR phenomenon vanishes abruptly.

![Diagram of an incident, reflected, and transmitted plane wave](image)

Figure 2.4. Schematic picture of an incident, a reflected and a transmitted transverse magnetic polarized plane wave at an interface between two different dielectric media. The subscripts stand for 'incident' (i), 'reflected' (r), 'transmitted' (t). The plane of incidence is determined by the wave vector of the incident beam and the surface normal. Note that the in-plane components of the wave vectors are equal.

However, the surface plasmon dip still appears at the same angle as if the extra layer of the substrate does not exist (cf. the reflectivity curve in panel (B)). In order to elucidate these phenomena, we refer to the reflection/transmission law at dielectric interfaces.

As shown in Figure 2.4, at the interface of two dielectric media with no adsorption \( n_1 = \varepsilon_1^{1/2}, \quad n_2 = \varepsilon_2^{1/2} \), the incident wave (e.g. a TM mode) is subjected to reflection and transmission. An important characteristic is the conservation of the in-plane wave vector \( k_{\parallel} \), i.e., the wave vector parallel to the interface.
\[ k_1 \sin \theta_1 = k, \sin \theta_1 = k, \sin \theta, \quad \text{(Eq. 2.22)} \]

This is schematically presented in the Figure 2.4. Although the modulus of \( \vec{k} \) for \( n_1>n_2 \) is smaller, it still yields for the transmission beam the same projection \( k_1 \) on the x-axis due to the increased \( \theta_1 \). Therefore, SPR resonance is possible at the same angle (cf. Equation 2.21).

Moreover, with Snell’s law:

\[ n_1 \sin \theta_1 = n_2 \sin \theta, \quad \text{(Eq. 2.23)} \]

for \( n_1>n_2, \ \theta_1=90^\circ \), total reflection (i.e. no transmission light) will occur at a critical angle \( \theta_c \) with

\[ \theta_c = \sin^{-1} \frac{n_2}{n_1}, \quad \text{(Eq. 2.24)} \]

Thus, with the same \( n_1 \), \( \theta_c \) increases with increasing \( n_2 \). Using this equation, those two total reflection angles can be precisely positioned.

### 2.1.4.2 Grating coupling

![Schematic representation of SPR coupling via a sinusoidal diffraction grating.](image)

Figure 2.5. Schematic representation of SPR coupling via a sinusoidal diffraction grating.
As is indicated mathematically by a Rayleigh expansion (approximation for shallow grating\textsuperscript{49}), the surface periodic structure can also enhance the wave vector of the incident light for resonance coupling\textsuperscript{20}. As shown in Figure 2.5, light ($k_{ph} = \omega/c$) from the dielectric ($\varepsilon_d$) hits a metallic grating ($\varepsilon_m$) with a grating constant $\Lambda$ at an incident angle $\theta$. Assuming the dispersion property of the SP wave is not disturbed by the corrugated surface, the momentum matching condition can be written as:

\[ k_x = k_{ph,x} \pm mg = \frac{\omega}{c} \sqrt{\varepsilon_d} \sin \theta \pm mg = \frac{\omega}{c} \sqrt{\frac{\varepsilon_d \varepsilon_m}{\varepsilon_d + \varepsilon_m}} = k_{sp,x} \quad - \quad 2.25 \]

with $m$ the order of diffraction and $g = 2\pi/\Lambda = k_\Lambda$, the grating wave vector. The resonance can be also observed as a minimum of the reflected light as a function of incident angle or wavelength. Note that the grating constant $\Lambda$ should be within the same order-of-magnitude as the wavelength of the incoming light, given the fact that SPR coupling via the first diffraction order is significantly more efficient than via higher orders. A shallow grating with amplitude of several tens of nanometers is proven to be sufficient for efficient SPR coupling.

2.1.5 SPR response to a thin film deposition

The evanescent field of a surface plasmon wave reaches the maximum at the metal-dielectric interface, and decays exponentially into the dielectric medium at a distance of several hundreds of nanometers. Therefore the surface plasmon resonance technique is extremely sensitive to the changes of the optical properties of the adjacent dielectric medium. This means that a variation of the $\varepsilon_i$ in equation (2.12) changes $k_{\text{app},x}$, and consequently, changes the surface plasmon resonance angle elucidated by equation (2.21). On the other hand, the addition of a thin (i.e. thickness $d$ much smaller than the SP decay length along the $z$ axis) and non-adsorbing layer with different dielectric constant ($\varepsilon_F \neq \varepsilon_i$) also induces a change in the overall dielectric constant integrated over the evanescent field, therefore shifts the SPR angle.

The mean refractive index within the evanescent field increases assuming $\varepsilon_F > \varepsilon_i$. Therefore, the dispersion relation of equation (2.12) shifts to larger wave vector,
depicted as curve SP-II in Figure 2.2. Consequently, a higher light incident angle $\theta' > \theta$ is required to fulfill the resonance criterion. For a thin, non-adsorbing layer defined by a thickness $d$ and a refractive index $n$, the resonant angle displacement $\Delta \theta$ is linear to the optical thickness $nd$ of the layer:

$$\Delta \theta \propto n \cdot d$$

There are various methods proposed, to separate the two distinct parameters ($n$ and $d$) within the optical thickness value, e.g., by measuring the SPR in a series of bulk media with different refractive indices$^{50}$ or under multiple light wavelengths$^{51}$. If the studied material forms a thick film, a number of waveguide modes can be excited which provide adequate information for resolving $n$ and $d$$^{52}$. However, for biomolecules, this optical picture is usually irrelevant. SPR angle displacement $\Delta \theta$ is directly correlated with the molecular surface concentration. For example, using radioisotope labeled protein, it has been found that $\Delta \theta$ depends linearly on the protein concentration (mass per area) and the dependence is irrelevant to the type of proteins studied$^{53}$.

Usually, a layer of biomolecules can be treated as non-adsorbing layer. However, if the biomolecules carries external labels (e.g. gold nanoparticles, fluorescent dye, etc.) the resulting thin layer becomes adsorbing, which more or less distorts the property of SPR$^{54}$.

Angle-scan SPR curves of three-phase systems (prism/Ag/air, prism/Ag/water, prism/Au/air, prism/Au/water) in the Kretschmann-Rather ATR configuration calculated by Fresnel equation are shown in Figure 2.6. All the curves show a critical angles $\theta_c$ and a resonance angle $\theta_{\text{SPR}}$. The critical angle $\theta_c$, whose value is determined only by the refractive indices of the prism and the dielectric medium, indicates the onset of the attenuated total reflection (ATR) phenomenon. The resonance angle, on the other hand, indicates the condition at which the wavevector of the incident light matches that of the surface plasmon wave supported by the metal. At the resonance angle, almost all of the energy of the incident radiation is converted into surface plasmon excitation and, thus, the electromagnetic field at the interface is greatly enhanced. As a result, a substantial attenuation of the reflectance is observed. The enhancement can be also depicted in a more obvious manner by applying energy conservation$^{20}$.
2.1.6 Field enhancement

![Graph showing field enhancement](image)

Figure 2.7. Simulation curves of the reflectivity and the relative field intensity as a function of the light incident angle on three-phase systems (prism/Ag/air, prism/Ag/water, prism/Au/air, prism/Au/water). Calculation parameters: right-angled prism ($c=3.7$), Ag ($c=17+0.7i$), Au ($c=12+1.3i$), air ($c=1$), water ($c=1.78$), HeNe laser (633 nm).

For p-polarized radiation, the electric field has two components (in $x$-direction and in $z$-direction) while the magnetic field has only one component (in $y$-direction). Since both fields are tightly correlated, for simplicity the field-enhancement estimation is calculated for the magnetic field. The angular dependent magnetic field intensities are normalized to the incident intensity and are plotted against their corresponding reflectivity curves in Figure 2.7. As shown, every field intensity reach a maximum near the reflectivity minimum. The enhancement factors are found to be 77, 58, 24, 17 for the interfaces of Ag/air, Ag/water, Au/air, Au/water, respectively.

The significantly higher enhancement factor on silver is attributed to its smaller imaginary dielectric constant $c''$, which results in lower dissipation of optical field. The SP field is pushed more into the metal by the optically denser dielectric (e.g. water) than...
the air, therefore is more dissipated. Consequently, the field enhancement is lower in metal/water than in metal/air.

A closer look reveals that the angle of the maximum field-intensity slightly shifts toward a smaller angle of the reflectivity minimum angle. This phenomenon can be explained by considering the system as a resonator driven by the incoming light. The imaginary dielectric constant $\varepsilon''$ of the metal is the lossy component of the resonator which separates the coincidence of the resonance. The larger is the $\varepsilon''$, the greater is the separation, which coincides with the factor that the angular separation is smaller on Ag than on Au.

2.1.7 Band-Gap effect

One of the key developments in photonics in the past 15 years has been that of photonic bandgap (PBG) materials. These synthetic materials use wavelength-scale periodic structures to manipulate the interaction between light and matter so as to build new photonic structures; a good example is that of the photonic crystal fibre. These developments have been predominantly made in periodically structured insulating and semiconducting materials. By making use of SPs, metals too can be used as PBG materials, this time in the form of photonic surfaces.

In its simplest form, a surface plasmon polariton (SPP) is an electromagnetic excitation that propagates in a wave-like fashion along the planar interface between a metal and a dielectric medium, often vacuum or air, and whose amplitude decays exponentially with increasing distance into each medium from the interface. Thus, a SPP is a surface electromagnetic wave, with its field confined to the near vicinity of the dielectric–metal interface. This confinement leads to an enhancement of the electromagnetic field at the interface, resulting in an extraordinary sensitivity of SPPs to surface conditions. This sensitivity is extensively used for studying adsorbates on a surface, surface roughness, and related phenomena. Surface plasmon polariton-based devices exploiting this sensitivity are widely used in chemo- and bio-sensors. The enormous strength of the electromagnetic field at the interface is responsible for surface-enhanced optical phenomena such as Raman scattering, second harmonic generation (SHG),
Theory

fluorescence, etc\textsuperscript{60}. However, significant limitations are imposed by this mode of excitation. The wave vector applicable to freely propagating photons in the air half-space is strongly limited by intrinsic metal dispersion. To fully engineer the electromagnetic confinement of the plasmon there are some means to reduce/augment the wave vectors of the SPPs to enable them to couple to light. One approach is to introduce wavelength scale microstructure into the device thereby allowing trapped SPP modes to be Bragg scattered and thus coupled the freely propagating radiation produce emitted light. This is essentially equivalent to what happens in pure dielectric photonic crystals.

A periodic arrangement of defects on a metal–dielectric interface exhibit the properties of a two-dimensional “plasmonic crystal” if the periodicity of the surface structure is comparable with the wavelength of the SPPs propagating on the interface. The simplest realization of plasmonic crystals is a metallic diffraction grating. The SPP propagation on a periodic structure results in changes of the dispersion relations due to the interaction with the periodic surface features. The SPP scattered by periodic features leads to the appearance of SPP band-gaps and SPP Bloch waves on a periodically structured surface. Only surface plasmons satisfying the Bloch wave condition can be excited and propagate on such a surface. A fundamental difference between the well known photonic and plasmonic crystals is a different electromagnetic field distribution. In plasmonic crystals the electromagnetic field enhancement at a metal surface can amount to several orders of magnitude. Such giant enhancement effects are absent in photonic crystals. Most importantly, active photonic elements based on nonlinear SPP optics, which allow for the control of optical properties with light, can be realized with suitably patterned metal surfaces.

Therefore, an important feature offered by the SPR is the possibility to increase the sensibility of the method taking advantage of the so called bang gap effect\textsuperscript{61}. In other words, a bidimensional pattern can stop the propagation of the Plasmon, differently from what happens with continuous metal films (Figure 2.7).
Periodic texturing of the metal surface can lead to the formation of an SP photonic bandgap when the period, $a$, is equal to half the wavelength of the SP, as shown in the dispersion diagram (Figure 2.8a). Just as for electron waves in crystalline solids, there are two SP standing wave solutions, each with the same wavelength but, owing to their different field and surface charge distributions, they are of different frequencies. The upper frequency solution, $\omega_+$, is of higher energy because of the greater distance between the surface charges and the greater distortion of the field, as shown schematically in Figure 2.8b. SP modes with frequencies between the two band edges, $\omega_+$ and $\omega_-$, cannot propagate, and so this frequency interval is known as a stop gap.

By providing periodic texture in two dimensions, SP propagation in all in-plane directions can be blocked, leading to the full bandgap for SPs. At the band edges the
density of SP states is high, and there is a significant increase in the associated field enhancement.

2.2 Surface diffraction

2.2.1 Diffraction from a smooth, sinusoidal surface

Ordered scattering from surface with a periodic structure is called diffraction. Figure 2.7 gives a schematic sketch of the diffraction of \( p \)-polarized light on a sinusoidal grating.

![Figure 2.7. Schematic sketch of a reflective diffraction of an incident light by a surface grating structure.](image)

The \( p \)-polarized light is incident in the \( x-z \) plane at angle \( \theta_0 \) with a wavelength \( \lambda \). The grating/air interface plane is oriented perpendicular to the \( x-z \) plane. The Rayleigh expansion\(^{49}\) gives the description of the diffracted electric field in the air region:

\[
E(x, z) = \sum_{m=-\infty}^{\infty} E_m e^{i(\alpha_m x + \beta_m z)}
\]  

---  

2.27
With

\[ \alpha_m = k_{ph} \sin \theta_m + mk_g \]

\[ \beta_m = \sqrt{k_{ph}^2 - \alpha_m^2} \]

\[ k_g = 2\pi / \Lambda \]

where \( \theta_m \) is the angle of the \( m \)th order of the diffraction peak relative to the \( z \)-axis, \( k_{ph} \) is the wave vector of the incident light with \( k_{ph} = 2\pi / \lambda \), and \( \Lambda \) is the wavelength of the grating.

If \( m \) is large enough, \( \alpha_m \) becomes larger than \( k_{ph} \). Consequently, \( k_z \) becomes purely imaginary, representing an evanescent wave \( E_m e^{-\beta x} e^{i\alpha_m x} \), propagating along the \( x \)-axis but damped along the \( z \)-axis. Otherwise, each term of the expansion represents a propagating plane wave, monitored as diffraction maximum. From Figure 2.7 one can see this by the superimposing of wave vectors. The \( x \) component of the \( k_{ph} \) gains discrete momentum \( mk_g \) from the periodic grating structure, which gives rise to the discrete deviation the angle \( \theta_m \) of the \( m \)th diffraction order from the zeroth diffraction order, i.e., the spectrally reflected beam. If \( k_{ph} \sin \theta_m + mk_g \geq k_{ph} \), no real \( z \)-component of the photon can exist. Consequently, the light couples into the grating.

Clearly, for those radiative diffraction orders, the positions of the diffracted orders are given by:

\[ \sin \theta_m = \sin \theta_0 + m\lambda / \Lambda \]

This is well known as the grating equation. Note that the spacing of the diffracted orders is dependent on the light wavelength and the grating periodicity, but is not on the grating amplitude and light power. For a grating with periodicity of \( \Lambda >> \lambda \) (note: this is different to the grating-coupled SPR case, where \( \Lambda \) and \( \lambda \) should be close for an efficient coupling), the spacing of the diffracted orders near the zeroth order is pseudo-periodic, i.e., \( \theta_1 - \theta_0 = (\theta_m - \theta_0) / m \).

The intensities (powers) of each diffraction order \( I_m \) depend on the grating amplitude \( \Lambda \) and the light power \( I_0 \) and are also found via the diffraction theory. Solutions may be divided into the classes of scalar and vector calculations that ignore and include the
effects of light polarization, respectively. Most scalar diffractions yield diffracted orders $I_m$ that are proportional to a summation of squared Bessel functions$^{62}$. Most of the calculations can be reduced to a simplified equation for the low-angle diffraction on ‘smooth’ gratings ($A<<\lambda$).

$$\frac{I_m}{I_1} \propto \left[ J_n\left(\frac{2\pi A}{\lambda}\right)\right]^2$$  \hspace{1cm} (2.29)

We can see that the diffraction intensity has a quadratic relationship with the grating amplitude $A$. A more accurate vector perturbation result, developed in the radar literature$^{63}$ and based on earlier diffraction calculations in 1907$^{64}$, has been introduced by Church$^{65}$. This relationship is commonly referred as the Rayleigh-Rice vector perturbation theory. The theory considers different situation of two orthogonal polarizations, i.e., TM and TE polarization, respectively. In either case, the quadratic relationship between $I_m$ and $A$ is preserved. In a more general picture, for periodic modulations with index-of-refractive amplitude ($\Delta n(\lambda) d$) and adsorption coefficient amplitude ($\Delta k(\lambda) d$), but with the material being only weakly adsorbing, the Equation 2.29 can be expanded for the first several diffraction orders$^{66}$:

$$\eta = \frac{I_m}{I_1} \propto \left(\frac{\pi \Delta nd}{\lambda}\right)^2 + \left(\frac{\Delta kd}{4}\right)^2$$  \hspace{1cm} (2.30)

where, $\eta$ is called the diffraction efficiency of the $m$th diffraction order.

### 2.2.2 Fourier diffraction optics

In the Fraunhofer approximation, a plane electromagnetic wave passing a transmission function $\tilde{T}(\tilde{v})$, the field distribution becomes:

$$\tilde{T}(\tilde{u}) = C \int \tilde{T}(\tilde{v}) e^{j2\pi(\tilde{u} \cdot \tilde{v})} d\tilde{v}$$  \hspace{1cm} (2.31)
which is the Fourier transform of the transmission function with respective to position \( \bar{u} \). For gratings, they can be considered as finite one-dimensional periodic optical modulations. The transmission function may be expressed as the convolution of a single gate function \( g(x) \) and a finite array of delta functions with periodicity of \( \Lambda^67 \).

\[
\tau(x) = \sum_{n=-(N-1)/2}^{(N-1)/2} \delta(x - n\Lambda) * g(x) \tag{2.32}
\]

with:

\[
\delta(x) = (x - \chi) = \begin{cases} 
0 & x \neq \chi \\
\infty & x = \chi
\end{cases},
\]

\[
\int_{-\infty}^{\infty} \delta(x - \chi) dx = 1,
\]

\[
g(x) = \begin{cases} 
0 & \text{if } |x| > a/2 \\
d & \text{if } |x| > a/2
\end{cases},
\]

and \( a < \Lambda \).

Its Fourier transform is:

\[
T(u) = d \frac{\sin(\pi au)}{\pi u} \left[ \sum_{k} \delta(u - k\Lambda) * \frac{\sin(\pi k\Lambda u)}{\pi k\Lambda u} \right]. \tag{2.33}
\]

The diffraction intensity is

\[
I(u) = T(u)^2 \propto d^2 \tag{2.34}
\]

Thus the height of each of the diffraction intensity maxima is proportional to the square of the gate height \( d \), equivalent to the grating amplitude. This also confirms the quadratic relation shown in Equation 2.30.
2.3 Fluorescence

2.3.1 Fluorescence process

Fluorescence is a result of a three-stage spontaneous process that occurs in certain molecules (generally polyaromatics hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. The process responsible for the fluorescence of fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram), as shown in Figure 2.8.

Upon the adsorption of a photon of energy $h\nu_{EX}$, the fluorophore is excited from the ground state ($S_0$) to one of the vibrational levels of a higher singlet state ($S_1'$) (Figure 2.8). The excited state only exists for a finite time (usually 1-10 nanoseconds). During this time, it undergoes changes such as conformational change and interacts with its environment in many different ways (Figure 2.8). Consequently, the energy from the excited state ($S_1'$) is partially dissipated, yielding a relaxed singlet excited state ($S_1$) from which the fluorescent emission occurs.

Figure 2.8. Simplified Jablonski diagram. The energies of some electronic ($S_0$ and $S_1$) and vibrational states of a molecule are sketched.
Finally, the reconversion to $S_0$ from $S_1$ emits a photon of energy $h\nu_{EM}$ (Figure 2.8). It is important to note that not all of the excited species return to the ground state via fluorescent emission. Many other processes can occur with their respective rate constants, such as fluorescence resonance energy transfer (FRET, $k_{ET}$), intersystem crossing (ISC, $k_{ISC}$), internal conversion (IC, $k_{IC}$) or collisional quenching (CQ, $k_{CQ}$), which can depopulate molecules from $S_1$. They all influence the fluorescence quantum yield $\Phi$, which is the ratio of the number of fluorescence photon emitted to the number of photon adsorbed.

Since the overall fluorescence probability is governed by the rate constants of the fluorescence process ($k_F$) and the depopulation processes ($k_D$), another expression of the quantum yield is:

$$\Phi = \frac{k_F}{k_F + k_D} \quad \text{--- 2.35}$$

where $k_D = k_{ET} + k_{ISC} + k_{IC} + k_{CQ}$. Due to energy loss during the excited state lifetime, $h\nu_{EM}$ is smaller than $h\nu_{EX}$. Therefore, the emitted fluorescence photons have longer wavelength (lower frequency) than the photons responsible for the excitation. This wavelength difference is termed the Stokes shift, which allows the emitted fluorescent photons to be easily distinguished from the excitation photons, leading to the possibility of a very low background in fluorescent studies.

### 2.3.2 Fluorescence quenching and self-quenching

Quenching refers to any process that causes a reduction in the quantum yield of a given fluorescence process. Quenching can be either collisional or static. The collisional quenching occurs if the quencher comes into contact with the excited fluorophore during the fluorescence lifetime and causes the dye to return to the ground state without emitting a photon, described by the Stern-Volmer Equation:

$$I_0/I = 1 + k_q[Q] \tau \quad \text{--- 2.36}$$
Where $I_o$ is the fluorescence intensity in the absence of quencher, $I$ is the intensity in the presence of the quencher at concentration $[Q]$, $k_q$ is the rate of collisional quenching, and $\tau$ is the observed lifetime.

Static quenching is due to the formation of a ground state complex between the fluorescent molecule and the quencher with formation constant $K_c$, described by:

$$\frac{I_o}{I} = 1 + K_c [Q]$$  \hspace{1cm} (2.37)

One of the interesting phenomena about fluorescence is that multiple labeling of a molecule with a fluorophore does not always lead to an increase in fluorescent intensity. The brightness of a fluorophore is defined as the product of the extinction coefficient ($\epsilon$) and the quantum yield ($\Phi$):

$$Brightness = \epsilon \times \Phi$$  \hspace{1cm} (2.38)

If we conjugate $N$ fluorophores to a molecule, the overall brightness can be described as:

$$Brightness = \epsilon \times \Phi \times N$$  \hspace{1cm} (2.39)

In many cases, as $N$ increases, the overall brightness does not increase linearly and sometimes even decreases. This is known as a phenomenon of inter-conjugate "self-quenching" of the conjugated fluorophores. Different fluorophores differ in their ability to self-quench. It has been confirmed that the more hydrophobic the fluorophore, the lower the ratio of fluorophores/conjugate to which quenching will occur. Intra-conjugate "self-quenching" is also observed if conjugates are densely packed. Generally, self-quenching is considered as a result of excited-state interactions (collisional quenching), or, of the formation of non-fluorescent complexes (e.g. dimers).

### 2.3.3 Fluorescence resonance energy transfer

Resonance energy transfer, often known as fluorescence resonance energy transfer (FRET) or Förster energy transfer, is the transfer of excitation energy from a donor to an
acceptor. An important consequence of this transfer is that the emission of light by the donor is greatly reduced. The acceptor may or may not be fluorescent.

![Figure 2.9. Schematic example of energy transfer efficiency dependent on the distance of donor-acceptor. The Förster radius (R₀) is shown.](image)

FRET is a distance-dependent interaction where the energy transfer occurs typically over a distance of 1-10 nm, making it useful over distances comparable with the dimensions of biological macromolecules (e.g., the molecular beacon technology\textsuperscript{70}). The efficiency of FRET is a key factor, which is dependent on the inverse sixth power of the intermolecular separation (R) of dipole-to-dipole interaction given by the following equation:

\[
\Phi_{ET(R)} = \frac{R^6}{R^6 + R_0^6}
\]

The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Förster radius (R₀).

### 2.3.4 Photobleaching

A typical fluorophore can undergo a finite number of excitation-relaxation cycles prior to photochemical destruction. This process is often referred to as photobleaching,
photofading or photodestruction. For a photostable fluorophore, e.g., tetramethylrhodamin, photobleaching occurs after about 105 cycles. In contrast, fluorescein photobleaches very easily. Generally speaking, the photobleaching involves the generation of reactive oxygen molecules, thus it is sometimes useful to introduce antioxidants or to use anoxic conditions. On the other hand, the rate of the photobleaching is often proportional to the intensity of illumination. Therefore, a simple practical way to overcome this is to reduce the time or the intensity of the excitation radiation.

### 2.3.5 Fluorescence at the metal/dielectric interface

![Figure 2.10. Schematic drawing of a fluorophore positioned close to a metal/dielectric interface. Different fluorescence decay channels take place at different fluorophore/metal separation distances.](image)

A fluorophore close to a surface can be excited by the evanescent wave. For that, the fluorophore should be placed within the decay length of the evanescent field for excitation, which is typically several tens to hundreds of nanometers for applicable wavelengths. This makes the detection to be surface sensitive and compatible to modern biosensor geometries. Surface plasmon fluorescence spectroscopy (SPFS) and total internal reflection fluorescence spectroscopy (TIRF) belong to this category. TIRF uses a dielectric sensor surface that is illuminated by an evanescent field created by
totally internally reflecting a laser beam and has been well developed to be a mature fluorosensor. Femtomolar sensitivity was reported with a channel-etched thin film waveguide fluoroimmunosensor\textsuperscript{72}. TIRF microscope has been even commercialized allowing for the observation of spatially resolved fluorescence images\textsuperscript{73}. Compared with TIRF, there are at least two advantages of using surface plasmon evanescent waves to excite the fluorophores locating at the interface. Firstly, the enhanced evanescent field excited by SPR exhibits greatly enhanced intensity more than the TIR case\textsuperscript{31}. Secondly, the semi-transparent metal film acts as an efficient blocker to reduce the background contribution from the excitation light source. However, introducing the metal layer alters the way an excited fluorophore loses its energy. As reviewed\textsuperscript{31,74}, there are additional decay channels which are contributing to the decrease of the radiative quantum yield of the emitters (fluorophores) and they take place in different dye-metal separations (summarized in Figure 2.10).

If a dye is positioned at a distance within 10 nm to the metal surface, the non-radiative decay of fluorescence is the dominating process. The excitation is assumed to be dipole-to-dipole, if it is due to the excitation of an electron-hole pair (exciton) in the metal. The standard Förster model gives a $R^6$ dependence of the transfer rate to the separation distance (cf. Figure 2.9), due to the co-effect from both near fields of the donor molecule and the acceptor molecule. However, the distance dependence of energy transfers involving a dye and a surface (could be rough) can be greatly compromised to be $R^3 \sim R^4$ dependent due to the integration over the enlarged number of effective acceptor sites. The transferred energy dissipated by the metal is converted into heat.

At an intermediate-distance regime (a few nm up to ~20 nm), a significant fraction of excited fluorescence couples back to surface plasmon polaritons, by fulfilling momentum-matching conditions. Unless SPP modes can be converted again into photons by a coupling-prism or a grating, which allows for the monitoring of SPP decay channel\textsuperscript{15}, it represents a significant loss of fluorescence yield.

At sufficient separation distances (>20 nm), free emission of the dyes dominates. However, the fluorescence yield cannot be directly obtained unless two effects are considered. Firstly, the fluorescence emission oscillates as the distance increases, since the metal reflects the fluorescence field and introduces light interference. Secondly, the excitation source, i.e., the evanescent field weakens as the distance increases.
3 MICRO AND NANOTECHNOLOGIES: AN OVERVIEW

There are two distinct approaches that can be taken toward fabrication of micro and nanometer scale structures:

- Top-down approach
- Bottom-up approach

The first approach is to pursue fabrication from the top down, shaping down from the large to the small: pattern transferring techniques, combined with additive and/or subtractive processes, are used to model existing materials into desired forms. The second approach can be defined synthetic, as micro and nanostructures built through the additive assembly of subunits: atom-by-atom, molecule-by-molecule from the bottom up. These methods are often inspired by natural processes (biomimetic) and include manufacturing options such as self assembled monolayers (SAM), self-repair, individual particle manipulation tools, Langmuir-Blodgett films and many others.

Top down miniaturization methods surveyed are micromachining and nanomachining. Micromachining, also defined as microfabrication or microelectromechanical systems (MEMS), refers to the fabrication of devices with at least some of their dimensions in the micrometer range that enable mechanical and electrical functionality. The potential of MEMS lies in the fact that miniature components are batch fabricated using the manufacturing techniques developed in the semiconductor microelectronics (IC, Integrated Circuit) industry enabling miniaturized and low-cost systems that integrate sensing and actuating functions. MEMS are now widely used in automotive (sensors are used, for instance, for crash detection and engine control), environmental and security areas. The application of MEMS technology to the health care has generated a new class of microdevices called bio-MEMS. Hot topics of bio-MEMS research are diagnostic (lab-on-a-chip) and drug delivery systems for clinical and home use. Microneedles, micropumps and microdispensers are examples of drug-delivering bio-MEMS under
study. Nanomachining, also nanoelectromechanical systems (NEMS), is a typical top down approach into submicron range, relying on advanced IC fabrication methods. Advanced nanofabrication techniques are described, along with the more common microfabrication techniques. Many top-down miniaturization methods are based on lithography, the technique used to transfer copies of a master pattern onto the surface of a solid material such as silicon wafer. Only the top-down miniaturization methods based on lithography will be surveyed here. Particular attention will be given to materials, processes and techniques employed for the fabrication.

3.1 Lithographic techniques

Each micro-nanodevice fabricated using a top-down approach based on lithography is built, layer by layer, alternating three fundamental processes: lithography, deposition and etching. A typical flow diagram is shown in Fig. 3.1. A film of metal, semiconductor or insulator material is deposited onto the substrate, usually a silicon wafer. Then, a thin layer of a polymeric material, named resist, is deposited onto the substrate by means of spin coating techniques. The resist is a material that changes its solubility into a particular solvent, known as developer, when exposed to radiation (UV, X-rays, electrons, ions). The resist is then exposed, through a mask in the case of UV-radiation and X-rays or directly in the case of electron or ion beam. The substrate is then developed. The developer solution removes the exposed or unexposed areas depending on the tone of the resist. Positive tone resists become more soluble when exposed to radiation while negative tone ones become more insoluble. The pattern can be transferred to the deposited film via etching techniques (wet etching or dry etching) generating a 3D structure. The resist is then removed (stripping process) and the steps described above are repeated to obtain multi-layer structures. Each step of the flow diagram will be described in detail in the following sections.
Figure 3.1. Basic processes for a micro-nanodevice fabrication via top-down approach based on lithographic techniques. Process steps include film deposition, resist spinning, exposure, development, etching and resist stripping.

Depending on the radiation source, we can distinguish between different types of lithographic techniques:

- UV lithography or photolithography
- X-ray lithography (XRL)
- charged particles lithography
  - electron beam lithography (e-beam lithography or EBL))
  - ion-beam lithography (i-beam lithography)
Here only electron beam lithography and X-ray lithography (XRL) will be described.

3.1.1 Electron Beam Lithography

Electron-beam lithography (EBL) is a high resolution patterning technique in which high-energy electrons (10 to 100KeV), focused into a narrow beam, are used to expose electron sensitive resist\textsuperscript{77}. The EBL tool consists of three components: an electron optical column, a mechanical stage that holds the substrate in the sample chamber and control electronics. The column is the part of the EBL system that forms the electron beam. It typically consists of an electron source and a beam alignment system: a mechanism for its deflection, a blanker for its turning on and off, apertures and lenses to shape and focus it (see Fig. 3.2). Electrons may be emitted from a conducting material either by heating it to the point where the electrons have sufficient energy to overcome the work function barrier of the conductor (thermionic sources) or by applying an electric field sufficiently strong that they tunnel through the barrier (field emission sources). Both the column and the sample chamber are in high vacuum (10–6–10–10 Torr) so to avoid electron scattering by gas molecules. The maximum area that the beam can write without aberrations due to the electro-optical system is named the maximum field size. This parameter is characteristic of each lithographic system. If a pattern bigger than the maximum field size has to be written, the substrate stage is moved. The stage movement is controlled at nanometric level via an interferometric system. The mismatching between adjacent fields is known as stitching error. Sophisticated computers and electronics precisely control all the system parameters.

The theoretical resolution limit of EBL corresponds to the spot size dimension, $d$, that is a function of the source dimension and the optical elements of the column. A full explanation of these phenomena is beyond the aim of this thesis; however, newer EBL tools have theoretical resolution limit lower than 5 nm. Unfortunately, the resolution of EBL tools is not simply the spot size of the focused beam. Resolution is affected also by scattering of the electrons inside the resist and substrate (forward scattering) and by
back scattering from the substrate that cause the resist exposure over a greater area than the beam spot size (see Fig. 3.3).

Figure 3.2. Schematic of e-beam system.

Figure 3.3. Schematic of electron scattering in electron resist exposure: small angle forward scattering, high angle backscattering and secondary electrons.
As the electrons penetrate the resist, some of them will undergo small angle scattering events (forward scattering), which can result in a significantly broader beam profile at the bottom of the resist than at the top. The increase in effective beam diameter, $d_f$, in nanometers, due to forward scattering, is given empirically by the formula:

$$d_f = 0.9 \left( \frac{R_t}{V_b} \right)^{1.5}$$  \hspace{1cm} (3.1)

where $R_t$ is the resist thickness in nanometers and $V_b$ is the beam voltage in kilovolts.

Forward scattering is minimized by using the thinnest possible resist and the highest available accelerating voltage. As the electrons continue to penetrate through the resist into the substrate, many of them will experience large angle scattering events. These electrons may return back (back scattering) through the resist at a significant distance from the incident beam, causing additional resist exposure. The range of the electrons (defined here as the distance that a typical electron travels in the bulk material before losing all its energy) depends on both the energy of the primary electrons and the type of substrate.

Contrary to forward scattering, back scattering range is maximized by using high accelerating voltage. The net result of the electron scattering is that the dose delivered by the electron beam tool is not confined to the shapes that the tool writes, resulting in pattern specific line width variations known as the proximity effect. For example, a narrow line between two large exposed areas may receive so many scattered electrons that it can actually develop away (in positive resist) while a small isolated feature may lose so much of its dose due to scattering so to obtain its incomplete development.

Many different schemes have been employed to minimize the proximity effect: some are based on the experimental adjusting of the required overall dose in order to obtain the desired pattern dimensions, some others are based on computational simulations. Minimizing or correcting the proximity effect, the newest EBL tools reach resolutions of about 5 nm.
3.1.1.1 Electron beam resist

Electron beam resist are radiation resist: polymeric materials that change their chemical and/or physical properties when exposed to ionizing radiation. Resist for e-beam lithography are usually good resist for other lithographic techniques based on ionizing radiation: i-beam lithography and XRL. When electrons strike a resist, there are two main physical reactions which take place. One is excitation, where an impinging electron’s energy is absorbed by an atom, thus leaving the atom in an excited state. A second reaction is ionization, where an incoming electron provides enough energy to cause an electron to be removed from an atom. A cascade of electrons is then generate into the resist layer where many different chemical reactions take place, broadly classified as either chain-scission (for positive electron resist) or cross-linking reactions (for negative electron resist). Although the energies involved in electron bombardment are so high that, in principle, any material can function as a resist, the chemical modification of the polymeric resist is very specific. Moreover, sensitivity, contrast and resolution capabilities of a polymer have to be considered in labeling it as EBL resist. Polymethylmethacrylate (PMMA) was one of the first positive radiation resists developed for EBL in 1968 and it remains to this date, one of the highest resolution resists available (about 10 nm). The main chain-scission reaction is shown in Fig. 3.4, where the first step is induced by electron-beam irradiation. One of the major disadvantages of PMMA is low sensitivity. PMMA is available in low-molecular weight form and high-molecular weight form, the former being more sensitive than the latter. A lot of positive and negative e-beam resist are commercially available but, in general, slow resist (low sensitivity) have higher resolution than fast resist. This sensitivity-resolution trade-off can be outlined as follow. The substrate to be exposed by electron beam has to be thought as subdivided into a grid. Each element of this grid is a pixel, the minimum resolution element that can be written, as previously defined. Pixels are combined to form pattern shapes.
To form a useful image in the resist, a minimum total number of electrons, \( N_m \), have to strike each exposed pixel. For a given resist sensitivity, \( S \), this minimum is:

\[
N_m = \frac{SLp^2}{q}
\]  

where \( L_p \) is the minimum pixel dimension in cm (in other words the maximum resolution), \( q \) is the electron charge. From the equation (3.8) it is easy to conclude that the product of sensitivity and pixel size is a constant fixed \( N_m \).^{79}

### 3.1.2 X-ray lithography

The use of X-rays in (semiconductor) lithography has been proposed by Smith in 1972\(^8^0\) as a simple proximity imaging system (i.e. there is no need for a demagnification optics). In spite of its strengths (minimum resolution about 30nm, no beam straggling in resist), the prerequisite of a powerful and reliable X-ray source (which can be practically obtained only at a synchrotron facility) has reduced the diffusion of XRL,
which is up to now only used as a research tool. Nevertheless XRL is a powerful tool that allows fast and accurate replicas of masks.

3.1.2.1 Physics of X-Ray Lithography

X-rays are electromagnetic waves with a wavelength \( \lambda \) in the range of 0.1-100, with a subset of 0.6-44 often called soft X-rays. Like all elementary excitations, they have to be treated as waves or particles, depending on the experiment that is performed. In general, it is convenient to think of them as photons (thus, particles) when considering the energy and the related issues (absorption, statistics) and as waves in: optical-like phenomena (diffraction, interference). An equivalent approach is to consider X-rays propagating as waves and interacting as photons.

3.1.2.2 X-rays matter interactions

In the soft X-rays, the adsorption is dominated by the photoelectric effect\(^{81,82}\). At the energies around 1 -2 keV (common in XRL), the electric field of the radiation couples most efficiently with the atoms' inner electrons. The adsorption of a photon result in the excitation of a photoelectron from a bound atomic state to the unbound continuum\(^{83}\), thus yielding an absorption spectrum characterized by edges (bound – unbound), rather than lines (bound – bound). A primary photoelectron, created by absorbing a photon, may have significant amount of energy. For example, the energy of a photoelectron created by absorbing a 1keV photon may be several hundreds of eV\(^{82}\). Obviously, such an electron ca, in the process of deceleration, produce several secondary excitations and ionization (electrons).

A different process leading to secondary electrons generation is the Auger effect. Since ionization by X-rays creates holes in the inner shell or valence band, the filling of these holes is accompanied by photo emission (fluorescence) or passes through a two Auger electron process: on electron fills the hole, lowering its energy, and the second electron carries off this excess energy, going into a delocalized state.
The ratio of the probability of fluorescence to the probability of an Auger process increases rapidly as the atomic number $Z$ increases (roughly as $Z^4$). Therefore, when material composed of light-weight atoms are irradiated by x-rays, the filling of a hole is most often accompanied by generation of Auger electrons which, just as high energy photoelectrons, can cause secondary excitations and ionizations that, in the end, are responsible for resist molecules disruption. The detailed description of the photoelectric and Auger effects are commonly found in many textbooks, as, for example, the Valiev book.82

### 3.1.2.3 Image formation

In order to have a better understanding of the XRL processes, it is useful to give a multilayer description of the mask-wafer system during the exposition. The lithographic apparatus can be thought as formed by a series of cascades subsystems, whereby the output of each element provides the input of the following one. This is particularly true with X-rays, here the low value of reflectivity gives not return signal.84

In general, it is possible to study an optical system by solving the wave equation on planes orthogonal to the optical axis, with particular attention given to the boundaries between different media. The input of each element is the electric field $\mathbf{E}(x, y; \omega)$, describing the image at the plane, and the result is the result of the transformation due to the optical element. The same is true with X-rays and the analysis is simplified by the impossibility of strong optics. Optically speaking, the X-ray lithography system is described by the incoming radiation (illumination), the image formation (mask), the propagation region (gap) and the absorbing material (resist). A sketch of the model is displayed in Figure 3.5.

In the case of a lithographic image, it has to be distinguished among different types of images that are created on the way towards the final developed resist:

- Mask output – The field intensity $I(x, y)$ immediately after the mask, units $mW/cm^2$
• Aerial image – The intensity of the electric field $I(x, y)$ after propagation through the gap to the resist surface, before absorption. It is independent on the resist and its units are $mW/cm^2$

• Dose image – The energy absorbed by the resist volume, $D(x, y, z)$, in $mJ/cm^2$

• Latent image – The distribution of the chemical species in the resist created by the absorption of X-rays

• Development image – The distribution of material left on the substrate after the development of the exposed resist.

These images are formed sequentially and are incoherent (in the image formation sense) with each other. Each is formed as the result of a complex sub process, which may or may not be linear. For instance, the formation of the latent image is highly nonlinear.
In order to obtain the true image, it is not sufficient describing the process in term of shadow casting, but it is necessary to use the full power of the diffraction theory. As a simple consideration, the role of diffraction effects on proximity lithography can be argued taking into account the diffraction pattern generated by a thin aperture. The displacement of the first satellite peak in the diffraction pattern is given by \( \delta = g \frac{\lambda}{d} \).

For typical values of the X-ray wavelength used in proximity lithography, \( \lambda = 1 \text{nm} \), of the aperture width \( d = 200 \text{nm} \), that we identify with a typical mask feature, and the fenditure-screen distance that in the example is the mask-substrate gap \( g = 15 \mu\text{m} \), the displacement \( \delta \) results to be 75nm. The linear dependence of \( \delta \) on the mask-substrate gap shows that only a zero gap eliminates any diffraction effect.

Using the Fresnel diffraction theory\(^5\), it is possible to numerically calculate the diffraction effects in XRL. An example is shown in Figure 3.6.

![Figure 3.6. Intensity of the diffracted field beyond a 0.25\( \mu\text{m} \) thick mask.](image)

Since some gap between the mask and the sample is physically unavoidable, some diffraction effect will be always present in a XRL process. Thus there are two different effects that concur in image blurring: the mean free path of electrons (similarly to the proximity effect in e-beam lithography) that increases inversely to the wavelength (the
electrons are more energetic if generated by more energetic X-rays) and the diffraction that instead decreases proportionally to $\frac{1}{\sqrt{\lambda}}$ (Rayleigh criterion). In order to find the more suitable wavelength for XRL, or to have a measure of the pattern ability of a XRL system, the function describing these two effect can be plotted. The result is shown in Figure 3.7.

3.1.2.4 LILIT Beamline

The proximity x-ray lithography processes have been performed at the LILIT beamline located at Elettra Synchrotron (Trieste). A schematic representation of the main optical and mechanical constituents of the beam line is shown in Figure 3.7.

Figure 3.7. Scheme of the LILIT beamline. Note the bottom inset showing the photon energy spectrum along the beamline after the main optical elements.
The beamline consists of one plane mirror (mirror 1) placed at 14nm from the bending magnet source and of a second toroidal mirror (mirror 2) placed 2m away from mirror 1. Both mirrors are gold-coated with an average surface roughness of 0.5nm. The total length of the beamline, calculated from the front-end port of the bending magnet, is 13.3m. Entry slits select the solid angle under which the synchrotron radiation is collected, fixing the maximum values at 6 and 0.83mrad (3σ) respectively in the horizontal and the vertical plane. The latter value corresponds to the natural divergence of the synchrotron radiation in the soft x-ray range under the standard operating conditions of the beamline. Just after the bending magnet front end, a 25µm thick beryllium window separates the vacuum between beamline and synchrotron ring. Additional energy filters are located before mirror 1. Further adjustable slits for final beam shaping are place after mirror 2 close to the end of the beamline where, after a photon shutter and a second 25µm thick beryllium window, the x-ray beam is delivered in a helium flux at atmospheric pressure to avoid undesired X-rays absorption by air. Both the mirrors operate at grazing angles (θ₁, θ₂) producing an image of the source at the wafer/mask location whose size is 3 x 50mm² (vertical by horizontal). Whereas the value of θ₁ can be freely chosen in the range from 0.3° to 2.8°, the amplitude of θ₂ and the height of the second mirror are, in contrast, determined by the constraints imposed by the fixed height from the ground of the source and of the final beam spot.

LILIT beamline is designed for performing both soft and hard x-ray lithography. The energy spectrum provided by the bending magnet of Elettra is quite wide, extending from a few hundred eV to 15keV, as shown in the inset in Figure 3.7. The sequence of the energy spectra along the beamline shows how the effects of the different optical elements allow to select the optimum energy spectrum. In general the rejection of the low energy part of the spectrum (100eV-2keV), responsible for diffraction effects in one-to-one deep x-ray lithography, is performed by a series of eight films of different materials (Be, C, Al, Cr, Si₃N₄) and thicknesses. These films can independently intercept the beam providing 16 combinations of different high band pass filter energy.
3.2 Deposition techniques

Lithography is only one of the fundamental steps of the micro-nanofabrication process. Once transferred into the resist material, the two-dimensional pattern has to be converted into a three-dimensional structure. Deposition and etching techniques make it possible via additive and subtractive approaches respectively. Deposition techniques allow depositing a thin layer of material onto the substrate, usually a silicon wafer. This section surveys the most important deposition techniques focusing on evaporation technique.

3.2.1 Physical Vapor deposition - Thermal evaporation

Figure 3.8. Contaminants compete with the intended source for deposition and may lead to undesired chemical reactions.
Many different kinds of thin films are deposited via thermal evaporation and sputtering, both of which are examples of Physical Vapor Deposition, PVD. Thermal evaporation represents one of the oldest deposition techniques of thin film.\textsuperscript{88} Thermal deposition works by creating a temperature gradient between a source of material to be deposited ("the source") and a surface to be coated ("the substrate"). By heating the source, the vapor pressure of the source material is increased; the evaporated material condenses on the substrate.

There are two main constraints to making this method practical, both kinetic in nature: 1) The deposition rate has to be high enough to achieve reasonable process time, and 2) other materials ("contaminants") may compete with the intended source material for deposition and/or may react with the source or substrate materials. Some contaminants, in particular organic matter, may thermally decompose at the heated evaporation source, generating a multitude of secondary contaminants (Figure 3.8.). Both constraints lead ultimately to the necessity of depositing in high vacuum (Figure 3.9).

![Figure 3.9. Block diagram of the evaporator setup.](image)
Evaporation is based on the boiling off or sublimation of a heated material onto a substrate in a vacuum chamber. The metal to be evaporated, target, is usually evaporated by passing a high current through a highly refractory metal containment structure (e.g. a tungsten or graphite boat). This method is called resistive heating. Resistive evaporation is simple but easily spreads contaminants that are present in the boat. Resistive heating has been surpassed by electron beam (e-beam) evaporation. In the e-beam mode, a high intensity electron beam gun (3 to 20KeV) is focused on the target material that is placed in a water-cooled boat. The electron beam is magnetically directed onto the evaporating material, which melts locally. In this way the metal forms its own crucible, resulting in fewer source contamination problems. e-beam evaporation also provides a higher evaporation rate. Thermal evaporation is limited to elements or simple compounds whose vapor pressure range from 1 to $10^{-2}$ Torr, typical pressure of an evaporation chamber, in the temperature interval from 600 to 1200 °C. Pressure and temperature of the evaporation chamber are related to the mean free path of gas species; by kinetic molecular theory:}

$$\lambda = \left(\frac{\pi RT}{2M}\right)^{1/2} \frac{\eta}{p}$$

where:

- $R$ = ideal gas constant
- $T$ = temperature
- $M$ = mass of the evaporating specie
- $p$ = pressure chamber
- $\eta$ = gas viscosity

An estimate for practical purpose is $\lambda \times p \approx 50 \times m$ Torr; for an operative pressure of $10^{-2}$ Torr, the mean free path is about 50 cm, that is a typical distance between substrate and target, $d$, in a evaporation chamber (see Figure 3.10).
The growth rate, $A$, of the film is proportional to:

$$A \propto \frac{\cos \beta \cos \theta}{d^2} \quad 3.4$$

where $\beta$ and $\theta$ are defined in Fig. 3.10. This has some consequences for evaporation on substrates with surface features such as steps or trenches. Locally the angle $\beta$ is changing, with the consequence that the film thickness will change accordingly. This implies a shadow effect which can be used in the so called lift-off process.

### 3.2.2 Lift-off processes

The lift-off is a technique that allows to define thin metallic pattern on a substrate with very good resolution (some tens of nanometers). In the lift-off process sequence, shown in Figure 3.11, a solvent, usually acetone, dissolves the photoresist underneath a deposited metallic film, starting at the edge of unexposed photoresist, and lifts off the metal.
Figure 3.11. Example of lift-off sequence.

Stripping of the resist and metal deposited on its top can be helped dipping the sample in a ultrasound bath. Resist thickness and profile play an important role in lift-off process. The resist layer has to be thicker than the metallic layer, at least three times. If the metallic layer is very thin, in the nanometer range, vertical resist profile can be sufficient to obtain good lift-off results. The poor adhesion of metal on resist layer can produce small defects, like holes, that help the metal lift-off allowing the solvent penetration in the resist layer. For thicker metallic layers, resists with undercut profiles are needed (see Figure 3.12.a) to produce a discontinuity in the metal deposit so that solvent can get at the uncoated resist wall. For this purpose, proximity effect for positive radiation resist in electron beam lithography or overexposure condition for positive photoresist in photolithography can be exploited. Gaps in metal deposit can be
produced also taking advantage of shadow effect in evaporation technique (see Figure 3.12.b).

Figure 3.12. Optimal lift-off conditions. (a) Undercut profile resist (b) Shadow effect in evaporation technique: vertical or inward sloping wall receive little or no metal deposit, leaving a gap for the resist solvent to dissolve the unexposed resist and lift off the metal on top of it. Rounded deposit profiles are also a consequence of shadow effect.

3.3 Etching techniques

Etching processes are the mostly used subtractive processes in micro and nanofabrication technology. In an etching process, one material has to be removed in specific areas not protected by a masking layer. Let us consider an hypothetic process in which a pattern has been transferred on a resist that coats a material film previously deposited onto the substrate (see Figure 3.13). The resist pattern must be transferred into the film material via etching process. In the situation described above, the patterned resist is a masking layer, the film deposited is the layer to be etched, and the substrate is the stop-layer. The etchants species can be in liquid phase (wet chemical etching) or gas phase (dry etching). Independently of the type of etching we are considering, dry or wet, two main etching process parameters have to be considered: etching bias or directionality and etching selectivity. The resolution of an etching process is a measure of fidelity of pattern transfer, which can be quantified by a parameter named bias, $B$. Bias is the difference in lateral dimension between the etched image, $df$, and the mask image, $dm$, defined as shown in Figure 3.13. A zero bias process produces a vertical edge profile coincident with the edge of the mask, as shown in Figure 3.13. In this case, there is no etching in the lateral direction and the pattern is transferred with perfect
fidelity: complete anisotropic etching. When the vertical and lateral etch rates are equal, the edge profile appear as a quarter-circle after etching has been carried just to completion, as shown in Figure 3.13. In this case the bias is twice the film thickness, $h_f$, and the process is named isotropic. Any edge profile, corresponding to etching just completion, which lies between the extremes depicted in Figure 3.13, results from an etch rate that is anisotropic. We can define the degree of anisotropy, $A_f$, by:

$$A_f = 1 - \frac{v_l}{v_v} = 1 - \frac{|B|}{2h_f}$$

where $v_l$ and $v_v$ are the lateral and vertical etch rate respectively. Thus, for isotropic etching $A_f = 0$, $A_f = 1$ for complete anisotropic etching and $1 > A_f > 0$ represents anisotropic etching. Etching selectivity is the ratio of vertical components of etch rate of target material and of other materials (mask layer and stop layer). Selectivity with respect to the resist mask has an impact on feature size control; selectivity with respect to the substrate can affect device performance.

### 3.3.1 Wet Etching

Many materials, conductors, semiconductors and insulators, can be removed exploiting the selective reactivity of some chemical agents. Wet etching proceeds by reactants transport to the surface (1), surface reaction (2) and reaction products transport away from the surface (3). If (1) or (3) is the rate determining step, etching is diffusion limited and the etching rate may be increased by stirring. If (2) is the rate determining step, etching is reaction rate limited and depends strongly on temperature, etching material and solution composition. Selectivity of the wet etching process involves the different chemical reactivity of materials in contact with the etching solution and has to be carefully evaluated every time.

Wet etching of amorphous materials is isotropic while the order due to crystalline arrangement of atoms in the space can introduce preferential etching directions making the etching process anisotropic.
Figure 3.13 The fidelity of pattern transfer can be quantified by the bias parameter, $B = d_f - d_m$, where $d_f$ and $d_m$ are defined as in figure. Complete anisotropic, isotropic and isotropic etching profiles are also shown.
4 THE BIOLOGICAL APPROACH

4.1 The DNA

Deoxyribonucleic acid (DNA) is a large organic macromolecule, that stores the genetic information in all living cells. DNA consists of a polymer of repeating units called nucleotides. The nucleotides are linked together by phosphodiester bonds to form a polynucleotide chain. Each nucleotide consists of a phosphate group, a five-carbon sugar deoxyribose and one of four different nitrogenous bases, i.e. Adenine (A), Guanine (G), Cytosine (C) and Thymine (T).

The native state of the DNA duplex is known as a double helix, which essentially resembles a twisted ladder structure. In the double helix, two polynucleotide chains combine via base-pairing between nucleotide units in the individual chains. The orientation of the two chains are antiparallel: one chain runs from 3' to 5' and the other runs from 5' to 3'. According to Chargaff's rule, the sum of the purine bases (A, G) in a duplex equals the sum of pyrimidines (T, C). The reason for this is the characteristic Watson-Crick's basepair formation between the A-T, and G-C by hydrogen bonds.

Figure 4.1 A cartoon of the DNA double helix (B-form).
Depending on base composition and physical conditions (hydration and/or salt contents), three different conformations (A, B, Z) of DNA helixes can be formed. Among them, the B-form is the common natural form, prevailing under physiological conditions. As described in Figure 2.1, B-DNA arranges 10 bases per helix tour and the distance between planes of bases is 0.34 nm. The plane of the bases is nearly perpendicular to the helix axis and the helix surface exhibits two prominent grooves (major and minor). The diameter of the helix is ~2 nm.

4.2 DNA hybridization

The key point while analysing DNA sequences is the hybridization and its properties. Hybridization is a term used to describe the specific complementary association due to hydrogen bonding, under experimental conditions, of single-stranded nucleic acids. It should more properly be referred to as "annealing", as this is the physical process responsible for the association: two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" helical molecule. One may make one's nucleic acid single-stranded for the purpose of annealing - if it is not single-stranded already, like most RNA viruses - by heating it in 0.01M NaCl to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling to ±0°C: this ensures the "denatured" or separated strands do not re-anneal.

Alternatively, one may denature DNA reversibly by treatment with 0.5M NaOH: this does not work for RNA, as this hydrolys under these conditions.

The complementary association of two strands of polynucleotides is the basis for replication of all organisms; the complexity inherent in the sequence of the molecules renders the association extremely specific for any molecule longer than sixteen nucleotides. This is easily understood if one considers the combinatorial possibilities of given lengths of "probe" sequence: there is a ¼ chance (4-1) of finding an A, G, C or T (U for RNA) in any given DNA sequence; there is a 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a sixteen base sequence will statistically be present only once in every 416 bases.
The biological approach

(=4 294 967 296, or 4 billion); this is about the size of the human genome, and 1000x
greater than the genome size of E. coli.

Thus, the association of two nucleic acid molecules - presumed to be at least a few
hundred bases long - is an extremely sequence-specific process, far more so than the
widely-used specificity of monoclonal antibodies in binding to specific antigenic
determinants. The correct annealing of two sequences to each other does, however,
depend on the physical and chemical solution conditions under which the reaction takes
place.

4.2.1 Melting temperature

All double-stranded nucleic acids - whether dsDNA, dsRNA or RNA:DNA hybrids -
have specific "melting temperatures", which depend mainly upon their specific
guanine/cytosine content, but also upon whether they are DNA, RNA, or a mixture
(RNA:RNA hybrids have the highest melting temperatures, followed by DNA:RNA
hybrids, then dsDNA), and upon the ionic strength of solution.

The melting temperature is also dependent upon the length of the sequences to be
annealed: the shorter the probe sequence, the lower the melting temperature. The degree
of sequence mismatch also determines the effective melting temperature of a hybrid:
Tm decreases by about 1°C for every 1% of mismatched base pairs. It therefore makes
sense to maximize probe length in order to minimize Tm reduction due both to length
and degree of sequence mismatch. Under standard conditions of annealing (0.8M NaCl,
neutral pH) one may calculate the melting temperature of any given DNA hybrid as
shown:

\[ T_m = 81.5°C + 0.41(%G + %C) - 550/n \]

where \( n \) = probe length (number of nucleotides).

One can see that the reduction in Tm becomes negligible for probes of length 200 base
pair or greater. Thus, one may vary the specificity of association of a specific single-
stranded "probe" and a target by varying the incubation temperature of the annealing
reaction: the higher the temperature, the higher the specificity of the reaction - and the lower the likelihood of annealing taking place.

4.2.2 Hybridization Stringency

The successful use of nucleic acids as probes for sequences of interest therefore depends upon certain reaction conditions which are in turn determined by the physical properties (ie. length and sequence) of the probe. This leads to the concept of stringency of hybridization: one increases the stringency by lessening the likelihood of non-homologous annealing. This can be done by simply increasing the temperature of incubation - bearing in mind that rate of hybridization/annealing is maximal at about Tm – 25°C, and too high a temperature results in very slow annealing. An acceptable compromise is to anneal at a standard temperature (eg. 65°C), and then wash the annealed and immobilized hybrid molecules to varying degrees of stringency: the extent to which one should wash can be assessed by repeated autoradiography, if the probe is 32P-labelled, or by repeated color assay of replicates in the case of non-radioactively labeled probe. Washing stringency may be increased by varying the ionic strength (from 1.0M NaCl to 0.02M), or varying the temperature (ambient to 65°C). One may also include SDS or other detergent in wash and in hybridization buffers in order to decrease non-specific attachment of probe to the adsorptive membrane. For this reason a blocking or pre-hybridization buffer is normally used before and during the annealing reaction, to block adsorptive sites, for examples on membranes, not occupied by target nucleic acid. This normally consists of buffer salts, detergent, protein, inert polymer material, and DNA.

It is possible to include various other constituents in annealing buffers, designed to increase the hybridization rate, or the stringency, or both. For example formamide is a helix destabilizer, and enables one to decrease annealing temperature.

Summarizing, a standard hybridization reaction, then, consists of probing a specific target in a solution, using the immobilized probe on a surface with a specific sequence matching the complementary target; this is done by annealing the probe to the target under (usually) standard hybridization conditions for a certain time.
The biological approach

Probes are usually molecules of DNA and should not be too long, as otherwise one needs very high concentrations of nucleic acid in order to guarantee a sufficient number of copies of the sequence in order to give a detectable "signal" for detection purposes.

4.3 Debye-Hückel theory and Debye length

The principle underlying the Debye-Hückel theory is of a tendency for anions to be found around cations in solution, and of cations to be found around anions. The ions are in ceaseless motion. Overall the solution is electrically neutral, but near any given ion there is an excess of counterions. Averaged over time, counterions are more likely to be found near any given ions. This time-averaged, spherical haze, in which counterions outnumber ions of the same charge as the central ions, has a net charge equal to in magnitude but opposite in sign to that on the central ion, and is called its ionic atmosphere.

The Coulomb potential at a distance r from an isolated ion of charge $ze$ in a medium of permittivity $\varepsilon$ is:

$$\phi_i = \frac{ze}{4\pi\varepsilon r}$$

--- 2.1

The ionic atmosphere causes the potential to decay with distance more sharply than this expression implies. Such shielding is a familiar problem in electrostatics, and its effect is taken into account by replacing the Coulomb potential by the shielding potential, an expression of the form

$$\phi_i = \frac{Z}{r} e^{-r/r_D}$$

--- 2.2

where $r_D$ is called the Debye length, which is a measure of the capacity of the ionic atmosphere arising from the central ion. If $r_D$ is large, the shielded potential is virtually the same as the unshielded potential. If it is small, the shielded potential is much smaller than the unshielded potential, even for short distance.

$r_D$ can be calculated by

$$r_D = \left( \frac{eRT}{2\rho F^2 I_b} \right)^{1/2}$$

--- 2.3
where $I$ is the ionic strength of the solution, $\rho$ is mass density, and $b = 1 \text{ mol kg}^{-1}$.

### 4.4 Electrical double layer

Electrical double layers form spontaneously whenever surfaces carrying ionizable groups are suspended in a polar solvent, most frequently water. The electrical double layer results from the buildup of the charge density (or “cloud”) of opposite sign to that of the surface charge, which tends to screen the surface electrostatic potential. The width of the double layer, which is a measure of its capacity, is determined by the competition between the thermal motion of the microions which tends to spread out or homogenize their distribution in order to increase their entropy, and the electrostatic interactions which attract the counterions towards the surface while repelling the coions. Two regions of charge must be distinguished. Firstly, there is a fairly immobile layer of ions that adhere tightly to the surface, and which may include water molecules (if that is the supporting medium). The radius of the sphere that captures this rigid layer is called the radius of shear. The electric potential at the radius of shear relative to its value in the distant, bulk medium is called the zeta potential, $\zeta$, or the electrokinetic potential. Secondly, the charged unit attracts an oppositely charged atmosphere of mobile ions. The inner shell of charge and the outer ionic atmosphere is called the electrical double layer.

### 4.5 DNA Polyelectrolytes

Polyelectrolytes are described as polymers composed of charged monomers by definition. If a polyelectrolyte is dissolved in water, it may acquire a certain amount of charge. Like strong polyelectrolytes, the monomers may be fully ionized so that the charge on the polyelectrolyte is permanent. If all ionizable groups are fully dissociated we speak of a quenched polyelectrolyte. The ions that are released into the solution are called counterions. The ionizable groups may also be weak acids or bases. This results in a partially ionizable polyelectrolyte. The fraction of ionized groups then depend on
the pH. This case is called an annealed polyelectrolyte. Of course, mixtures of different types of monomers may be present in polyelectrolytes.

From a chemical point of view, a DNA molecule is a linear condensation polymer of nucleotide sub-units. The nucleotide phosphate groups have two ionizable protons whose pKa values depend only slightly on the nature of the base and on the position of the phosphate on the sugar ring. The equilibriums are as follows:

\[
\begin{align*}
\text{R-O--P-OH} & \rightleftharpoons \text{R-O--P-O}^- \\
\text{pK}_a = 1 \\
\text{OF} & \rightarrow \text{OH} \\
\text{pK}_a = 6.7
\end{align*}
\]

Therefore, above pH 7 a nucleotide phosphate primarily carries a doubly negative charge; below pH 6 it has one net negative charge. The phosphodiester in an oligonucleotide or polynucleotide has only one ionizable proton; its pKa is about 1. Thus, above pH 1 each phosphate group in a polynucleotide (except for terminal phosphates) is singly charged. Therefore, DNA can be viewed as a strong polyelectrolyte with a high charge density.

4.6 Electrostatic interaction of metal cations with nucleic acids

As highly charged polyanion, the DNA double helix in solution tends to be screened by counterions. A great deal of studies, both experimentally\(^{92}\) and theoretically\(^{93}\), have demonstrated that a large fraction of the phosphate charge is screened by counterions residing very close to the DNA surface.

According to Manning's proposition\(^{92}\), the high negative charge density of DNA induces a concentrated cloud of mobile and hydrated counterions within \(\sim 7 \text{ Å}\) of the DNA surface. For monovalent cations, the ionic strength of the cloud approaches 1 M and is independent on the bulk cation concentration, if the latter is above a threshold value. This "condensed" layer of counterions is sufficient to neutralize \(\sim 76\%\) of the phosphate charge, but no further, and thus reduces the charge of each phosphate (in a thermodynamic sense) to \(-0.24\) e. Under physiological ionic conditions the Debye screening length of the residual phosphate charge, \(r_D\), is \(\sim 10 \text{ Å} (\sim 3 \text{ bp})\). For divalent and
trivalent counterions, the phosphate charge is reduced to $-0.12\ e$ and $-0.08\ e$, respectively. Therefore, multivalent cations are more effective than monovalent cations in screening the DNA charges. In general, monovalent cations provide highly uniform screening, whereas each multivalent cation creates a local region of excess positive charge surrounded by a region of excess negative charges from the phosphates.

It has been identified that different classes of metal ions have preferred sites of interaction in contacting with nucleic acids. The binding sites for the metal cations are the electronegative oxygen and nitrogen atoms of the nucleic acid constituents. Some metal cations can bind to more than one site, forming cyclic chelate compounds.

### 4.7 Summary of the strategy of the bio-protocol

The architecture chosen to perform the detection of the DNA targets has never been seen in the literature and is a method for the indirect labeled pTNT detection. A fluorescent labeled oligonucleotidic probe is hybridized on the plasmid pTNT, which is previously denatured, on a specific site.

![Diagram](image)

Figure 4.2. Scheme of the architecture used. a. Cy5 labeled oligonucleotide; b. pTNT plasmid; c. molecular recognition layer; d. LaSFN9 slide covered with 50nm of evaporated gold.

This allows avoiding any labeling step of the plasmid itself, and in general of the target if this is long enough. The indirect labeled pTNT is then directly hybridized on the surface of the sensor, which is the molecular recognition layer, where the fluorescence
The biological approach

detection takes place. The hybridization of the indirect labeled pTNT occurs at a plasmid's site opposite to where the fluorescent labeled oligonucleotide is hybridized on the pTNT. Figure 4.2 briefly shows the architecture of the system.
5 EXPERIMENTAL

5.1 Instrumental

This study is based on a surface plasmon resonance set-up in the Kretschmann-Reather configuration, which is also commercially the most implemented configuration. Almost all of the components of this set-up are modular, which directly enables the convenient instrumental upgrade, e.g., combining the electrochemical probing/manipulating or fluorescence detection abilities.

The schematic of the set-up is depicted in Figure 5.1. The beam of a Helium-Neon (HeNe) laser (Uniphase, 5 mW, \( \lambda = 632.8 \) nm) passes through a chopper (frequency = 1331 Hz) that is connected to a lock-in amplifier (EG&G). The modulated beam then passes through two polarizers (Glan-Thompson), by which the intensity and the plane of polarization of the laser can be adjusted. A programmable shutter is installed such as to constantly block the laser (unless data points are recorded), thus minimizing the photo-bleaching effect of the fluorescent dyes. Next, the beam is reflected off the base of the coupling prism (Schott, LASFN9, \( n=1.85 @ 633 \) nm) and is focused by a lens (\( f = 50 \) mm, Owis) onto a collection lens and a photo-diode detector, connected to the lock-in amplifier. The prism/sample and the photo-detector are mounted on two co-axial goniometers respectively, enabling an independent tuning of respective angular positions.

The fluorescence detection unit is mounted towards the base of the prism, rotating together with the prism (sample) at \( \theta \), while the photo-diode detecting the reflected light rotates at 20. The fluorescence emission from the sample surface is collected by a lens (\( f = 50 \) mm, Owis) and passes through an interference filter (\( \lambda = 670 \) nm, \( \Delta \lambda = 10 \) nm, LOT, 80% transmittance) into a photomultiplier tube (PMT, Hamamatsu), which is connected to a photon-counter (Agilent) unit via a home-built electronic interface. Note that the interference filter is specifically designed for commercially versatile fluorophores such as Cy5 (Cyanine 5, from Amersham Pharmacia Inc.) and Alexa Fluor 647 (from Molecular Probes Inc.). Occasionally, a neutral filter (attenuator) is used to
Experimental

attenuate the fluorescence in the case of strong fluorescence intensity, in order to keep the PMT working in the linear input-versus-output range (<1–2 million counts per second). Eventually other optical component can be mounted to allow the use use of different dyes like Cy3 when using a different laser (like a green laser with $\lambda = 543\text{nm}$). Custom programs accomplish the data acquisition and the controlling of the system electronics.

Figure 5.1. Schematic of the SPFS set-up.

5.2 Alignment of the SPR spectrometer

5.2.1 Alignment of the irradiance

Rough alignment

The laser beam has to be aligned parallel to the optical bench. The laser and the reflecting mirror have to be adjusted first in height and tilt on the optical bench so that the laser beam is impinging on the mirror and that the ray direction is approximately
parallel to the optical bench 2. This is possible by turning and moving the laser holder and also by moving the reflecting mirror on the optical bench 2. To do this, special alignment screws are placed at the laser holder and mirror (Figure 5.3). The laser can be aligned in the horizontal direction and also in its tilt. The reflecting mirror can also be tilted horizontally and vertically and turned round horizontally.

Figure 5.2: SPR set-up with particular optical components (for completeness a PC, step motor control and a Lock-In amplifier are used).

Figure 5.3: Rough alignment of laser and reflecting mirror
Installing of iris diaphragm 1
The 1. iris diaphragm (pinhole 1) is positioned as near as possible in front of the reflecting mirror on the optical bench. The height of the iris has to be adjusted to the height of the sample. The frame has to be exactly perpendicular to the laser beam. Now the optical axis is defined as the position of the iris aperture, as it is moved on the optical bench 2. The laser has to be adjusted so that the laser beam impinges on the iris diaphragm 1.

Establishment of a copy
A copy of iris 1 is established for defining the optical axis through 2 fixed iris diaphragms without moving any optical elements. For this one have to move another marking holder to iris 1 to define the exact position on the optical bench. In another step the iris 2 (pinhole 2) has to be positioned instead of iris 1 at the marking holder and aligned in this way that the laser beam passes through iris 2.

Definition of the optical axis
Iris 1 is fixed as near as possible in front of the reflecting mirror, iris 2 far away from iris 1 on the optical bench 2.

Figure 5.4: Position of the iris diaphragms
Experimental

Adjustment of "position" and "direction"
The aim is to adjust the laser beam defined by iris 2 on the optical axis. The misalignment of the laser beam on iris 1 has to be adjusted by moving and tilting the laser (alignment of "position"). By tilting and moving the reflecting mirror the direction of the beam through iris 1 is changing, while the beam should be adjusted to hit the centre of iris 2. Minor deviations from iris 1 could be adjusted by tilting and moving the reflecting mirror.

Final test
Iris diaphragm 1 has to be moved on the optical bench in a way that the laser beam is always positioned in its centre. If this is not the case, the position of the iris diaphragm 2 is not completely correct.

5.2.2 Adjustment of the displacement tables
The displacement tables have to be aligned so that the laser beam is hitting the goniometer axis.

Attachment of a marking
Image 5.5 shows the goniometer set-up. Displacement table 1 and 2 and the tilting tables are presented. At the tilting table holder a rail carrier with a hole and a mark will be fixed (label with a vertical line). Later the prism is positioned there.

Attachment of a marking
Image 5.5 shows the goniometer set-up. Displacement table 1 and 2 and the tilting tables are presented. At the tilting table holder a rail carrier with a hole and a mark will be fixed (label with a vertical line). Later the prism is positioned there.

Figure 5.5: Set-up on the goniometer
**To adjust the optical axis to the goniometer axis**

With a marking label fixed on a rail carrier with a hole, it is checked if the optical axis is lying on the goniometer axis by positioning the marking label with the displacement table 1 for $\theta = 0^\circ$ into the laser beam. The laser beam is hitting the goniometer axis correctly if the laser beam is also hitting the marking label for $\theta = 180^\circ$. If this is not the case, you have to move the optical bench 2 with both displacement tables A and B so that the beam hits the mark. Repeat this procedure until the goniometer axis is reached.

![Figure 5.6: Sample angle theta=0° and 180°](image)

Figure 5.6: Sample angle theta=0° and 180°

![Figure 5.7: Alignment of the optical axis](image)

Figure 5.7: Alignment of the optical axis
**Alignment of the impinged laser beam rectangular to the goniometer axis**
Instead of the marking label an object holder is fixed on the rail carrier. The laser beam must pass through the object holder (Figure 5.7).

**Adjustment and final check**
The laser beam has to be adjusted in that way that it is reflecting back in itself at $\theta_{\text{sample}} = 0^\circ$ and $\theta_{\text{sample}} = 180^\circ$. You can check it with the aid of the iris diaphragms. If this is not the case the incline of the excitation axis has to be changed by the screws 1 and 2 for the height adjustment on the optical bench 2 (Figure 5.7).

### 5.2.3 Alignment of the prism

**Sample preparation and assembly of prism and sample**
For a SPR measurement a gold layer of 50 nm thickness is evaporated onto the substrate (LaSFN9).

For every measurement an index-matching liquid with the same refraction index as the substrate has to be used, (for LaSFN9 substrates the index-matching liquid has a refractive index of $n=1.7$). A very small amount of the liquid is dripped very carefully onto the non-evaporated sample side and the prism base is positioned free of bubbles onto the liquid film. The prism and sample will be positioned for a measurement in solution as shown in the following figure:

![Assembly of prism and sample for measurements in solution](image)

Figure 5.8: Assembly of prism and sample for measurements in solution (schematic)
**Rotation of the prism around its horizontally axes**

The aim is to place all sides of the prism in a vertical position.

The prism holder with prism and sample (Figure 5.8) will be fixed above the displacement tables. The sample goniometer has to be unscrewed, so that you can turn the goniometer bidirectionally without any resistance.

Afterwards the sample goniometer has to be screwed tightly, making sure that the gears are interlocked and that the motors are tightly in place.

Both surface planes of the prism have to be hit by the laser beam while turning round the sample goniometer. If this is not the case, the sample displacement table has to be adjusted again. The tilting tables 1 and 2 have to be aligned in that way that the back reflex is backsliding after reflection of both cathetus planes in itself by choosing appertained angles of $\theta_{\text{sample}}$ (see also Figure 5.5).

Alignment algorithm:

a) For the reflection at cathetus plane 1: the half of the bypassed tilting has to be covered a distance by tilting table 1 and 2

b) Turning the sample goniometer: for reflection at cathetus plane 2 step a) has to be repeated

c) Steps a) and b) have to be checked again till the adjustment fits exactly

**Displacement of the prism**

The aim is that the laser beam is hitting the centre of the prism base and the position is constant while turning the goniometer.

**Method 1**

The laser spot will be observed at the backside of the prism. By turning the sample goniometer it can be seen if the laser spot is moving or not.

**Method 2**

It is possible that the laser reflex can be observed from the top of the prism.
Alignment
First, the sample goniometer will be unscrewed and pivoted by hand through the relevant angle area. The laser reflex has to stay in the middle of the prism base at all angles θ (see figure 5.9).

![Diagram of a glass prism with labeled cathetuses and angles](image)

Figure 5.9: Schematic of the optical path through a glass prism

Alignment procedure:
- a) both displacement tables have to be adjusted
- b) Tip: at smaller sample angles θ alignment of displacement table 1, at larger sample angles alignment of displacement table 2 forward and backward (not all set-ups are the same)
- c) Finally the alignment of both displacement tables has to be checked. To do this the sample goniometer has to be rotated from smaller to larger angles

Both displacement tables are aligned as long as the incoming laser beam corresponds to the pivotal point at the prism base.

Calibration of the inner goniometer
Aim: The sample 0° has to be defined
Method: Set reference point (45°) for the sample goniometer

The sample goniometer has to be aligned in a way that the reflex of the 1. cathetus plane of the prism is perpendicular to the incoming beam. Now the sample goniometer should be screwed tightly, so that the gear is fixed.
Set the reference point (45°)
Next, the sample goniometer has to be positioned by the motor control unit as follows: the directly reflected beam has to pass through the centre of the iris diaphragm. For this position a reference point of 45° will be defined. Finally open the iris, so that does not disturbing the incoming laser beam.

5.2.4 Alignment of the detector arm

Adjustment of the detector goniometer
Aim: The optical axis of the outer goniometer (detector goniometer) has to be parallel to the out coming beam
Method: Set reference point (90°) for the detector goniometer

Rough alignment of the detector goniometer, so that the detector arm is positioned parallel to the out coming beam. The sample goniometer (θsample) is fixed to an angle in the middle of the located measurement range, but a perfect alignment is not possible. An iris diaphragm will be positioned on a displacement table rail carrier on the detector arm and will be aligned with the laser beam. It should be possible to move the pinhole along the optical bench without “loosing” the laser beam. If the beam is lost it has to be corrected with the motor control unit and the displacement table of the pinhole (see figure 5.10 moving direction 2).

Set reference point (90°)
If the description described previously its applicable the reference point for the detector motor in the software the 90° reference point can be set..

Installation of the photodetector
The photodetector is positioned directly behind pinhole 3, so that the laser beam is hitting its centre. It is possible that you have to install it at a displacement table (see
Experimental

figure 5.11). To avoid back reflection from the detector onto the cathetus plane of the prism, the detector should be tilted a little bit.

![Figure 5.10: Topview of the set-up at the detector arm with possible moving directions](image)

**Installation of the lens and the polariser**

The lens is positioned by visual judgement in front of the detector vertically to the laser beam. Afterwards it will be moved on the optical bench 2 to the detector without diffracting the beam, to focus it. The reflection of the beam mustn’t hit the cathetus plane of the prism.

![Figure 5.11: Top view of the set-up with photodetector and lens](image)
5.2.5 Final operations

Polarisation of the incoming laser beam
To excite a plasmon, the incoming laser beam has to be polarised transverse magnetically (TM). For this, 2 polarisers are positioned onto the optical bench 2: the first one to fix the intensity, the second to define the direction of the polarisation.

Installation of the light chopper
Finally the light chopper, which has to be arranged to the beam height, will be positioned behind the reflecting mirror.

Connection of the electronic circuit
A lock-in amplifier and a step motor control are used and both devices are connected to the measurement PC. The same goes for the light chopper, the photo detector. By using the computer software “Wasplas” (written by Andreas Scheller, Max Planck Institute for Polymer Research - Mainz) the sample motor and the detector motor can be controlled and measurements can be done.

5.2.6 Setup test

The setup has been tested through a large angle area and the data fitted, according to the following parameters:

- Measurement in air
- LaSFN9 substrate and prism (n = 1.85 Schott glass catalogue)
- Index matching liquid: n = 1.7
- Evaporated gold thickness: 43 nm
- Thickness of the chromium: 1.5 nm
- Chromium: $\varepsilon' = -1.28; \varepsilon'' = 20.46$ (calculated with $n=3.1$ and $k=3.3$ from the internet: “Refractive index and extinction coefficient of materials”)
5.3 Flow cell and liquid handling

A well-designed flow cell and a simple fluidic system have been used in order to operate with the biological samples. These fluidic systems are advantageous in:

1) accelerating mass-transport limited interaction kinetics, in the case of low analyte concentration;
2) minimizing sample consumption;
3) enabling automatic sample mixing, delivery, etc.

The schematic of the flow cell designed for our sensing system is shown in Figure 5.13. It consists of a thin polydimethylsiloxane (PDMS) spacer (300 μm, with a 5mm*7mm ellipse hole) and a quartz cover slide (Herasil glass) through which two holes are machined and two steel needles are glued, serving as inlet and outlet, respectively. The flow cell is attached, via Tygon® tubing with an inner diameter of 0.76 mm, to a peristaltic pump (Ismatec, Switzerland) and the sample tube, forming a closed
circulation loop. Buffer and sample solutions can only be manually exchanged, however, with little trouble from air bubbles. Once the exchange is completed, the whole loop is closed and completely sealed allowing for a long interaction time (>48 hours). The loop volume is around 300 µL, with a minimum sample consumption of around 400–600 µL to assure the desired analyte working concentration. The highest linear flow rate of 100 mm/sec (at 9 mL/min) at the sensing point compares favorably with that of Biacore’s microfluidic system\(^9^6\), which one of the most sophisticated/advanced fluidic system in the market.

![Figure 5.13. Sketch of a homemade flow cell with a thin PDMS spacer (300 µm) and a sealed circulation loop.](image)

5.4 Sensor Surface

In this study we use a functional system based on the gold-sulfur self-assembly technique\(^9^7\). The schematic drawing is shown in Figure 5.14. The self-assembled monolayer (SAM) is composed of 6-mercapto-1-hexanol (MCH - Sigma) and the thiolated DNA probes (Sigma) which are bonded on the surface with a spacer which is similar to the MCH (indeed these probes are so called 6-mercapto hexyl-oligonucleotides). The probes have to be 10nm far away from each other in order to have the best efficiency of the system\(^9^8\).
Figure 5.14. A schematic presentation of the sensor surface architecture: the thiolated DNA oligonucleotides are bonded to the surface through a S-Au bond. The surface is then passivated with a self assembled monolayer constituted by 6-mercapto-1-hexanol.

5.4.1 Gold substrate preparation

LaSFN9 substrates (n=1.85 @ \( \lambda = 633 \text{ nm} \)) are cleaned by being sonified for 15 minutes subsequently in 2% detergent solution (Helmanex), Milli-Q water, absolute ethanol. After drying with a stream of nitrogen, the slides are coated with a 45–50 nm gold layer by thermal evaporator (Edwards) at a deposition rate of \(~1 \text{ Å/second} \) under vacuum condition \((10^{-7}–10^{-6} \text{ mbar})\). The prepared substrates were stored under Argon atmosphere until use.

5.4.2 Self-assembled monolayers

Molecular self-assembly phenomena are widely seen in surface modification strategies. Self-assembled monolayers (SAMs) are highly ordered molecular assembles of long chain alkanes that chemisorb on the surface of solid materials.
The structure of SAMs, effectively two-dimensional crystals with controllable chemical functionality, makes them ideal model systems for the investigations and applications in wetting, adhesion, corrosion, protein adsorption, surface functionalization, micro-/nano-fabrications, etc. SAMs of alkanethiolates on gold (RSH/Au) are one of the most attractive system for their: (i) ease of fabrication; (ii) degree of perfection; (iii) chemical stability; (iv) availability of materials; and (v) flexibility in chemistry and thus surface functionality. SAMs of alkanethiolates on Au (111) form quasi-crystalline structures \((\sqrt{3} \times \sqrt{3})R30^\circ\) for \(n>6\) with the driving forces coming from lateral van der Waals forces and the strength of the sulphur-gold bond (Figure 5.15)\(^9\). Formation of MCH SAM on a gold surface functionalized with 6-mercapto hexyl-oligonucleotides has been previously reported\(^9\).

Experimentally, the LASFN9 slides (Schott, \(n = 1.85 \at \lambda = 633\text{nm}\)) are carefully cleaned by detergent/water/ethanol and coated with 50 nm of gold by thermal evaporation. SAMs were prepared by simply dipping the Au substrates into the 6-mercapto hexyl-oligonucleotides solution first, and in the MCH solution afterwards. The need to passivate the surface with MCH is due to the fact that the amino groups of the DNA have an affinity for the gold surface, therefore an unspecific adsorption may be observed without this step. In addition MCH ensures no aspecific binding of probes on the surface, the probes lying on the surface are removed, the aspecific binding of targets is prevented and the overall efficiency of the system is increased due to the fact
that the probes can't collapse on the surface (Figure 5.16). The surface of the sensor, that is the molecular recognition layer, is then ready to be used.

![Diagram of probe self-assembly, substrate passivation, and hybridization](image)

Figure 5.16. MCH treatment removes aspecific bonded probes on the surface while makes the surface no more available for aspecific binding of the targets.

### 5.5 The pTNT plasmid

As a model for further studies, a plasmid has been used as the DNA target. Indeed, due to the numerous restriction sites, the plasmid can be manipulated in order to obtain, later on, fragments of different length to model the behavior of histopathological samples.

The pTNT plasmid has been chosen as a target in order to have a well known DNA target to work with, with known sequences. It is important to note in the first place the β-Lactamase (Amp<sup>+</sup>) coding region which gives the bacteria carrying this plasmid the resistance to the antibiotic ampicillin which is used as the screening parameter during the bacterial transformation to select the cells which have incorporated the plasmid.
5.17. pTNT Vector circle map and sequence reference points.

T7 RNA polymerase promoter 16-34
SP6 RNA polymerase promoter 40-56
5' β-globin leader sequence 57-83
Multiple cloning region 84-130
Synthetic poly(A)30 region 131-160
T7 transcription terminator sequence 161-208
Phage fl region 287-742
β-Lactamase (Amp') coding region 1179-2039

5.5.1 Bacterial transformation

Transformation is the genetic alteration of a cell resulting from the introduction, uptake and expression of foreign genetic material (DNA). This is a common laboratory technique in molecular biology. The effect was first demonstrated in 1944 by Oswald Avery, Colin MacLeod and Maclyn McCarty, who showed gene transfer in Streptococcus pneumoniae. Avery, Macleod and McCarty call the uptake and incorporation of DNA by bacteria transformation.
In bacteria, transformation refers to a genetic change brought about by taking up and expressing DNA, and competence refers to the state of being able to take up DNA. Two different forms of competence should be distinguished, natural and artificial.

5.5.1.1 Natural competence

Bacteria of many species (perhaps most) are naturally capable of taking up DNA. Such species carry sets of genes specifying machinery for bringing DNA across the cell's membrane or membranes. The evolutionary function of these genes is controversial. Although most textbooks and researchers have assumed that cells take up DNA to acquire new versions of genes, a simpler explanation that fits most of the observations is that cells take up DNA mainly as a source of nucleotides, which can be used directly or broken down and used for other purposes.

Most naturally transformable bacteria express their competence genes and develop competence only under specific conditions, often in response to a nutritional stress. Once the DNA has been brought into the cell's cytoplasm, it may be degraded by cellular nucleases, or, if it is very similar to the cell's own DNA, enzymes that normally repair DNA may recombine it with the chromosome. Natural transformation is very efficient for linear molecules such as fragments of chromosomal DNA but not for circular plasmid DNAs.

5.5.1.2 Artificial competence

Artificial competence is not encoded in the cell's genes. Instead it is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature. These procedures are comparatively easy and simple, and are widely used to genetically engineer bacteria. Artificially competent cells of standard bacterial strains may also be purchased frozen, ready to use.
Chilling cells in the presence of divalent cations such as Ca$^{2+}$ (in CaCl$_2$) prepares the cell walls to become permeable to plasmid DNA. Cells are incubated with the DNA and then briefly heat shocked (42°C for 30-120 seconds), which causes the DNA to enter the cell. This method works well for circular plasmid DNAs but not for linear molecules such as fragments of chromosomal DNA. An excellent preparation of competent cells will give ~$10^8$ colonies per µg of plasmid. A poor preparation will be about $10^5$/$µg$ or less. Good non-commercial preps should give $10^5$ to $10^6$ transformants per microgram of plasmid.

Electroporation is another way to make holes in cells, by briefly shocking them with an electric field of 100-200V. Now plasmid DNA can enter the cell through these holes. Natural membrane-repair mechanisms will close these holes afterwards.

A plasmid DNA molecule contains sequences allowing it to be replicated in the cell independently of the chromosome. Plasmids used in experiments will usually also contain an antibiotic resistance gene which is placed in a bacterial strain that has no antibiotic resistance. Therefore, only transformed bacteria can grow on a media with the antibiotic (this is known as a selection medium).

One example of this is putting in a plasmid that contains the encoding for the protein β-lactamase, which makes bacteria resistant to ampicillin. This is called the *bla* gene. The bacterial colony is then treated with ampicillin, thus weeding out those bacteria who did not take up the plasmid with the *bla* gene. Another selection medium is bioluminescence, using a gene taken from jellyfish.

In bacteria the term transformation is not normally applied to genetic changes arising by Transduction or Conjugation, in which transfer of DNA is mediated by genetic parasites (phages and conjugative plasmids respectively).

### 5.5.2 Preparation of pTNT

The first step to obtain a sufficient amount of plasmid is to replicate it using the bacterial transformation. This has been done using the MultiShot StripWell TOP10 Chemically Competent E. coli cells (Invitrogen). The process has been performed as follows:
1. A MultiSho StripWell plate is removed from the freezer and the required number of wells is collected. Any unused wells are replaced into the freezer for further use. The collected wells are placed in a container with ice. Cells thaw within 1 minute.

2. Carefully, the caps from each well are removed and kept for further use.

3. 2-5 µl pTNT are added to the wells. The volume should be kept around 2 µl for uniform results.

4. After adding the pTNT, the wells are covered with the caps and the cells are incubated with the plasmid on ice for 30 minutes.

5. The wells are transferred to a water bath for the heat-shock step for 30 seconds at 42°C.

6. The wells are transferred back to the ice and allowed to cool for 1 minute.

7. 250 µl S.O.C. medium (2% Tryptone; 0.5% Yeast Extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl2; 10 mM MgSO4; 20 mM glucose) are added to each well.

8. The wells are incubated at 37°C for 1 hour with shaking (225 rpm).

9. The appropriate volume from each well is then plated on LB solid medium containing ampicillin and incubated overnight.

A single colony taken from the incubated plate is then used to obtain a liquid culture of the E. coli cells that have taken the pTNT (they are selected due to the ampicillin resistance the plasmid gives to them) in order to produce a large amount of cells. These are then processed in order to extract the purified plasmid. To purify the pTNT, the GenElute Five-Minute Plasmid Miniprep Kit (Sigma) has been used as follows:

1. 40 µl of reconstituted and pre-chilled Lysis Reagent are added to 400 µl of overnight culture in a 2-ml Collection Tube and then mixed. The solution is incubated at room temperature for at least 2 minutes.

2. The Binding Column is prepared. A GenElute Miniprep Binding Column is inserted into a 2ml Collection Tube and 500 µl of Column Preparation Solution are added to
each column; the columns are spinned for 10 seconds. The flow-through is decanted and the column back inserted back into the Collection Tube for subsequent use.

3. The DNA has to be bonded on the columns, therefore 400 μl of Binding Solution are added to the lysate and mixed thoroughly by inverting at least 15 times without vortexing. Approximately 780 μl of the mixture is poured to a pre-washed Binding Column seated in a 2ml Collection Tube and spinned for 20 seconds. The flow-through is then decanted.

4. To wash the column, 700 μl of diluted Wash Solution are added to each column and spinned for 20 seconds and afterwards the flow through is decanted. Another 200 μl of diluted Wash Solution are added to each column and spinned for 30 seconds to wash and dry the filter.

5. The Binding Column is then transferred to a clean 2ml Collection Tube and 40 μl of Elution Solution (or water if desired) are added directly to the surface of the filter and spinned for 30 seconds to elute. Plasmid DNA is now present in the eluate and ready for immediate use or storage at -20 °C.

pTNT preparation

Figure 5.18. Scheme of the pTNT preparation.
5.6 Sequence design

The sequences used in this study have been designed using software, freely available for research purposes, listed here:

- Vector NTI (Invitrogen)
- Oligo Design (University of Maryland)
- Oligo Analyzer (Integrated DNA Technologies)

Several sequences have been designed, based on the pTNT plasmid’s sequence, but only three have been used. The first one has been used as the probe on the surface and corresponds to the position 383 on the pTNT sequence:

- Name of the sequence: **383a**

Sequence: CGAGAAAGGAAGGGAAGAAAGC
Length of Oligo: 22
GC Fraction: 0.5
Melting temperature: 53.5 °C
dG: 25.7 (kcal/mol)
**Potential Hairpin Formation:**
No potential hairpins detected
**Potential Self Annealing:**
No potential self-annealing detected

Two sequences have been then selected as the secondary fluorescent labelled probe, in position 1191 and 1577, which means as near as possible to the opposite site of the first sequence used as the surface probe, in order to avoid steric hindrance:
- Name of the sequence: 1191

Sequence: ATAAGGCGACACGGAAATG
Length of Oligo: 20
GC Fraction: 0.5
Melting temperature: 52.6°C
DG_with_24.0 (kcal/mol)

Potential Hairpin Formation
No potential hairpins detected

Potential Self Annealing

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = 0.39
3' GTAAAGGCACACGGGAATA 5'

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = 0.39
3' GTAAAGGCACACGGGAATA 5'

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = -4.94
3' GTAAAGGCACACGGGAATA 5'

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = -2.06
3' GTAAAGGCACACGGGAATA 5'

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = -2.19
3' GTAAAGGCACACGGGAATA 5'

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = -0.37
3' GTAAAGGCACACGGGAATA 5'

5' ATAAGGGCGACACGGAAATG 3' dG (kcal/mol) = -3.76
3' GTAAAGGCACACGGGAATA 5'

- Name of the sequence: 1577

Sequence: TCGTTGTCAGAAGTAAGTTG
Length of Oligo: 20
GC Fraction: 0.4
Melting temperature: 47.6°C
Experimental

DG¢ 21,6 (kcal/mol)

Potential Hairpin Formation
No potential hairpins detected

Potential Self Annealing

\[
\begin{align*}
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -1,03 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5' \\
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -1,43 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5' \\
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -0,79 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5' \\
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -2,12 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5' \\
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -1,88 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5' \\
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -1,88 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5' \\
5' & \text{TCGTTGTCAGAAGTAAGTTG} & 3' & \text{dG (kcal/mol)} = -2,12 \\
3' & \text{GTTGAATGAAGACTGTTGCT} & 5'
\end{align*}
\]

5.7 Hybridization on gold surface of radiolabeled targets

To confirm that the probe sequence was bonded to the surface, a radiolabeling protocol has been used.

This method is a modification of the common radiolabeling protocol used for the dot-blot technique. It consist in the hybridization of a radiolabeled target on a gold surface which has been previously functionalized with a probe which sequence is complementary to the radiolabeled target.

There are six different steps involved:

a) pre-hybridization
b) target radiolabeling
c) purification of the radiolabeled target  
d) hybridization  
e) washing  
f) visualization of the results  

The pre-hybridization in this context is realized binding a DNA probe on a gold surface for 30'. This is realized using a thiol-modified DNA oligo (500 nM). The gold surface is then passivated immersing the sample in a solution of 6-mercaptop-1-exanol 1mM for 30', which covers the area of the gold not occupied by the probes. The target is then radiolabeled with $^{32}$P using the T4 polynucleotide kinase which substitutes a P in the 5' position as shown in Figure 4.4.

![Figure 5.19. Oligonucleotides or double stranded DNA molecules can be phosphorylated by T4 polynucleotide kinase. This enzyme transfers the gamma phosphate from ATP to the 5' end of the oligonucleotide chain. The picture shows the 5' end of a single strand of DNA. The strand on the left has a 5' OH group and the one on the right has a 5' phosphoryl group added by T4 polynucleotide kinase.](image-url)
Target labeling solution:

<table>
<thead>
<tr>
<th></th>
<th>[initial]</th>
<th>[final]</th>
<th>µl/Eppendorf</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O sterile</td>
<td></td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>PK buffer</td>
<td>10X</td>
<td>1X</td>
<td>3.0</td>
</tr>
<tr>
<td>DNA probe</td>
<td>250ng/µl</td>
<td>750ng TOT</td>
<td>3.0</td>
</tr>
<tr>
<td>γ³²P</td>
<td>10 µCi/µl</td>
<td>50 µCi</td>
<td>5.0</td>
</tr>
<tr>
<td>PK</td>
<td>10U/µl</td>
<td>15U TOT</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The final volume is 30µl.

The PK buffer (polynucleotide kinase) is prepared as follows:

- 70M Tris HCl (pH: 7.6)
- 10mM MgCl₂
- 5mM DDT (dithiothreitol)

The solution is incubated at 37°C for 60-90 minutes. The enzyme is then deactivate adding 5 µl of EDTA 0.5M and the target has to be purified from the [γ³²P]dATP not bonded. This step is easily accomplished but a chromatography on a micro-column (Sephadex G25) after diluting the sample up to 100 µl adding sterile water. The micro-column is prepared using a Costar test tube (Costar, Cambridge, MA) with filter and connected to a vacuum pump, loading the Sephadex G25 resin which must be already equilibrated with water.

The column must be uniform in order to work properly. 100 µl of water are added to the column and then it is spinned at 14000rpm for 15 seconds. This step is repeated until the excluded volume is 100 µl. The column is then ready and the target (100 µl) can be loaded. The column is spinned again (14000rpm for 15 seconds) and the excess of [γ³²P]dATP binds on the column. 1 µl of solution not purified is spotted against 1 µl of purified solution on filter paper in order to determine the efficiency of the enzymatic reaction. If the efficiency is less than 30%, the reaction have to be repeated. If the efficiency is higher than 70%, the chromatography has to be repeated.

The target is then hybridized with the probe on the gold surface immersing the gold slide in the target solution overnight at a suitable hybridization temperature ($T_h = T_m -$
15°C). The target solution is previously denatured in hot water for 5 minutes. After the hybridization, it is necessary to wash the gold surface to remove the excess of target. This is done using by SSC 6X + 0.1 % SDS solutions. The amount of immobilized target is then revealed using a Phosphor Imager (Packard Instrument Co.) or a Cyclon Storage Phosphor System (Packard Instrument Co.). The values, depending on the instrument, are expressed in c.p.m. (counts per minute) or D.L.U. (digital light units).

The results of the experiment are shown in Figure 5.20. According to the measurements, the probe is bonded to the surface in a robust way since washing it with the buffer don’t affect the system. In addition, as shown on Figure 5.20 2), the washing solution effectively removes any aspecific binding of the target on the gold even if the surface haven’t been passivated. The same applies for the BK7 glass surface. Unfortunately, even if high sensitivity is possible with $^{32}$P because this isotope has a high emission energy, the sensitivity is accompanied by low resolution because of signal scattering. Therefore it is not possible to quantify the probe density on the surface.

Figure 5.20. 1) Positive Control: Gold with thiolated DNA nucleotides (500 nM). The surface has been passivated with 6-mercapto-1-hexanol (Counts/mm²: 1900); 2) Negative Control a: naked gold with 6-mercapto-1-hexanol (Counts/mm²: 0). 3) Negative Control b: the left half of the surface is naked gold, the right half of the surface is BK7 glass without gold (Counts/mm²: 0).
5.8 Polymerase chain reaction (PCR)

In order to verify that the designed sequences were working properly, hybridizing with the plasmid, a PCR have been performed.

5.8.1 Principle of PCR

The polymerase chain reaction (PCR) was firstly reported by Kary B. Mullis in 1985\textsuperscript{101}, who was awarded a Nobel Prize for the discovery. It is virtually an \textit{in vitro} method for oligonucleotide primer-directed enzymatic amplification of a specific DNA sequence of interest.

<table>
<thead>
<tr>
<th>PCR target or template</th>
<th>The segment of nucleic acid (DNA or RNA) that is to be amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotides</td>
<td>Building blocks from which nucleic acids are constructed: adenine, guanine, cytosine, thymine and uracil</td>
</tr>
<tr>
<td>Primer</td>
<td>A short sequence of nucleotides complementary to, and building (annealing) to, known sequences of the target nucleic acid; essential for priming the amplification reaction</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>A heat-stable enzyme that makes a new complementary copy of the target nucleic acid by adding nucleotides to the annealed primer</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>A enzyme that converts RNA into a complementary DNA sequence (used in reverse transcription PCR)</td>
</tr>
<tr>
<td>Thermocycler</td>
<td>The equipment in which PCR reaction occur; it is able to change rapidly to the different temperatures required for repeated PCR cycles</td>
</tr>
</tbody>
</table>

Table 5.1 Reagents and equipments required for PCR.

The essential materials, reagents and equipment required for nucleic acid amplification by PCR were summarized in Table 2.1. A prerequisite for PCR amplification is to have known, unique sequences flanking the segment of DNA to be amplified so that specific primers can be made. It is not necessary to know anything about the intervening
sequence between the primers. The PCR product is amplified from the DNA template using a heat-stable DNA polymerase from Thermus aquaticus (Taq DNA polymerase) and using an automated thermocycler, which is an instrument that can hold the assay's reagents and allows for the reactions to occur at the various temperatures required. The target sequence to be amplified is ideally 200-400 bp in length, with an upper limit probably around 3 kb.

![PCR: Polymerase Chain Reaction](image)

Figure 5.21. Schematic of the PCR process.

Typically, the PCR is carried out in 3 steps (Figure 5.21.):

1) **Template denaturation:**
During the denaturation, the double strands melt open to single stranded DNA. The initial denaturation of template is accomplished at 95–100°C. If supercoiled plasmids are served as the template, it may require boiling for several minutes, or may be initially denatured by using base (NaOH, followed by pH neutralization) in order to be melted completely. The denaturation during the PCR experiment (i.e. from the second cycle
onward) is usually accomplished at temperatures of 92–95°C, which is empirically
determined.

2) Primer annealing:
At primer annealing temperature, primers are allowed to bind to their complementary
sequences on the single-stranded DNA template. The determination of the primer
annealing temperature is crucial in the success of the PCR experiment. The exploration
of the ideal primer annealing temperature can start from the calculated melting
temperature of the primer oligomers.

3) Primer extension:
The *Taq* polymerase begins adding deoxynucleotide bases to the ends of each primer
and eventually makes a complementary copy of the template DNA. Primer extension is
usually performed at 72°C, which is the optimum working temperature of the *Taq*
polymerase.

Theoretically, PCR can amplify a single molecule of target DNA. Thus, at the end of
the first cycle, we would have 2 molecules of the targeted DNA segment. The three
steps in the PCR process take less than 2 minutes. But the cycle can be repeated at least
30 more times before the *Taq* polymerase loses activity from the repeated heating. Each
newly synthesized DNA sequence acts as a new target for the next cycle, so after 35
cycles, 34 billion copies of the original target DNA can be produced. Taking into
account the time it takes to change the temperature of the reaction vial, 34 billion copies
can be ready in about 3 hours.

Because after each cycle the newly synthesized DNA strands can serve as templates in
the next cycle, the number of DNA copies doubles at every cycle. Thus, for a given
number of cycle 'n', we obtain $2^n$ x total possible duplexes, where x is the concentration
of the original template. However, the *Taq* polymerase adds dNTP's always from 5' to
3', starting from the 3'-end of each primer, which makes the consequence that the new
strands have defined 5' ends (the 5' ends of the oligonucleotide primers), whereas the 3'
ends are potentially ambiguous in length.
Figure 5.22. The first 4 cycles of a PCR reaction in detail.

Fortunately, the number of DNA fragments of indeterminant length increases linearly, represented by \(2(n+1)\). Therefore, the total concentration of a desired product (duplexes with a length defined by the PCR primers) can thus be represented by the formula:

\[
(2^n - 2(n+1))x
\]

This is often abbreviated to a simple rule of thumb for the amplification: \(2^n - 2n\)\(x\).

The formula gives a theoretical amplification value, however, it can never be achieved in practice owing to the following factors:

1) Competition of complementary DNA fragments with primers for reannealing (i.e. two DNA fragments reannealing results in no amplification)

2) Loss of enzyme activity due to the thermal denaturation, especially in the later cycles

3) Even without thermal denaturation, the amount of enzyme becomes limiting due to the strikingly expansion of DNA fragments in amount in later cycles (i.e. after 25 ~ 30 cycles too many primers need extension)

4) Possible second site primer annealing and non-productive priming
In our study we have used the 1191 sequence as the forward primer and the 1577 sequence as the reverse primer in one case, the 382a as the forward primer and the 1577 sequence as the reverse primers in another test using standard PCR conditions. The results of the PCR can be seen performing a gel electrophoresis of the PCR amplicons.

5.9 Gel electrophoresis

Nucleic acids in solution generally have a negative charge because their phosphate groups are ionized; thus they migrate toward a positive electrode. However, nucleic acids molecules consisting of long chains have almost identical charge-to-mass ratios, whatever their length, because each residue contributes about the same charge and mass. Therefore, if the electrophoresis of nucleic acids were simply carried out in solution, little or no separation of molecules of varying lengths would occur.

Molecules are now most commonly subjected to electrophoresis in a gel, rather than a liquid solution. The size of the pores in such gels limits the rate at which molecules can move through them. Migration in gels is inversely proportional to the size of the nucleic acids. The structure of the nucleic acids (e.g., linear, circular, double-stranded, or single-stranded) will also affect migration in gels. It is possible to distinguish DNA fragments that differ by as little as 1%. A wide range of sizes (<10 bp to >20 kb) can be analyzed by gel electrophoresis.

Agarose gel electrophoresis is the earliest and most common way of separating and analyzing DNA. The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragments that cannot be separated by other sizing procedures. Furthermore, the DNA can be visualized in the gel by addition of EtBr (ethidium bromide). EtBr is a fluorescent dye that binds strongly to DNA by intercalating between the bases, and transmits the energy as visible orange light if excited by invisible UV light. As little as 1 ng of DNA can be detected by direct examination of the gel. Size determination of the PCR product can also be realized by electrophoresing size markers on the gel.
The gel electrophoresis of the PCR products (Figure 5.23), performed in standard conditions, has shown that an aspecific hybridization can be observed for the sequence 1191. Therefore this sequence hasn’t been used. Single specific hybridization has been seen for the 1577 sequence with the expected length. Consequently, this is the sequence used as the secondary fluorescent labeled probe. It is important to note that the hybridization in PCR condition differs from that one performed with the setup, since our setup cannot be heated.

5.10 Plasmonic crystal sample preparation

An analysis of the literature show that is missing so far an experimental demonstration of the potentiality of SPP control for light emitting device in:

1) the control of the electromagnetic field enhancement in periodic plasmonic crystals
2) the control of the deposition and the properties of light emitting nanoparticles
3) the coupling of the previous factors i.e. the possibility to integrate with nanometric precision and accuracy these nanoparticles in the proximity of the metallic thin layers.

The possibility to combine all the previous elements developing a currently very important thematic.

The lithography has been performed exploiting an already exiting X-ray mask characterized by 200nm holes with a triangular lattice parameter of 400nm. The process protocol is quite simple (Table 5.2). A Si/SiO substrate is cleaned with acetone and isopropyl alcohol (IPA) and then a 300 nm PMMA 671.025 is spun on the surface (3000 rpm x 1; bake at 180°C x 10'). The X-ray exposure is the performed (Dose: 5000mJ/cm²) with the X-ray stepper at the LIL1 beamline. The PMMA 671.025 is then developed (MIBK 1:3 x 15-20°). The next step is an Electron Beam Metal Evaporation of Cr and Au and the final step is the lift-off with hot acetone, in an ultrasound bath, of the metals evaporated on the resist. The bidimensional pattern is then obtained on the substrate surface.

<table>
<thead>
<tr>
<th>Operation</th>
<th>Materials</th>
<th>Conditions</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Si/SiO₂</td>
<td>Ultrasound bath</td>
<td></td>
</tr>
<tr>
<td>Cleaning</td>
<td>Hot Acetone/IPA</td>
<td>Ultrasound bath</td>
<td>Slow heating/cooling rate</td>
</tr>
<tr>
<td>Spinning</td>
<td>PMMA 671.025</td>
<td>RPM 3000 x 1' Bake 180°C x 10'</td>
<td></td>
</tr>
<tr>
<td>X-ray exposition</td>
<td></td>
<td>Dose ~ 5000</td>
<td>High res. mask (Ø 200 nm)</td>
</tr>
<tr>
<td>Development</td>
<td>MIBK 1:3 Risciacquare in IPA</td>
<td>Time 15-20°</td>
<td></td>
</tr>
<tr>
<td>Metal evaporation</td>
<td>Cr 5 nm Au 25 nm</td>
<td>Glow Discharge 120° NO rotation</td>
<td>Keep low evaporation rates</td>
</tr>
<tr>
<td>Lift-Off</td>
<td>Hot Acetone</td>
<td>Ultrasound bath (50 °C)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. Summary of the plasmonic crystal sample preparation protocol using one resist (PMMA)

Previously, the protocol used was base on two different resists, PMMA (671.025) and PMGI. The use of PMGI below the PMMA layer should have given better results. The protocol used in this case in summarized in Table 5.3.
Experimental

<table>
<thead>
<tr>
<th>Operation</th>
<th>Materials</th>
<th>Conditions</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Si/SiO₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaning</td>
<td>Hot Acetone/IPA</td>
<td>Ultrasound bath</td>
<td></td>
</tr>
<tr>
<td>Spinning</td>
<td>PMGI 70 nm</td>
<td>RPM 4000 · 1'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bake 170 °C · 5'</td>
<td></td>
</tr>
<tr>
<td>Spinning</td>
<td>PMMA (671.025) 100 - 200 nm</td>
<td>RPM 4500 · 1'</td>
<td>Slow heating/cooling rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bake 180°C · 10'</td>
<td></td>
</tr>
<tr>
<td>X-ray exposition</td>
<td></td>
<td>Dose ~5000</td>
<td>High res. mask (Ø 200 nm)</td>
</tr>
<tr>
<td>PMMA development</td>
<td>MIBK 1 : 3 Wash in IPA</td>
<td>Time 15”</td>
<td></td>
</tr>
<tr>
<td>PMGI development</td>
<td>PMGI Developer Wash in IPA</td>
<td>Time 20”</td>
<td></td>
</tr>
<tr>
<td>Metal evaporation</td>
<td>Cr 5 nm Au 25 nm</td>
<td>Glow Discharge 120” NO rotation</td>
<td>Keep low evaporation rates</td>
</tr>
<tr>
<td>Lift Off PMMA</td>
<td>Acetone caldo</td>
<td>Ultrasound bath (50 °C)</td>
<td></td>
</tr>
<tr>
<td>Lift Off PMGI</td>
<td>PMGI Developer caldo</td>
<td>Ultrasound bath (50 °C)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3. Summary of the plasmonic crystal sample preparation protocol using two different resists (PMMA and PMGI)

5.10.1 Sample characterization

The purpose of this experiments was the realization of metallic plasmonic crystals: 2D nano-patterning Au plasmonic crystal nanostructures and of metallic-dielectric plasmonic crystal (2D nano-patterning of gold-polymer plasmonic crystal nanostructures).

The X-ray lithography experiments have given very good results in terms of quality of the pattern realized as shown in Figure 5.24. A triangular array (400 nm lattice parameter, 200 nm diameter) with areas as large as 2 mm free of defects have been obtained.
Experimental

Figure 5.24. SEM image of the X-ray lithography after the exposure: the experiments have given very good results. The quality of the pattern realized is very good over very large areas of the samples without defects.

Figure 5.25. Magnification of the previous sample. The cracks on the surface are due to the sputtering process necessary to realize the SEM image and are not a defect of the sample.
It is important to note that the lift-off process has to be done in an ultrasound bath in order to be complete. Without this step, the samples appear as shown in Figure 5.26 and 5.27.

Figure 5.26 (left) shows an incomplete lift-off of the resist. The reason of this behavior is due to a non-completely orthogonal evaporation. A step in a ultrasound bath is needed. Figure 5.27 (right) shows a magnification of the previous picture.

In addition, the protocol using two resist should have given better results making easier the lift-off process, but the results shown the opposite behavior (Figure 5.28 and 5.29). In particular, it can be observed that the "caps" covering the holes are less displaced than those seen in Figure 5.27, suggesting that an ultrasound bath step won’t probably be enough to complete the lift-off. It is possible anyway that implementing a step with a Reactive Ion Etching machine could solve this issue.

Figure 5.28. (left) The lift-off process using the PMGI under the PMMA leads to worse results. Indeed in Figure 5.29. (right) it can be seen that the holes are more covered if compared with figure 5.5.
The samples have been also characterized with AFM in order to obtain the profile image, which is consistent with what we were expecting in terms of dimension (Figure 5.30).

Figure 5.30. AFM image of the plasmonic crystal sample. In this case the AMF confirms that the metal layer is 25nm meaning that the removal of the resist is complete.

The high-resolution bidimensional patterns realized have been characterized for SPR applications. The strong dependence of the resonances can be seen as a function of the variable angle dependence in an energy dispersive spectrum (Figure 5.31).

A probable plasmonic structure has been identified in the samples fabricated using only the PMMA (Figure 5.32), in particular with and angle $\theta$ of 70° but the effect of the interference measured is too high. This is probably due to a non-optimal thickness of the metals (10 nm Cr, 100 nm Au). To separate the interference fringes from the plasmonic contribution it will be necessary to tune the thickness on different values.
Figure 5.31. Reflectivity measurement as a function of photon energy collected at different incident angles on the two dimensional arrays of gold.

In Figure 5.32 is clearly shown the correct interference through the gold layer (the dielectric layers are both air and SiO₂) and the min/max entanglements show a diverse trend which can be considered a proof of the presence of a plasmonic gap.

Figure 5.32 This sample show a probable plasmonic structure in particular at a θ of 70° but the interference observed is too intense.
6 SPR/SPFS studies on planar surfaces

6.1 Motivations

Surface plasmon field-enhanced fluorescence spectroscopy (SPFS) uses the greatly enhanced electromagnetic field of a surface plasmon mode for the excitation of surface confined fluorophores. At the commonly used gold/water interface, the evanescent field of a surface plasmon is enhanced by a factor of 16 compared to the incident field at the respective resonance angle, and then decays exponentially into the dielectric medium, with a penetration depth of approx. $L_z = 250$ nm. In this thin layer, the accumulation of the analyte molecules alters the optical properties of the interface and can be sensed by an angular shift in the SPR resonance minimum. If the analyte molecule carries a fluorescent dye, fluorescence emission will be excited by the SPR field. In this sense, SPFS can be considered as a combined tool, which simultaneously conveys the information of local refractive index change and the fluorophore concentration. By this, it offers a potential for a more detailed surface investigation than SPR.

As has been discussed theoretically in chapter 2, the fluorescence yield of fluorophores near a metal exhibits a highly pronounced distance-dependent behavior. Briefly, within a short separation distance range (approx. 20-30 nm), the fluorescence of the fluorophores can be 'quenched' by a reduced fluorescent lifetime or, can be 'back-coupled' as red-shifted re-radiation. Both effects reduce the effective fluorescence emission. Beyond this range, as the distance increases further, the fluorescence yield weakens with the fading of the evanescent field. On one hand, this strong distance behavior of fluorescence causes problems in the quantification of surface analyte, whose fluorescence yield may depend on its unpredictable position/conformation at the surface. On the other hand, it results in a much more pronounced distance information than the surface plasmon evanescent field ($L_z = \sim 150$ nm), which may offer a clearer understanding of the distribution of the bound molecules. Obviously, a planar functional surface (or two-dimensional surface) is experimentally desirable for pronouncing such a distance effect. In this study, gold surfaces were functionalized with DNA.
oligonucleotides. Specific hybridizations occur if the target solution passes over the surface, driven by the fluidic system.

6.2 Materials

The molecular recognition layer on the surface has been prepared functionalizing the surface with the 382a thiolated oligonucleotide (Sigma) dipping the evaporated gold substrate in a 500nM solution (in PBS or NaCl 100mM) of the probe for 30'. The surface has been then passivated using the 6-mercapto-1-hexanol (1mM - Sigma) dipping the functionalized gold surface for 30' in the MCH solution. The pTNT plasmid (Invitrogen) has been indirectly labeled with the secondary fluorescent labeled probe 1577 (Sigma) after denaturation (95°C for 5') for 60’ prior hybridization on the molecular recognition layer. The robustness of the molecular recognition layer has been already tested during the hybridization on gold surface of radiolabeled targets experiments and it has been seen that the functionalized surface is resistant to washing buffers such as SSC 6X + 0.1 % SDS solutions.

6.3 Surface Regeneration

The robustness of the molecular recognition layer enables the repetitive removal of the bound targets by NaOH. This means that the functionalized surface can be used several times without affecting the measurements. This is an important feature that could lead in the future to a reusable chip, reducing the costs of this DNA analysis platform. This robustness have been proved using as targets DNA oligonucleotides (all targets were 0.04M in PBS) functionalized with polystyrene with different molecular weights performing a kinetic SPR analysis at a fixed angle corresponding to the minimum of the SPR spectra for the sample used. The targets have been firstly bonded on the surface (Figure 6.1) and then the surface has been regenerated with NaOH 0.01M (Figure 6.2) allowing more hybridizations on the same substrate. The deep minimum observed is due
to the change of the refractive index induced by the NaOH solution compared to the PBS buffer refractive index. After replacing the PBS solution in the microfluidic channel, the reflectivity is increased but the value is lower than before the surface regeneration. This tells that the target has been removed. Comparing this value of reflectivity with the one recorder prior hybridization of the target, it is possible to state that the target has been removed completely.

Figure 6.1. Kinetic measurement of the target binding.
Figure 6.2. Surface regeneration with NaOH 0.01M. The target is removed and a great reduction of the reflectivity is observed due to the refractive index of the NaOH. Replacing the PBS solution in the microfluidic channel, the refractive index is restored.

Figure 6.3. The robustness of the molecular recognition layer proved that the regeneration of the surface is possible multiple times using the same substrate multiple times continuously by simply regenerating the surface after every hybridization.
6.4 Indirect detection of unlabeled pTNT

Combining the enormous surface plasmon field enhancement with the sensitive fluorescence technique, SPFS has shown a remarkable sensitivity in detecting oligonucleotide targets. However, as shown in many efforts\textsuperscript{102}, avoiding the dye (or other) label still remains a desirable aim. The label-free assay is expected to provide us with complementary information in the understanding of interfacial hybridization mechanism. In this study, we try to detect the unlabeled pTNT by a 2-step indirect detection strategy by using SPFS were the pTNT is firstly hybridized with a fluorescent labeled secondary probe (1157) and then the complex pTNT-secondary probe is hybridized on the functionalized gold surface.

6.4.1 Differences between passivated and non-passivated surfaces

Preliminary experiments have been performed on a non-passivated gold surface were only the probe (382a) was present on the molecular recognition layer. Therefore, the substrate is functionalized as previously described without passivating the surface with MCH. A reference SPR (Figure 6.4) spectra is then taken in order to study the behavior of the system after adding the pTNT indirectly labeled with the secondary probe Cy5 labeled. Figure 6.5 shows that after exposing the surface to the pTNT indirectly labeled, the SPR spectra get broaden, indicating that the plasmid is indeed interacting on the surface. This anyway is not sufficient to tell any information about the specificity of the hybridization. In order to have such information, the surface has been exposed to a solution of NaOH (0.01M) for 60 seconds, in order to test the possibility to regenerate the surface. Figure 6.6 shows that it is not possible, after the surface regeneration step, to restore the surface and obtain SPR spectra comparable with the reference spectra. This mean that there is surely and aspecific binding of the pTNT with the gold surface due to the aminic groups of the DNA, that cannot be reversed completely. In addition, it is possible to observe a roughening of the SPR curve in correspondence of the critical angle, as it happens with a slight misalignment of the
optical setup. This is probably due to an effect induced by the great quantity of plasmid that sticks on the surface in a non homogeneous way. Therefore in such system and with this kind of architecture, it is necessary to passivate the surface in order to prevent aspecific binding.

Figure 6.4. SPR spectra of the thiolated probe. This is the reference spectra.

Figure 6.5. Adding the pTNT indirectly labeled with the secondare Cy5 labeled probe determines a broadening of the curve.
6-mercapto-1-hexanol (MCH) is commonly used (1μM for 30’) to passivate gold surfaces in order to prevent any DNA aspecific binding. MCH ensures no aspecific binding of probes on the surface, the probes lying on the surface are removed, the aspecific binding of targets is prevented and the overall efficiency of the system is increased due to the fact that the probes can’t collapse on the surface. A first reference spectra has been taken after functionalizing the surface with the thiolated probe and passivating the surface with MCH (Figure 6.7). Consequently, the surface has been exposed to the pTNT indirectly labeled (Figure 6.8.). In this case, it can be observed, as a clear proof of the pTNT binding on the surface, an increase of the reflectivity value in correspondence of the SPR minimum. This is due to the fact that the plasmid hybridized on the surface causes a reduction of the light coupling efficiency (roughly 18%) if its concentration is high, as in this first experimental test (1μM). The high concentration has been used in order to have a clear evidence that no aspecific binding on the surface occurs. Nevertheless, a surface regeneration with NaOH (0.01MM) is possible as shown in Figure 6.9. Indeed, after the NaOH treatment, the coupling efficiency is restored, suggesting a complete removal of the pTNT from the surface.
Figure 6.7. SPR spectra of the thiolated probe and MCH on the surface. This is the reference spectra.

Figure 6.8. After the addition of the pTNT on the passivated gold surface, a coupling efficiency reduction is observed.
6.4.2 Unexpected quenching of the fluorescence

Unfortunately, the architecture designed for the detection of the indirectly labelled pTNT has proven to be not working to obtain an SPFS spectra (Figure 6.10)
Indeed, even if the pTNT is sufficiently long to allow the secondary Cy5 labeled probe to be outside the Foerster radius, no fluorescence has been detected. The only explanation is that the plasmid, even if it can’t interact with the gold, is nevertheless laying on the surface. Therefore the secondary probe and its fluorescent dye are too close to the surface, so a complete quenching of the surface is observed due to the Foerster transfer that causes the energy to be dissipated into the adjacent metal.

### 6.4.3 Modification of the surface architecture

To prevent the complete quenching of the fluorescence, still avoiding using the biotin self assembled monolayer system used in previous works that requires a time consuming overnight process step, it has been chosen to modify the architecture using a polystyrene layer on top of the gold. A streptavidin layer is then realized above the polystyrene and this will be the attaching point for a biotinilated DNA probe with the very same sequence of the 382b. These experiments have been performed at the IMRE in Singapore using a setup mounting a green laser (543nm) therefore some changes have been made, since at this wavelength bare gold has a high absorption, preventing the possibility to perform SPR measurements. The first change made is the metal surface: with the green laser, the optimum substrate is silver with a thickness of 37nm. To prevent oxidation, the silver layer is covered with 8nm of gold. Another change needed has been the substitution of the Cy5 label with a Cy3 fluorescent dye on the secondary probe. The last change made is the filter before the fluorescent detector that has been changed in order to detect the fluorescence emitted by the Cy3 fluorescent dye. The polystyrene layer is simply realized by spinning, in a two steps process, a 0.5% solution at 1000rpm for 15 seconds and at 3000rpm for other 45 seconds, in order to obtain a 30nm thick layer. The streptavidin layer above the polystyrene is formed exposing the polystyrene surface to a 1μM solution of the protein (in PBS) for 40’.

This process can be monitored in real time (Figure 6.11)
Figure 6.11. Kinetic measurement of the streptavidin binding on the polystyrene surface.

Figure 6.12. Kinetic measurement of the biotinilated probe binding on the streptavidin layer. The fluctuation on the measurement is due to the laser.
The biotinilated probe is then bond on the streptavidin layer exposing the surface to a 1μM solution of the probe for at least 30’. The process can be monitored in real time as shown in Figure 6.12.

Once the molecular recognition layer is ready, after functionalizing the surface with the probe, it is possible to hybridize the pTNT plasmid indirectly labeled with the secondary probe Cy3 labeled. With this new architecture it has been proven that the SPFS can be detected, as shown in Figure 6.13, where a 0.04 μM pTNT solution has been used.

Figure 6.13. SPFS detection of pTNT indirectly labeled with Cy3. The deep corresponds to the SPR spectra; the peak corresponds to the SPFS signal.

6.5.4 Future works

The present project has opened a great number of possibilities for further development. Indeed, acquiring the knowledge to perform SPR and SPFS studies allows to exploit the possible improvements of the technique.
This will be done using a new powerful tool recently introduced in our laboratory, a VASE® Research Spectroscopic Ellipsometer (J. A. Woollam Co., Inc.) (Figure 6.14) which key features, compared to the SPR setup used in this study, are the possibility to control the polarization of the light e and the wavelength in a wide range (from hundreds of nanometers to the IR range).

Therefore, the normal SPR spectra can be obtained at different wavelength and multiple polarization values, while with the ellipsometry it is possible to obtain immediately the refractive indexes and the dielectric constant (imaginary and real part) of the analyzed samples. The substrates for these experiments are gratings on BK7 slides which have been metal plated (Ag 37nm, Au 8nm) kindly provided by Prof. Knoll (Max Planck Institute for Polymer Research, Mainz).

The detection method that is going to be developed is based on the ellipsometric analysis of the plasmonics grating. In figure 6.15 the SPR spectrum of the uncoated plasmonic grating is compared with the SPR spectrum of an area exposed to a solution of pTNT. These reflectivity spectra have been obtained for three different wavelength (600, 650 and 700 nm) using the p polarization (the electrical field of the incoming light is therefore perpendicular to the sample surface). It clearly appears that the reflectivity deeps of the bare grating, in correspondence with the Plasmon excitation, are deeper than those collected for the coated grating. This is the typical signal coming from a SPR
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Figure 6.15. Comparison between a bare grating (red lines) and the same grating exposed to a solution of pTNT (green lines). At different wavelength.

Figure 6.16. Refractive index comparison between bare grating (black lines) and the same grating coated with pTNT (colored lines) at different wavelength.

analysis. The SPR observed with this configuration are quite similar (Figure 6.15) and they can be easily used to evaluate the refractive index that shows the changes of the refractive index when a dielectric material (the pTNT in this case) is introduced on the surface even in low concentration. However a first difference must be noticed: in this
case the use of different wavelengths multiplies the spectra and allows both to have a larger number of meaningful signal deeps and also allows to select specific energies, for example, in correspondence of the plasmonic crystal band gap, where the density of states is greater with a consequent increase of sensitivity. It must be noticed that in SPR mode the signal appears as a small difference at the bottom of reflectivity deeps with respect to a reference system that in this case is represented by the bare grating.

A completely different approach can be obtained exploiting ellipsometric analysis that ultimately can directly measure the index refraction of the coated film. An example is shown in Figure 2 where the index of refraction singularities in correspondence of the plasmonic-polaritons excitation for the different wavelength have been collected. As it appears in this case we don't have to compare peaks related directly to the quantity that we are interested to measure. It is possible to compare the peaks provided by the bare and by the coated areas. The light polarization in this case is selected in order to optimize the signal.

Figure 6.17. Absorption coefficient comparison between bare grating and the same grating coated with pTNT at different wavelength.

The plasmonic excitations are exploited to detect a index refraction increase. Corresponding also the imaginary part of the refraction index of refraction, i.e. the
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absorption extinction can be collected as well. Changes of index of refraction and absorption coefficients can provide the full information for the extraction of the film coated on the top of the grating. The energy dispersion again will be used for tuning the wavelength at the plasmonic band gap edge.

This method has not been already developed but clearly show potentiality for higher plasmon based sensing.
7 CONCLUSIONS

An optical sensor is a sensing device which, by optical means, converts the quantity measured (measurand) to another quantity (output) which is typically encoded into one of the characteristics of a light wave. In SPR sensors, a surface plasmon is excited at the interface between a metal film and a dielectric medium (superstrate), changes in the refractive index of which are to be measured. A change in the refractive index of the superstrate produces a change in the propagation constant of the surface plasmon. This change alters the coupling condition between a light wave and the surface plasmon, which can be observed as a change in one of the characteristics of the optical wave interacting with the surface plasmon. Based on which characteristic of the light wave interacting with the surface plasmon is measured, SPR sensors can be classified as SPR sensor with angular, wavelength, intensity, phase, or polarization modulation.

In this study, Surface Plasmon Resonance’s potential for nucleic acid analysis has been explored in order to develop the knowledge for new microarray DNA platforms. In particular, Surface Plasmon Fluorescence Spectroscopy (SPFS) has been investigated with a new indirect labeling system that allows avoiding the labeling of the targets but still making use of the high sensitivity allowed by SPFS. Moreover, we have strengthen the believe that optical methods, and especially those surface plasmon based, are well suited for DNA analysis due to the speed of the measurement, the possibility to have label-free and indirect-label methods, the possibility to work in liquid, change the temperature and, last but not least, doing a non-destructive measurement allowing the sample to be eventually further analyzed.

In this study the interfacial hybridization of a plasmid has been investigated using SPR and SPFS. Two different architectures have been used for the molecular recognition layer, direct binding of the probe on the gold surface via sulfur-gold bond and functionalization of the surface using a biotinilated oligonucleotide bonded on polystyrene. The second architecture has been proven to be able to avoid the fluorescence quenching observed with the former one. Moreover, this architecture can be easily produced faster than other architectures found in literature. The detection of the plasmid, being indirect, has a very
Conclusions

good feature: there is no need to directly label the target. Therefore no labeling efficiency issue is encountered, since the secondary probe can be purified after labeling.

The work done shows that there is still a huge potential to be uncovered, in order to improve the current state of the art. These improvements are based on the nanofabrication of plasmonic crystals.

Substrates to improve the SPR technique have been indeed produced using the x-ray lithography. The bidimensional patterned substrates have shown the characteristic to induce the coupling of the light with the metal layer and they will be useful in further studies to improve the technique. Indeed, preliminary experiments performed using the new VASE® Research Spectroscopic Ellipsometer (J. A. Woollam Co., Inc.) show that using plasmonic crystals coupled with this setup may lead to an increase of sensitivity of one order of magnitude of the label-free SPR.

In addition, this study had the fundamental importance to introduce Surface Plasmon Resonance (SPR) and Surface Plasmon Fluorescence Spectroscopy (SPFS) techniques in our laboratory, opening countless opportunities for a great number of new projects and research lines.
BIBLIOGRAPHY

Bibliography


10 ACKNOWLEDGEMENTS

It is difficult to overstate my gratitude to my Ph.D. tutors, Prof. Giorgio Stanta and Dr. Filippo Romanato, who have supported the work all the way along. They gave me the opportunity to work on such fascinating and pioneering topics. With their enthusiasm, their inspiration and their great efforts, they have managed to guide my work at the right points through many insightful conversations.

My gratitude also goes to Prof. Wolfgang Knoll who hosted me in his group at the Max Planck Institute for Polymer research and to Dr. Eva Kathrin Sinner who helped during my work there. I also want to thank Prof. Wong Chee Cheong who gave me the opportunity to be an exchange student at the Nanyang Technological University in Singapore.

I also want to thank all the helpful colleagues that I have met on the way during this three years in Italy (ICGEB and Lilit), Germany (MPI-P) and Singapore (NTU and IMRE). I'm sorry: you are too many to be listed here.
Special thanks

Un ringraziamento speciale alla mia Piccola Stella.
Continua a tenere il filo...