Executive summary:

Future breakthroughs in the understanding of fundamental biological processes and of molecular events causing major diseases require a nano-chemical probe, which could be moved around and inside a cell, able to detect and identify a few or even single molecules. The SingleMoleculeDetection (SMD) proposal integrates on the same device an atomic force microscope or an optical tweezers set up, with optical spectroscopy so to perform simultaneously and in a dynamic way force and Raman/SERS/InfraRed(IR)/Terahertz measurements. Focus of the project is to design and fabricate novel devices for the generation of plasmon polariton from noble metal, consisting of a combination of photonic crystals and novel plasmonic nanolenses. These new devices will be able to detect few/single molecules in a near-field configuration/near-field configurations through force spectroscopy in combination with Raman, IR and Terahertz with a spatial resolution in the range of 10 nm or smaller. The chemical and mechanical information originating from all those spectroscopies represents an unambiguous signature of a single (unknown) molecule which will be applied to determine processes of biological and medical interest: i - in cancer, proteins released during cancer development that can diffuse in the body and be exploited as an early marker of tumoral activity or can become part of the extracellular matrix, playing a fundamental role in the cancer cell seeding; - ii in neurobiology, proteins and small molecules released by differentiating cells and stem cells responsible for the differentiation process itself. These new devices can also be used to recover structural information of membrane proteins and to understand their conformational changes upon ligand binding. These new devices, not only will they provide major scientific breakthroughs but they will also open new avenues for diagnosis and therapy.

Project Context and Objectives:

Project Objectives

The SMD project aims at building new devices which are able to combine different experimental techniques for label free chemical characterization at the single molecule level. This includes new materials for use in bridging between spectroscopic and analytical based research as well as specific, purpose built experimental setups capable of single molecular detection.

The possibility to perform single molecule spectroscopy opens new avenues for pharmaceutical companies and industries interested in health care. In particular a greater understanding of how individual molecules behave in biological systems has significant implications in healthcare due to the ability to intervene in a well understood system.

The SMD project is highly interdisciplinary and sits at the interface between physics, chemistry and biology to be a genuinely collaborative nanoscience project.

The SMD project aims at building a novel generation of nanobiosensors based on novel nanoparticles conjugates nanotransducers, nanoantennas and functional AFM tips.

Single molecules can be detected and their motion followed when they are labeled with an appropriate tag, such as a fluorescent label i.e. the green fluorescent protein (GFP) and/or quantum dots, combined with the molecule of interest by genetic engineering or chemical manipulation. Manipulation of single molecules as well as the measurements of several important mechanical properties can be performed with optical tweezers (OT) and atomic force microscopy (AFM). SMD works on the Biological dream of a miniaturized chemical probe or microscope which could be moved around and inside a cell, able to detect, visualize, characterize and identify a single or a small number of molecules. Such a device could allow both the characterization of the chemical composition of proteins in their native environment and the study of the conformational changes of these proteins in situ.

Membrane proteins are usually hard to crystallize and it is very difficult to obtain structural and chemical information on how these proteins change their conformation in response to ligands, agonists and drugs. In order to understand fundamental biological processes, the project investigates on the 3-dimensional structure of membrane proteins, such as receptors and ionic channels and how they change their conformation following the binding of transmitters, hormones, drugs and signaling molecules.

In order to detect and identify previously unknown single molecule and to obtain structural information on membrane proteins it is necessary to manipulate the unknown molecule, measure its mechanical properties, obtain its chemical signature, correlate all these information at a molecular level.

By combining manipulation of a single molecule – as already been achieved – with the Raman and IR detection, it is possible to make substantial progress in achieving the " Biologist Dream " of a chemical microscope at single molecule level.

Therefore, within the SMD project the plan is:
1. to design and fabricate novel devices that allow on the same instrument and at same time: topographic measurements, binding force measurements and chemical measurements through Raman/IR-nearfield and TeraHertz spectroscopy;
2. to characterize the chemical composition and physical properties of some membrane receptors, such as rhodopsin, odorant receptors and ionic channels;
3. to explore the possibilities of THz nanoimaging for the characterisation of skeleton motions and network vibrations in biological tissues.

The nanotechnological component of the present project is involved in developing new devices in a surface operational mode as in AFM, or in the free space mode as in OT. In both configurations the tip is used to anchor the molecule and it acts as an antenna able to amplify the relevant signal. This amplification is one of the key components of the entire project.

The present project is based on two recent achievements obtained by Enzo Di Fabrizio's group on optical trapping and plasmonics [The group of Enzo di Fabrizio before starting the project designed and miniaturized single-fibre OT able to create a 3D trap and to provide manipulation capabilities. This was achieved thanks to the fabrication of an array of micromirrors on the head of an optical fibres bundle: in this way, trapping of 10-mm-diameter polystyrene beads was demonstrated. Photonic crystals and plasmonic antennas were added at the ends of the optical fiber so to concentrate the light in a very confined region.

It was also demonstrated that an hybrid photonic/plasmonic device consisting of a photonic crystal and a plasmonic antenna (PCPA) allows high efficient light coupling via plasmon-polariton interaction between far field radiation and local evanescent field allowing tip enhanced Raman spectra (TERS) to be performed with a sensitivity of few tens of molecules. Similar plasmonic antennas (APs) have been fabricated on top of commercial AFM tips in order to test their suitability for imaging. In planar configuration, this device has been tested for the detection of Raman spectra from very few molecules depositing single quantum dots (QD) covered by an organic coating composed of amino-terminated poly-ethylen glycol. The spectrum of a bulk suspension of particles (blue line) is compared with the spectrum of a single particle deposited on top of the PCPA structure (red-line) and it is possible to observe a signature of the amino group at about 3600 cm-1, in spite of the very small number of molecules – estimated to be less than 80 - present in the system.

Within the SMD project the focus is on the design, fabrication and use of novel devices combining photonic crystals and plasmonic antennas (PCPA) to achieve spectroscopic sensitivity for single molecule detection. These new devices allow Raman and Infrared spectroscopy to provide an excellent chemical signature of the molecules under observation.

The new devices developed within SMD are meant to be used to address some major neurobiological issues: the determination of conformational changes during channel gating in cyclic nucleotide gated channels and the determination of the structure of seven TM G protein coupled receptors - in photoreceptors and olfactory sensory neurons - and of their conformational changes upon activation.

During the project the groups functionalize the tip of the new device either with gold and/or hydrophobic sylane so to harvest proteins and molecules secreted by neurons under investigation. Harvested proteins are characterized by measuring their length, mechanical properties by force spectroscopy, either by a bead manipulated by an OT or by the cantilever of an AFM, following previous approaches. Their chemical composition and secondary structure are determined by IR and Raman spectroscopy.

The new devices will be used with the aim to:
- Determine the 3D structure of membrane proteins, such as ionic channels and membrane receptors, and their conformational changes upon ligand binding.
- Characterize the function and role of molecules involved in cancer and metastasis

These measurements provide a signature of their mechanical and chemical composition, quantifying their content of a-helices, b-sheets, hydrophobic-hydrophilic domain and of other major chemical constituents.

The aim of this passage is to obtain structural information on proteins which are hard to crystallize almost in their native environment. These measurements do not require the purification and crystallization of the protein under investigation and will be performed almost in situ. If successful, the present project will be a major biological breakthrough able to open major new perspectives. Therefore the project SMD provides an effective convergence between nano and bio technologies.

Project Results:
The first point in the work plan of SMD project is the "design and fabrication of novel devices capable of detecting a single or few molecules". The idea is to develop a structure capable of resolving details at extremely high resolution with the final goal of identifying a single molecule. A possible approach is to realize an artificial tool with the property of confining a high electromagnetic field in an extremely small portion of space which can be used as probe for chemical mapping at nanoscale level. Quasiparticles known as surface plasmon polaritons (SPPs) have the characteristic of being confined just around the interface (few tenths of nanometers) between metal and dielectric, which makes SPPs an ideal candidate for our purposes.

In order to understanding the physics behind the device realized at IIT and University of Magna Graecia it is necessary to provide some background on the field of plasmonics. Surface plasmon polaritons are bosonic quasiparticles originating by the coupling of photons and plasmons. The former are associated to the electromagnetic light usually originating by a laser (even though they are also commonly obtained from natural light sources) whereas the latter are related to ensembles of electrons in the metal. When light (photons) impinges on a metal (plasmons), under the appropriate conditions, can create SPPs. The physical laws followed by these quasiparticles are the Maxwell equations. In a classical way, we can picture the SPPs as an electromagnetic wave confined on an interface between metal and dielectric. The simplest situation where this phenomenon can be observed is on a flat surface. Actually exact theoretical studies can describe a perfectly metallic flat surface with infinite extension. The fundamental conditions that must be satisfied in order to realize the coupling between incoming photons and surface plasmons are conservation of the energy and the conservation of the component of the wave vector parallel to the surface.

The curve describing these relations is called dispersion curve and mathematically is described by the expression w(k), with w the frequency (or energy) and k the component of the wave vector parallel to the surface. Ought to the di erent shape between the dispersion relations of the incoming light and the surface plasmons this coupling is generally not possible. However there exist some "tricks", such as the use of a coupler, a corrugate surface or near-field excitation which allow an overlap between the two dispersion curves, which in turn means creation of SPPs. Once the SPPs have been generated they will travel along the surface with an oscillatory slow exponential decay, while a strong exponential decay will be manifested along the direction orthogonal to the surface. This characteristic, even though necessary in order to fulfill our goals, is yet not enough because the signal would extend on an infinitely wide area such as the surface. For single molecule detection we need an electromagnetic field possibly confined in the three spatial coordinates, contrary to a flat surface which is capable of providing just one-dimensional confinement. A conical-like structure have been introduced [1, 2] which o ers the possibility of localizing light in proximity of the apex of the pillar. Improvements in the fabrication techniques have lead to a perfectly nano-dimensional conical structure [1] which allows both better resolution and higher localized field.

A sub-10nm resolution was demonstrated. Here the team designed and fabricated different devices compatible with the AFM scanner available the labs constituting the consortium. In particular were fabricated structures capable of resolving details at extremely high resolution with

the final goal of identifying few/single molecule. The devices consist in nano probes with the capability of confining a high electromagnetic field in an extremely small portion of space which can be used as probe for chemical mapping at nanoscale level. The devices were delivered to the partners of the consortium for performing their characterization and measurement activity.

The second issue of the project was the "Fabrication of Photonic Crystal on Plasmonic Antenna (PCPA) on AFM cantilever"

PCPA tips have been continuously produced since the beginning of the project. Several tip aspects ahve been optimized. In particular the process to deposit the metal coating used to sustain the propagating polaritons has been optimized and the possibility of introducing a tilt to enhance the photon coupling has been evaluated.

The following process has been used to fabricate Photonic Crystal plasmonic antennae on Commercial AFM cantilevers:
The whole device was fabricated using an FEI Nova Nanolab 600 dual beam system. The photonic crystal consisted of a triangular lattice of air holes (lattice constant! 250 nm, hole diameter! 160 nm) patterned on a 100-nm-thick Si3N4 membrane. Three missing holes in the centre generated a photonic crystal cavity, termed L3, tuned at l! 532 nm. To fabricate the cavity on the AFM cantilever with a thickness of 100 nm, we thinned locally a commercially available Si3N4 cantilever (typically 600 nm thickness) around the area of the photonic crystal cavity. Ion-beam milling was then used to define the photonic crystal. Unfortunately it is not possible to use thinner slabs as starting material since the resulting mechanical properties of the AFM cantilevers would be too soft for proper imaging.

A silver tapered waveguide was grown in the centre of the cavity using electron beam induced deposition from a gas precursor containing a platinum–carbon polymer (CH3)3Pt(CpCH3) (ref. 16). A thin film of silver was deposited on the surface of the device (30 nm thick), and then silver was removed locally from the photonic crystal surface. The cone apex was machined with a radius of about 5 nm using low-current ion milling (current, 10 pA). Finally, the sample was immersed in diluted fluoride acid for a few seconds to remove a 10- nm layer of silicon nitride implanted by gallium during the milling process. PCPA tips have been fabricated on a large number of Si3N4 cantilevers of different shape and from different producers. Chips with PCPA devices have been sent on several occasions to the interested SMD partners namely: CBM UMG CNR IIT and RUB. The procedure for fabricating the PCPA tips has been transferred between partners and, in particular IIT will flank UMG for the fabrication activity starting in June 2011, for a beneficial of the whole consortium.

Resolution and durability tests have been performed within the first task of WP9 and will be treated in detail in that section. Briefly, a proper geometry, with cone growth angle tilted at 10 to 20 degrees with respect to the normal of the cantilever, has been selected, that increased significantly the robustness of the tips. Following these results we concluded that tapping mode in liquid is the best imaging condition and no further developments are required to increase durability and reproducibility of PCPA tips. Moreover, we observed that tip damage occurs during the engage process, which does not depend on the imaging technique. Therefore the above mentioned development of new high

frequency or shear force cantilever in order to minimize tip substrate interaction has been discarded.

For the task of fabrication of plasmonic antennas on optical tweezers beads we started with the idea to create conical plasmonic lenses on standard latex spherical beads.

The team succeeded on this task by using the following procedure. The start is from a glass substrate where 6 µm polystyrene beads are deposited. A 10 nm gold layer is then sputtered on the sample to avoid electrical charging effects. The Pt\C nanocones are fabricated by means of electron-beam-induced deposition using Pt gas precursor and a 0.15 nA current. The fabricated cones have 2 µm heights, 300 nm diameter bases and about 10 nm diameter tips. Afterwards, a 20 nm Au layer is deposited on the structures and then Focused Ion Beam (FIB), with a 50 pA current, is used to remove gold all around the cones. Afterwards, following what indicated by the simulations carried out in WP 1, for final best coupling of the excitation focused laser beam into an adiabatic plasmon polariton on the nanocone, a grating is fabricated by using a FIB milling. Although we succeeded on the fabrication of plasmonic nanocones on standard spherical beads, when moving to the Optical Tweezers (OT) we immediately faced some very limiting technical issues related to the "standard" spherical beads approach. First of all, as already mentioned in the risk assessment, we found that, due to the limited number of plasmonic beads that can be created with the previous procedure, it was extremely difficult to retrieve the small number of useful beads once they were suspended again in water for OT operation. A second important drawback of the spherical beads is related to the lack of an effective mechanism to fully control in the 3D space the orientation of the nanocones. This aspect is very important when it will be realized the physical approach of the plasmonic bead to the sample to be measured. A third important issue is comes from the observation that, when an optically trapped bead is close to, e.g., an in suspension cell, the latter will be also attracted by the optical trap due to the closeness of the trapping point and the plasmon excitation point, preventing an accurate positioning of the nanocone with respect to the sample.

Then, for the task of manipulating plasmonic beads with optical tweezers, to simplify the experimental procedure and to have more degrees of freedom on the bead-plasmonic antenna geometry and orientation, we choose to adopt an approach based on the direct fabrication of the beads through a 3D fabrication technique.

As a first step, we choose to create an asymmetric bead with a larger main body, where it is optically trapped, and a thin arm which will be the plasmonic part, which extends away from the position of the trapping laser. The asymmetrical structures are fabricated by using the two-photon lithography (TPL). The advantages of this technique, which is intrinsically a 3D structuring process, since the photo-ploymerizable resist is exposed only in the focus spot of an intense laser beam, rely on the straightforward process that allows creating structures that would be very complex or even impossible to fabricate with conventional lithographic methods. Moreover, as recently demonstrated, the spatial resolution attainable using TPL is sub-100 nm using laser wavelengths around 800 nm, which is well below the diffraction limit.

A 100 femtoseconds pulsewidth, 80 MHz Ti:Sapphire laser oscillator (Tsunami, Spectra-Physics) is used as the excitation source for two-

photon photopolymerization, and its central wavelength is tuned to around 720 nm. The output laser power at the back focal plane of the microscope objective is controlled by using a variable attenuator made by an achromatic half-waveplate and a polarizer. The exposure time for each pixel is controlled through a computer-driven mechanical shutter (Oriel Electronic Fast Shutter, Newport). The beam is expanded and collimated by a telescope in order to obtain overfilling of the focusing microscope objective. Further, laser beam is reflected by a 45° dichroic mirror directly onto the objective back aperture. The dichroic mirror reflects most of the NIR laser beam and transmits part of the visible spectrum (400 – 550 nm) enabling real-time monitoring of the sample surface with a CCD camera. We choose to use a dry semi-apochromatic microscope objective (Olympus, LUCPlanFLN 60x, N.A.=0.70) equipped with a spherical aberration correction collar to demonstrate the achievement of good results even without using more expensive oil immersion objectives.

A suitable coverslip holder is mounted on a xyz piezo-stage (Tritor 101, Piezosystem Jena) for positioning in horizontal and vertical directions. The travel range of the piezo-stage is 80 "m in each of the x, y, and z directions. A dedicated software, developed by our group, translates the structure-points to piezo stage positions and controls the synchronization of the movements with the mechanical shutter, in order to achieve the desired local dose.

A tube lens images the sample onto the CCD, which is used to check the position of the beam focus and for real-time monitoring of the photopolymerization process.

The photopolymer chosen for fabrication is a commercial UV curing adhesive (NOA 63, Norland) with optimum sensitivity in the 350-400 nm range.

We start out by depositing a drop of NOA 63 on a coverglass and letting it to achieve a mechanical equilibrium. A pre-exposure with a UV lamp is done for a few seconds in order to increase the resin viscosity. The laser power (measured before the objective) is set to approximately 6.5 mW with a pixel exposure time of 20 ms. For optical manipulation of the fabricated beads we use an optical tweezers setup based on an inverted microscope, with infinity-corrected optics, (Nikon ECLIPSE TE 2000-U) and a high numerical aperture objective (Nikon Apo TIRF 60X, 1.49 N.A., oil immersion, or Nikon Plan Apochromat 100X, 1.3 N.A., oil immersion). The laser source is CW Ytterbium fiber laser (YLM-5 from IPG Photonics) emitting at 1064 nm, linearly polarized. In order to generate and to move in the three-dimensional space more than one optical trap from one laser beam, a spatial light modulator (SLM - Hamamatsu X8267-15) for the dynamic generation of diffractive optical elements (DOEs) is employed in the optical setup. This system becomes truly efficient and user-friendly once the SLM is automatically operated through software control. For this purpose, a computer user-interface is accomplished in Lab view environment. The main core of the Lab view program is the algorithm for a fast calculation of DOEs, based on spherical-waves approximation. The DOE resolution is 768!768 pixels, which matches exactly the SLM resolution, and it is coded in a 256 gray scale. A real-time CCD camera acquisition is also displayed on the interface and it allows observing both the sample and the generated spots. In order to make easy the positioning of one laser spot at a desired point, a "click and generate" function has been implemented for the real-time acquisition.

The TPL fabrication of microscopic objects able to be optically trapped was already reported in literature: the 3D structures were fabricated not in direct contact with the glass surface but are separated by a thin layer of unsolidified material. The possibility to lose the structures, during the removing process of the unsolidified material is quite high. Also, to find microscopic object floating in a large liquid volume is difficult. A matrix of asymmetrical beads are fabricated on the coverglass surface, and for each bead two small "legs" were added in order to keep the beads attached to the coverglass after development process. After washing out the unpolymerized resin, the coverglass with the beads is introduced into a petri dish containing a Phosphate-Buffered Saline (PBS) solution. Afterwards, while viewing the sample under a low magnification microscope objective, one of the beads is detached and aspired with a glass capillary mounted on a mechanical micromanipulator (InjectMan NI 2 from Eppendorf) and connected to a manual piston (CellTram Oil) by a silicon rubber tube. The diameter of opening at the end of the capillary (Eppendorf - custom designed for our needs) is about 10-µm. The maximum travel range of the manipulator arm is 20 mm for each of the 3 axes with a resolution of about 40 nm.

Further, the microscope objective is changed with the one used for optical trapping (a high numerical aperture is needed). The collected asymmetrical bead is then carried under the optical tweezers action using the micromanipulator and here is released from the capillary to be optically trapped. The transfer of the beads, from the capillary to the trap is made with help of the piston pump. To ensure controlled, constant pressure transmission, the cylinder, pressure tube and part of the capillary are filled with water.

To this point we demonstrated the possibility of custom bead fabrication, their efficient delivery to the OT operation field and their successful trapping, As a further step, to obtain true full 3D spatial orientation control of the plasmonic antenna carried by the custom fabricated bead, we finally choose to adopt a bead design with three different trapping points and a protruding tip. The geometry will be the best one to guarantee both full 3D orientation control and spatial separation between trapping points and Raman excitation points. Another interesting feature of this structure is the fact that it will probably suffer less Brownian motion- related displacement noise with respect to smaller structure as the spherical bead.

After the TPL asymmetric bead fabrication, as a second lithographic step, a nanocone and a grating are fabricated on top of the protruding arm of each bead by using the FIB-SEM dual beam system, following the same process previously reported with the spherical beads As a further step, we delivered these fabricated beads on the OT operation field to assess the ability to trap and effectively orientate them. By using our OT-control software we were able to generate and move as a rigid frame a set of three optical traps, spatially arranged as the three as-a-bead parts of the fabricated structure.

These experiments finally confirm us what is the best final geometry for the bead-nanocone structure, in order to avoid the technical issues described at the beginning of this report.

Next steps that are currently going on, will be the experimental verification of the effective coupling of the plasmonic nanocone by illuminating the grating structure while the bead is optically trapped,

the development of a suitable video-tracking algorithm for detection of bead displacement and, finally, a full-system Raman/OT experiment with the described asymmetric beads\nanocone structure.

In WP4 "fabrication and testing of plasmonic antennas on AFM cantilever in order to combine force measurements with IR and THz spectroscopy". The team did a strong effort in order to find the best suited cantilevers for supertip modification as well as utilization in AFM and infrared (IR) microscopy and THz. Different kinds of cantilevers were modified at UMG and were tested at RUB. The reason is that it was necessary to make compatible the general architecture, the geometry, the detection system with the new cantilever developed for this project. The majority of this deliverable has been completed by the integration of supertips at the end of AFM cantilevers. The novel PCPA tips designed and fabricated were tested and preliminary results on polymers and proteins show already a good improvement compared to the best results obtained by using commercial cantilever.

The fabrication of supertips on AFM cantilever was performed by Francesco De Angelis in the group of Prof. Enzo Di Fabrizio at the UMG. In order to find the best suited cantilevers for supertip modification as well as utilization in AFM and infrared (IR) microscopy, different kinds of cantilevers were modified at UMG and were tested at RUB. Scanning electron microscopy (SEM) micrographs 1-2 depict a carbon/platinum nanocone grown on a tipless NSC12 Cr/Au cantilever from MikroMasch. Image 3-4 displays an ArrowTM CONTR silicon cantilever from NanoWorld with a tetrahedral tip with a curvature radius of less than 10 nm before and after modification with a silver coated carbon/platinum nanocone. Micrograph 5-6 show our standard gold coated cantilever NSC16 Cr-Au from MikroMasch with pyramidal tip with a tip radius of less than 50 nm before and after growth of a silver coated carbon/platinum nanocone with a tip curvature radius of less than 10 nm on top of the pyramidal apex. First step for testing the supertips with our IR microscope was to implement them in our Nanotec AFM.

The AFM provides the basis of our IR microscope and the first aims of our studies have been:
1. Obtaining a "good" deflection signal and resonance curve"
2. approach a sample surface without damaging the tip "
3. scanasurface"
4. test of focusing our infrared beam onto the tip"

For first experiments gold coated carbon/platinum nanocones were attached to tipless cantilevers (NSC12/Cr-Au form MikroMasch).

In our near-field measurements, we perform phase-sensitive lock-in detection on the resonance frequency of the AFM tip. In order to avoid interfering signals we use special frequency filters (320, 170, 75, 10 kHz) before feeding the cantilever frequency output into the lock-in reference input. The tipless cantilevers are much shorter (l = 90 to 130 "m) compared to the cantilevers we commonly use (l = 230 "m), resulting in much weaker optical power at the 4-quadrant photodiode. Nevertheless, we successfully record a resonance curve with a sufficient resonance peak amplitude of approximately 1 V.

Approaching a sample surface was successful, but we were faced with the challenge to achieve stable imaging conditions (e.g. having topography profiles of forward and backward scan tracking each other). Measurements

on a silicon calibration grid with a periodicity of 3 "m show a variation in the image with a periodicity of about 1.5 "m. Changing the scan angle resulted in a rotation of the pattern. Scanning the same calibration grid with a commercial tip To monitor changes in the supertip due to use, we recorded electron microscopy (EM) images of each tip before and after approaching the sample. The EM micrographs recorded after approaching the surface confirmed that the tip at the cantilever is still intact. Most likely, the difficulties during scanning result from the large cantilever width- to-nanocone length ratio. If the sample or the cantilever are tilted by greater than 4.5°, the cantilever might touch the sample earlier than the tip or influence at least the tip-sample interaction. Since our BerMad2000 AFM from Nanotec Electronica works with three stepper motors, a slight tilt of the sample cannot be entirely excluded. Another issue that became obvious during EM are small additional features on the cantilevers. Some of our EM micrographs clearly show "satellite" features similar or even larger in height than the nanocone itself. These features might disturb or influence the tip-sample approach, as well.

Since the nanocone is much smaller than the cantilever, only a small gap between cantilever and sample remains after approaching. Therefore the nanocone illumination is difficult. Only a small part of the incoming light illuminates the nanocone. Furthermore the scattered near-field signal from the nanocone might also be disturbed by the cantilever.

All of the above outlined problems have been solved by growing the nanocones on top of the pyramidal apex of our "standard" cantilevers (NSC 16 Cr/Au or NSC 18-Cr/Au from MirkoMasch) The optical power at the 4-quadrant photodiode is almost at a maximum value.

Approaching sample surfaces is successful, and also scanning a sample surface in contact as well as in dynamic mode is possible. Using the nanocone grown on AFM tips, we achieved both high resolution large-scale and small-scale imaging with better than 50 nm lateral resolution. Additional features can now be seen on the porous aluminum test sample that is otherwise not observed using commercially available tips.

During scanning, we encountered problems with long-term stability. Continuously scanned images shared a sudden decrease in lateral resolution with a later sudden increase in lateral resolution. This might result from a bending of the nanocone or contamination with dirt. Therefore, optimal stabilization of the tips for high resolution scanning is the subject of future work.

When a blurred image is recorded, it is important to determine if the tip got damaged, requiring an easy way to characterize the tip. A simple and convenient method to prescreen AFM tips are tip characterizing samples exploiting reverse imaging.

We tested different kinds of tip characterizing samples:
- a 1-D array of triangular silicon steps (TGG01, MikroMasch
- Tip Check sample (Budget Sensors) - porous aluminum (PA01, MikroMasch)

The 1-D array of triangular silicon steps can be used for 2-D tip characterization. Since the edge of the triangle has a curvature radius of less than 10 nm, a reverse imaging of the AFM probe is possible. We have tested this grid with a standard gold coated tip with a curvature radius of less than 50 nm and a standard silicon tip with a curvature radius of less than 10 nm. When estimating the tip curvature radii from

the recorded line profiles, a good agreement with the given radii is found. However, this does not hold for dynamic mode measurement.

Currently, the evaluation of the tip characterization using a blind reconstitution algorithm is under way. The blind reconstruction method assumes that sharp features in the AFM image are the result of self-imaging of the tip, or equivalently, objects on the sample surface with dimensions smaller than the tip radius of curvature act as a probe to image the AFM tip. In order to confirm the results from the tip characterization samples, SEM images of the tips have been recorded Additionally, these images demonstrate that the nanocones are still at the apex of the standard tips, even after scanning different samples in contact as well as dynamic mode. In addition to confirming the improved lateral resolution of the supertips with standard triangular gratings and the porous alumina test samples, we have also successfully accomplished the first near-field (NF) infrared measurements at 9.22 "m using the supertips from Prof. Enzo Di Fabrizio. The collection of measurements confirm the laboratory prototype device, which uses a modified version of a Nanotec AFM has been demonstrated and by using silver- coated supertips on AFM pyramids, we can achieve improved resolution compared to commercially available gold-coated AFM tips.

We have demonstrated the supertips show much better lateral resolution than the commercial tips. Results of recent simulations – which were undertaken together with the group of Enzo di Fabrizio, indicate that by changing the geometry of the supertips we can further improve the plasmonic-induced field enhancement at the tip apex in the wavelength range (9 to 11 "m) used in our SNIM setup. Due to excessive computation time, our simulation so far could not take into account the geometry of the nanocone in combination with a commercial pyramidal tip since this complex structure contains length scales ranging from micrometer down to nanometer. To speed-up calculations, we have purchased a computing cluster and a finite-element method based electromagnetic solver, which uses an adaptive discretization of the computation domain, thus considerably reducing the memory and time consumption. This allows us to decrease the computation time and more efficiently optimize the nanocone/AFM tip geometry.

We have successfully demonstrated IR enhancement on polymer brushes [1], and proteins embedded in reconstituted artificial membranes [2]. We showed that SNIM has an enhanced detection sensitivity and is capable of probing volumes as small as 80 nm # 80 nm # 30 nm [1]. SNIM measurements on polymer brush surfaces show the high sensitivity of this technique in being able to distinguish nanophase-separated domains of polystyrene (PS) and polymethylmethacrylate (PMMA) in complicated Y-shaped polymer
a) b)
c) d)

We have additionally accomplished Task 4.2 of WP4 by imaging proteins in membranes. Correlation between simultaneously recorded topography and infrared near-field measurements between 1600 cm-1 and 1800 cm-1 allow a 3D chemical reconstruction of the artificial membrane with a resolution of 80 nm.

Then we started measurements on nanostructured DNA samples provided by the Dortmund group. DNA-protein conjugates provided by the group of Prof. C. Niemeyer (TUDO) are ideal test samples for high resolution imaging as well as spectroscopic characterization. The molecules consist of a

double-stranded DNA strand with a Streptavidin attached at each end of the DNA).So far H. Schmitte from TUDO synthesized two conjugates with different DNA lengths (255 base pairs, corresponding to a distance of 86.7 nm, and 486 base pairs, corresponding to a distance of 165.24nm).

Preparing the DNA dumbbell samples for successful use in the near-field microscope was more challenging than initially expected since we cannot use the same protocol for sample preparation as the TUDO group. This originates from the fact that we need to use flat, gold surfaces for scanning near-field infrared measurements instead of mica.

First, we had to overcome topographic artifacts resulting from dumbbell aggregation. In addition to causing artifacts, this aggregation increases the chance of tip contamination. To avoid aggregation, we used different preparation methods. Specifically, we decreased the concentration of conjugate solution, as well as MgCl2 buffer solution needed to activate the gold surface. However, reducing the MgCl2 buffer also limits binding of the dumbbells to the surface. Nonetheless, parallel processing of samples allowed us to obtain a sample containing a sufficient amount of DNA dumbbells that didn't aggregate.

Using near-field probes with a curvature radius of about 50 nm, we were able to acquire AFM images Using conventional NSC 16 silicon cantilevers (NSC-16 AlBs, MikroMasch) with a radius of curvature of 10 nm, we were able to obtain high resolution AFM images of the dumbbell DNA samples The smaller curvature tips can more clearly resolve the DNA dumbbell structures. We have also completed the task of sensing nano-structured DNA chips, while we have already demonstrated the ability of "Simultaneous recording of topographical information by the AFM and IR spectra with nm resolution" and "IR and THz spectroscopy measurements." And we worked on further optimization of the supertips for use in the IR, as well as improved robustness during AFM imaging. We decided to first finish the optimization of the current supertip design due to 1) the integration of the new investment parts from Nanotech (closed loop-feedback for AFM) shifted additional testing of the supertips, and 2) the CO laser had to undergo extensive repair. These issues required us to shift the schedule of D4.3. We anticipate that initial results will be obtained in January/February 2011.

The task of combining the p-Ge laser with the near-field microscope with the p-Ge has been re-designed due to the limitations of the laser source. The p-Ge laser operates at 100 Hz pulse rate which requires a lock-in integration time of at least 10 ms per point for a SNIM image resulting in data collection times exceeding one hour for a single SNIM image. This excessive time is not feasible and additional strong laser sources in the same wavelength range are currently not available. To achieve the goal of THz SNIM, we will use the newly installed THz/IR Nanoscope at the synchrotron light source ANKA.

The use of synchrotron radiation has three primary benefits:
1) since the pulse rate of the synchrotron is 500 MHz (2 ns between light pulses), the beam operates in a "quasi-cw" mode and we can use similar lock-in integration times of 1 ms, thus ultimately reducing our data collection times to only 20 minutes per SNIM image;
2) the peak pulse power of 1-2 W is comparable to the p-Ge laser system. Indeed, Amarie et al. have shown that with 0.005 mW quasi-cw power distributed over the wavelength band from 9 to 12 "m, it is possible to make near-field measurements [3]; and

3) the broadband nature of the radiation will help us to more easily pinpoint known resonances of the supertips.

The last point is probably the most critical for efficient coupling of THz radiation to the supertip. As discussed previously, we are in the process of running computer simulations to determine optimal supertip geometries for improving IR and THz radiation coupling to the supertips and anticipate initial results in 6-8 weeks.

To address parts of Task 4.5 of WP4, we utilized the ANKA Nanoscope in combination with a CO2-laser emitting radiation in the long MIR regime (wavelength range 9 to 11 "m, 1111 to 900 cm-1) to provide measurements on nanostructured DNA. In this wavelength range, DNA exhibits a strong absorption band at 1090 cm-1 that is assigned to the symmetric PO2- stretching vibration. We have successfully obtained near-field images on microstructured printed line-patterns of monomolecular films of DNA and 1- octadecanethiolate (ODT) using commercial gold-coated cantilevers (NSC 16 Cr/Au from MikroMasch). More complex DNA structured surfaces, in the form of DNA chips, have also been imaged with SNIM [4]. The DNA chips consist of a monolater of 6-Mercapto-1-hexanol (MCH) as a linker molecule to immobilize single stranded DNA (ss-DNA) molecules to a gold substrate. Using an AFM-assisted nanografting technique, patches of ss-DNA were removed and replaced by double stranded DNA (ds-DNA). When going through the absorption band associated with ds-DNA, it's clear in both the spectra and SNIM images the ability to differentiate between the two types of DNA. The lateral resolution of our current measurements is about 125 # 125 nm2, or about 103 to 106 DNA molecules, which is almost 104 times better sensitivity than typical far-field FTIR microscopy techniques.

As a crucial step towards utilising SERRS to obtain nanoscale distance-dependent information at single- molecule level, we investigated the first example of the parallel hybridization of dye-labeled LNA silver nanoparticle probes to double stranded DNA (dsDNA) bridges of different lengths via hoogsten hydrogen bonds to form a triplex assembly that provides SERRS enhancements directly related to the interparticle distance imposed by the high structural rigidity of the double stranded linker. To acquire the necessary knowledge to tackle the challenging application at single molecule level, we first investigated the triplex-assembling ability of DNA-conjugated silver nanoparticles within an ensemble of nanoparticles in suspension. In this manner, we managed to gain new important insights into the correlation between the averaged time-dependent plasmonic and SERRS responses in these complex systems which pave the way for improving the reproducibility and robustness of SERRS signals within the complex environment of a colloidal solution along with a variety of applications such as the study of DNA in its natural double stranded state and, ultimately, using SERRS to obtain nanoscale distance- dependent information in challenging biological environments using specially designed optically active materials based on hybrid biological-inorganic systems with programmable properties. These results laid the foundations for the second part of the study, which is in progress at the moment and deals with the application of SERRS at single nanoparticle and single molecule levels.

When two nanoparticles supporting localized surface plasmon resonances (i.e. coherent oscillations of the metal electrons in resonance with light of a certain frequency; LSPRs) are brought into close proximity, new plasmon resonances arise from the coupling of the individual LSPRs

whose shift, for a specific size and composition, is largely dependent on the interparticle separation. This plasmon coupling is also at the heart of high electromagnetic fields concentrated at the interparticle gap which provide dramatic enhancement of the Raman scattering for molecules located at these junctions down to single-molecule detection capability. The use of single stranded DNA (ssDNA) as a powerful molecular tool for controlled assembly of NPs functionalized with complementary sequences is widely known and takes advantage of the extraordinary properties of DNA, such as molecular recognition, structural plasticity and programming capabilities to precisely direct the assembly [DNA is naturally found in the double stranded form and this is the first example of targeting such a structure to provide discrimination of binding by SERS. Triplex formation requires certain criteria to be met. Firstly, the target strand of the double-stranded DNA target must be of polypurine sequence due to the unavailability of hydrogen bonding sites from cytosine and thymine in the major groove, where the triplex-forming oligonucleotide (TFO) binds. Limited hydrogen bonding also requires triplex forming cytosines to be protonated. Indeed, Jung et al. exploited this fact by manipulating the pH of the system to reversibly assemble oligonucleotide gold conjugates into a triplex [16]. Cytosine protonation may also be achieved by modifying the bases to incorporate a methyl group at the 5 position. 5-Methylcytosine has an increased pKa therefore triplex formation can be performed at neutral pH. Another consideration is that hybridisation to form tri-molecular species is entropically unfavorable and so preorganized DNA analogues are often employed to reduce loss of entropic energy. One such analogue is LNA; a conformationally restricted structure that incorporates a methylene bridge across the ribose sugar. However, the increased thermal stabilities afforded by incorporation of LNA modifications has been attributed predominantly to improved base stacking interactions [17]. In addition, dsDNA offers high structural rigidity as a molecular linker, overcoming flexibility and bending properties that limit the ssDNA application when a strict control of interparticle distance is required [15].

Experimental Chemicals. DNA oligonucleotides were purchased from ATD Bio, Southampton, UK. LNA/DNA chimera oligonucleotides were purchased from Eurogentec, Belgium. X-rhodamine-5-(and-6)-isothiocyanate (ROX-ITC) was purchased from Invitrogen, Paisley, UK. All other materials were purchased from Sigma- Aldrich and used without further purification.

Nanoparticle synthesis. Silver nanoparticles (Ag NPs) were prepared according to a modification of the method presented by Lee and Meisel [18]. An aqueous solution of AgNO3 (53 mM, 2 mL) was added to Milli-Q water (100 mL) that had been heated to 40°C. Following further heating to 97°C, sodium citrate (43 mM, 2 mL) was added. The temperature of the reaction mixture was maintained at 97°C for 90 minutes. The colloid was then submitted to slow centrifugation (1k rpm, 20 min) to eliminate larger irregular nanostructures and aggregates. Deposits at the bottom of the falcon tube were eliminated. Ag NP concentration in the supernatant was determined by UV-Vis spectroscopy using an extinction coefficient of 3.0x1010 M-1 cm-1 at 408 nm. The average particle diameter measured from SEM analysis was 48 ± 5 nm.

Nanoparticle functionalization and Triplex assembly. 5' Thiolated (Oligo 1) and 3' thiolated (Oligo 2) oligonucleotides were conjugated to NPs by addition of the oligo-nucleotide solution (2.5 nmoles) to the colloidal supernatant (0.12 nM, 1 mL) to give probe 1 and probe 2, respectively. NPs functionalized with oligonucleotides were incubated overnight at room

temperature, followed by the addition of phosphate buffer (60 mM pH 7) giving a final concentration of 10 mM phosphate. Following 24 hours at room temperature, the salt concentration was progressively raised to 0.1 M by successive additions of NaCl 1.0 M over 48 hours. Afterwards, labelling with the resonant Raman reporter Carboxy- X-Rhodamine Isothiocyanate (ROX-ITC) was carried out on Ag NP functionalized with Oligo 2 by addition of 7.0 ROX-ITC 10-6 M methanol solution to 1 ml of NP sample (Probe 2) was performed in the presence of unbound oligo 2 in the bulk solution to preserve NP monodispersity. The solution was then incubated for 16 hours in the dark. 3 steps of centrifugation and resuspension in PBS 0.3 M were carried out on both probe 1 and probe 2 samples to eliminate both excess of oligonucleotides and dye molecules (final probe concentration 0.20 nM each). Each step of separation consisted of two subsequent centrifugations: (i) 4k rpm, 15 min. Nanoparticles loosely sitting at the bottom of the eppendorf were collected whereas the supernatant was subjected to a second centrifugation (ii) 6.5K, 15 min. The second precipitate was then combined with the first one, and the sample then subject to the subsequent centrifugation step. Head-to-head nanoparticle assembly was initiated by addition of complementary double stranded target DNA. Specifically, 10 of dsDNA 1 µM solution in PBS 0.3 M was added to a nanoparticle mixture consisting of 42 µL of each probe 1 and probe 2 solutions (0.20 nM) and 306 µL of PBS 0.3 M.

SERS, UV-Vis Absorption, Dynamic Imaging, DLS measurements and SEM microscopies. SERS was recorded using a Renishaw Probe spectrometer with a 532 nm argon ion laser equipped with a 50 x long working distance objective, 0.6 N.A. UV-Vis analysis was performed on a Varian Cary Bio 300 spectrometer. Extinction triplex melting experiments of oligonucleotide-modified NPs were recorded at 408 nm and the temperature was programmed to a heat/cool rate of 0.3°C/minute. UV duplex melting experiments of dsDNAs were recorded at 260 nm and the temperature was programmed to a heat/cool rate of 1°C/minute.

Rayleigh scattering image and tracking analysis was per-formed using a Nanosight LM10 instrument and accompanying Nanoparticle Tracking Analysis (NTA) software version 2.1. Videos of 90 seconds duration were analyzed using the NTA software. The experimental set-up used for particle imaging is reported in detail in reference [19]. Aliquots of the investigated sample (NP probes assembled via dsDNA15 addition) were taken at various time intervals (t = 0, 12, 32 and 60 minutes during the aggregation process) and diluted 25 times prior to image analysis to drastically slow the assembly process during video capture.

Dynamic light scattering (DLS) measurements were per-formed with a High Performance Malvern Zetasizer.

Results and Discussion

Ag NPs (48 ± 5 nm) were modified with thiol-functionalized oligonucleotide sequences 1 and 2 to create probes 1 and 2, respectively. Each sequence has LNA modifications incorporated every 2-3 bases and 5-methylcytosine to aid triplex formation as well as three hexaethylene glycol (HEG) units as a spacer adjacent to the terminal thiol group. In addition, probe 2 was also labeled with a resonant Raman reporter, ROX-ITC, which covalently binds to the silver surface via the isothiocyanate group [20]. The two sets of oligonucleotide-modified nanoparticle probes (total concentration 40 pM, ratio1:1) were dispersed in PBS buffer (0.3 M) and assembled by addition of dsDNA (final concentration 25 nM) of different lengths at room temperature. The target dsDNA includes an internal sequence with no complementarity to the NP probes, formed by 0, 5, 10 and 15 base pairs (dsDNAx, where x= 00, 05, 10 and 15) corresponding to overall duplex lengths of approximately 4.6, 6.3, 7.9 and 9.6 nm, respectively.Triplex formation is revealed by a change of the probes extinction profile. The LSPR of monodispersed NPs with a max at 408 nm is progressively weakened, broadened and red-shifted as the aggregation proceeds, eventually reaching a self-limiting plateau.

Extinction melting experiments were also performed to determine the melting temperatures, Tm, of the triplex-to-duplex transition [21]. The Tm for triplexes incorporating 0, 5, 10 and 15 base pair spacers were found to be approximately 44°C, 41°C, 40°C and 38°C, We suggest that the observed aggregation delay and decrease in Tm for longer dsDNA targets could be associated with higher interparticle salt concentrations resulting from larger overlaps of electric double layers surrounding the oligonucleotide-functionalized NPs for short interparticle distances. In fact, it is known that increased ionic strength and local dielectric constant stabilizes the hybridization event and, in turn, increases the temperature required to achieve NP dissociation.

To investigate the correlation between the ensemble-averaged plasmonic and the SERS responses of dsDNA-driven NP assemblies, we monitored the aggregation process in the 0-80 min time range. High- throughput Rayleigh-scattering imaging and tracking analysis of many individual particles/clusters in suspension was also used to characterize the heterogeneity of the NP-assembly in suspension and provide statistically robust information correlating particle size distribution with the bulk optical responses. The accurate evaluations of the relative density and size of particles in the detection volume cannot be achieved by common microscopic techniques such as TEM because such investigations provide limited statistics and suffer from drying effects during the process of immobilization that may lead to unspecific aggregation. Plasmon resonances arising from plasmonic coupling of interacting NPs (referred to here as "gap- plasmon resonances") are largely responsible for SERS enhancements in aggregated colloids. To reveal more clearly the red-shifted gap-plasmon resonances, difference extinction spectra were obtained by subtracting the initial extinction spectrum prior to the addition of dsDNA from each time-dependent spectrum of the dsDNA-driven assembling nanoprobes The spectral behaviour of these two features resembles that observed for quadrupolar (Q) and gap-plasmon (G) resonances in the case of dimer extinction spectra for longitudinal incident polarization with respect to the dimer axis. This analogy suggests that the G band may be considered as an ensemble-averaged "gap-plasmon-like" resonance gathering most of the plasmonic contributions that arise from the coupling between LSPRs of individual NPs. To prove this point, the peak position of the G band was monitored during the NP

assembling process for each dsDNA linker. More interestingly, the relative position of the plateau onset shows to be dependent on the dsDNA length as its absolute red-shift is larger for shorter interparticle spaces. Similarly, the relative magnitude of the G resonance is also dependent to the extent of the assembly.

In particular, it is possible to recognize three main regimes during the aggregation process:
(i) an initial rapid increase of G intensity) followed by
(ii) an intermediate plateau which eventually evolves into
(iii) a slow intensity decrease associated with a large band broadening.

Similar time-dependent profiles were obtained for all the dsDNA linker sizes, except for the time range over which each of the identified aggregation regimes occurred. As previously mentioned, this is due to the increasing rates of cluster growth imposed byduplexes of shorter length. A high-throughput and real time tracking of cluster formation in solution was carried out via dynamic imaging analysis to correlate the optical bulk response to subpopulations of particles in solution and, thus, gain a deeper understanding of the dsDNA-driven assembly of NP probes. Dynamic imaging analysis prior to assembly (t=0) shows a monodispersed NP distribution with an average hydrodynamic radius of ca. 80 nm, which matches well with values obtained by DLS (ca. 77 nm, data not shown). When dsDNA15 is added to the sample, a progressive decrease in particle concentration is observed as the NP assembly proceeds, which is associated with an increased polydispersity of the size distribution. In particular, after 12 minutes the NP distribution is still mostly dominated by the monomer population but a broadening of the particle distribution is observed. At minute 32 (i.e. ca. maximum G band intensity), an approximately 5-fold reduction in particle density is revealed and it is clearly possible to distinguish separate contributions at 79 nm, due to monomers, at 107 and 148 nm, ascribed to dimers and trimers, and 181 nm which can be assigned to clusters of 4-6 units. Interestingly, even for extended NP aggregation (t=60 min) and correspond-ing large broadening of the initial extinction spectrum , dynamic imaging measurements indicate the presence of residual monomers and dimer/trimer populations in significant amounts.

Based on the dynamic analysis of individual particles, we can deduce that in the early stages of the NP assembly, the G band increases in intensity since dimers/trimers are formed mostly to the detriment of individual NPs (i.e. interparticle junctions are generated in the colloidal dispersion). Progressively, the population of small aggregates (2-6 NPs per clusters) increases to a certain point where these clusters are themselves significantly involved in aggregation events to yield larger features with broader and weaker plasmonic contributions. This corresponds to the change in trend of G intensity which first reaches a plateau and then, for more extended aggregation, undergoes a marked weakening associated with a simultaneous broadening of the band. The G intensity trends also suggest that shorter dsDNA duplexes promote faster aggregation dynamics. Interestingly, the maximum G intensity is achieved when the unsubtracted extinction intensity of the initial NP probes at 408 nm (no dsDNA) is approximately halved, regardless of the dsDNA length. It is also worth noting that the dynamic of the NP assembly differs from the one observed for salt-induced aggregation of citrate- capped silver NPs, where large clusters of 150-350 nm hydrodynamic diameter were formed on a fairly homogenous scale, with a very small fraction of remaining unaggregated NPs [19]. This result highlights the different cluster assembly dynamics

of the two systems: the electrostatic-induced aggregation equally destabilizes all NPs in solution as soon as the salt is added to the colloid, whereas the dsDNA- driven assembly leaves the particle unperturbed unless successful hybridization events occur forming a larger cluster.

In addition to colour change, plasmonic coupling of individual NPs provides a strong enhancement of the Raman scattering from molecules located at or near the interparticle junction [14]. The time- dependence of the ensemble-averaged SERS intensity during the aggregation process was therefore investigated by monitoring the peak height of the ROX-ITC band at 1646 cm-1 to determine possible correlation with the plasmonic G feature. The SERS intensity response shows a rapid increase in the early stages of assembly then, once a maximum is reached, a slow and moderate decrease, previously re-ported in the literature for dynamic NP assemblies [28,29]. More importantly, our results indicate that the SERS response follows similar trends to those observed for G band intensity, reaching maximum values at approximately the same aggregation times (more specifically, max. SERS intensities consistently occur at slightly longer times at the same dsDNA length). Based on the detailed characterization of the aggregation process, we can interpret this result in terms of optimal balance between the assembling of poorly enhancing monomeric NPs to form interparticle junctions, and the consumption of highly SERS active enhancers (2-6 NP units per clusters) to yield larger aggregates. The latter process decreases the cluster density in the colloidal volume interrogated by the laser in suspension without providing counterbalancing benefits in terms of overall SERS enhancement [30]. Similarly, relative SERS intensity and corresponding position of the G band correlate very well in terms of interparticle distance dependence: the shorter the interparticle gap, the higher the SERS intensity and more red-shifted the G band.

These results constitute clear evidence of the direct correlation between the SERS and the extinction properties of randomly aggregated NPs with defined interparticle distances in suspension. Moreover, the simple and straightforward method provided in this work to monitor the aggregation degree for chemically-driven assembly of NPs by following the "gap-plasmon like" band via the extinction plot, allows to take into account uncontrollable changes in experimental parameters that limit the SERS reproducibility by affecting the aggregation dynamic (such as NPs and linker concentration, temperature, sample preparation etc.). This becomes a key factor when accurate and reproducible correlation between the magnitude of the SERS response and the interparticle distance needs to be determined.

Within the project, the Technical University Dortmund (TUDO) group worked on the development of DNA-protein and DNA-nanoparticle conjugates With respect to the set of DNA oligonucleotides it required for conjugation to proteins and nanoparticles comprise the fundamental building blocks for all described objectives. We therefore initially focused on the design of oligonucleotide sequences, which allow for efficient and highly specific nucleic-based assembly of DNA-protein and DNA- nanoparticle conjugates. DNA directed immobilisation (DDI) approach allows a sequence specific and efficient hybridisation of complementary single-stranded DNA (ssDNA). The DDI method is a chemically mild procedure for highly parallel and reversible attachment of multiple proteins or nanoparticles to a range of solid supports [3-4]. In previous projects, we had established a library of 14 oligonucleotides [5], which was generated with DNA sequence design

software[6] and examined in vitro using DNA microarray technology. This library was now tested for the specific requirements of the SMD project. The respective work included further optimisation in silico, chemical conjugation of oligonucleotides to model proteins, in particular fluorescent proteins (see below), reconstituted Myoglobin (Mb) containing an artificial photocatalytic heme group, or monovalent streptavidin (mSTV; see below). Experimental assessment of the most promising subset of oligonucleotides in microarray- and nanoparticle- (AuNP and MNP; see below) based DDI experiments were carried out. It was found that special requirements of SMD are met best by a subset of oligonucleotides of the previous established "F-library", i.e., F1, F5, F9 and F10 which are supplemented by oligonucleotide A24. These oligonucleotides reveal almost identical hybridisation efficiency and complete orthogonality with respect to cross-hybridisation.

The following sequences of oligonucleotides were therefore selected: F1: 5'- CCT GCG TCG TTT AAG GAA GTA C -3' F5: 5'- GGT CCG GTC ATA AAG CGA TAA G -3' F9: 5'- GTG GAA AGT GGC AAT CGT GAA G -3' F10: 5'- GGA CGA ATA CAA AGG CTA CAC G -3' A24: 5'- TCC TGT GTG AAA TTG TTA TCC GCT -3'.

With respect to the Design and synthesis of DNA-fluorescent protein conjugates a methodology for DNA-conjugation and immobilisation of fluorescent proteins (FP) onto surfaces for the planned AFM and plasmonic device was developed. To this end, TUDO group synthesised six novel DNA oligonucleotide-FP conjugates, based on the mutant variants of native FPs, using molecular cloning and heterologous expression. In particular, the fluorescent proteins ECFP, a cyan mutant of jellyfish Aequorea victoria GFP, EGFP], E2GFP [9], mDsRed, a monomeric variant of tetrameric DsRed available from Clontech, photoswitchable Dronpa,and mCherry were cloned with an N-terminal hexahistidine sequence and a C-terminal cystein residue. All six FPs were fully characterised, including electrophoretic and spectroscopic analysis. All details are summarised in the accompanying reprint of the resulting publication.

The cystein residue of the cloned set of FPs was then used for site-selective covalent attachment of oligonucleotides using the heterospecific crosslinker Sulfosuccinimidyl-4-(N- maleimidomethyl) cyclohexane-1-carboxylate (sSMCC) The resulting conjugates were purified, chemical conjugation yields determined and the conjugates were fully characterised by biochemical techniques, namely gel electorphoresis and hybridisation studies (see below). This work has already been published in an international leading journal on bioconjugation chemistry and specific results of this study are summarised in the attached reprint.

With respect to the Protocol for DDI of fluorescent proteins and hemoproteins, the aforementioned FPs were used for DNA-directed immobilisation (DDI). To this end, ssDNA-functionalised gold nanoparticles (AuNP), as versatile models for the planned AFM and plasmonic devices, were employed for the hybridisation of the FP-DNA-conjugates. The hybridisation efficiency was studied by fluorescence quenching of the FP resulting from the close coordination to photoactive AuNP. Additionally, different quenching effects due to the FP distance to the AuNP surface were studied. Altered coordination of the FP- DNA conjugates was achieved by the use of 23 nm AuNPs coated with different sets of ssDNA. For instance, 5'A24-AuNP contained the complementary oligomer A24 bound via its 5'-end, while 3'A24-AuNP contained the A24 sequence attached via its 3'-end. Due to the directionality of the DNA double helical spacer, the distance between the gold surface and the FP

is greater in the case of 5'A24-AuNP than for 3'A24-AuNP. This work has already been published in an international leading journal on bioconjugation chemistry and specific results of this study are summarised in the attached reprint.

Then hemoproteins were immobilised by DDI. To this end, the TUDO-group first focused on the design of a hemoproteins-DNA-conjugate. We used a methodology of reconstituting a DNA-modified heme group into apoenzymes, which was developed by TUDO group. [17] With this approach, apo-Myoglobin (Mb) was reconstituted with an artificial photocatalytic heme group that was linked to a DNA oligonucleotide. The obtained semisynthetic Mb-DNA-conjugate was hybridised on magnetic microbeads, containing complementary capture oligonucleotides. The DDI efficiency was measured by quantitative determination of the enzymatic activity of the immobilised conjugate using Amplex Red as a fluorogenic substrate. Results of this study have already been published in international chemistry journals. Specific details of this study are summarised in the attached reprint. Furthermore, the validity of published results were confirmed for other types of particles, such as smaller magnetic nanoparticles (MNP) and AuNP.

In addition to the above mentioned work on FP-DNA and hemoprotein-DNA-conjugates, we started the design of appropriate protein systems for single molecule investigation First systems were comprised of novel ssDNA-streptavidin conjugates, to be used as model for development of scanning near field infrared microscopy (SNIM) by SMD-partners at Ruhr-University Bochum (RUB). Following to a project meeting of the TUDO and the RUB group in February 2010, the participants agreed to initially develop a synthetic route to obtain novel DNA-protein conjugates as model systems for SNIM established at RUB. TUDO decided to synthesise a conjugate, comprised of double-stranded DNA with two streptavidin (STV) molecules attached to the end of the double helix. To achieve this goal, it was necessary to use monovalent streptavidin (mSTV)protein, which has previously been described by Ting and co-workers. The use of mSTV enables generation of well defined conjugates for SNIM development, which can be synthesised without formation of unwanted STV-DNA oligomer conjugates containing more than one strand of DNA per protein. The latter are usually formed during the assembly of bis-biotinylated dsDNA and wild-type STV [22]. The heterotetrameric mSTV contains one single femtomolar biotin binding site (Kd=5.4 x 1014 M) and three mutated subunits with negligible biotin binding (Kd=1.2 x 103 M), while, in contrast, wild-type STV is able to bind four biotin molecules with high affinity (Kd=4.0 x 1014 M). TUDO was able to facilitate the cloning, heterologous expression, and subsequent assembly of mSTV following the method described in the literature.

The use of mSTV and two bis-biotinylated dsDNA fragments, generated by PCR, enabled the successful synthesis of the desired conjugate. This well-defined nanostructure was delivered to RUB partners in March 2010 to be used for further SNIM measurements.

As a second system, a nano dumbbell conjugate of fluorescent proteins was prepared consisting of EGFP and mDsRed to be used as model for development of tip enhanced fluorescence mapping by SMD-partners at Consiglio Nazionale delle Ricerche (CNR-INFM-TASC). Development of this model system was agreed upon during the annual SMD project meeting in London in July 2010. TUDO and CNR groups decided to develop the fluorescent nano dumbbell, consisting of two fluorescent proteins

covalent coupled by hybridization of complementary ssDNA linkers. The length of the hybridised oligonucleotides allows control over the distance between the FPs. All aforementioned FPs can be used to form the fluorescent nano dumbbells. The conjugates can be formed by hybridisation of two different or else two identical FPs. CNR partner decided to use EGFP and mDsRed proteins, because the existing laser equipment allowed their excitation (EGFP: exc = 488 nm and mDsRed: exc = 558 nm). For the first tip enhanced fluorescence mapping experiments fluorescent nano dumbbells with a distance of about 8.5 nm (22 bp) in between the two FPs were targeted.

The FP-DNA-FP nano dumbbell conjugates were characterised by PAGE-gel electrophoresis and atomic force microscopy (AFM).

As a suitable model system to explore single molecule detection methodologies, a nanoparticle-protein hybrid was envisaged consisting of EGFP-DNA conjugates, DNA-modified Au- NP (about 23 nm diameter) and Ag-NP about (40 nm diameter) and oligonucleoitde linkers to enable the assembly of individual components. The NP-hybrid was designed to provide a sensitive platform for SERRS measurements conducted in the course of WP 5 (STRATH-AC). Development of this model system was agreed during the annual SMD project meeting in Genua on 27th January 2011. TUDO and STRATH-AC groups prepared modified Au-and AgNP, respectively, the assembly and the characterisation was done at TUDO and the SERRS measurements will be conducted at STRATH.

As per "analysis and characterization of ionic channels in situ". we needed of a good method to obtain suitable samples useful to harvest CNG channels. After developing an appropriate technique for the preparation of pure plasma membranes and an appropriate CNG construct, the task of this deliverable was the anchoring of single molecule necessary to obtain structural and conformational information of this protein in their native environment. These measurements do not required the purification and crystallization of the protein under investigation and were performed in presence of the plasma membrane. For these reasons this methodology was a revolutionary way to study the proteins that were hard to crystallize; moreover the idea to study the proteins in their natural environment representing a strong progress into the analysis of these ion channels in order to obtain structural information and to characterize the conformational changes during gating.

In this multidisciplinary project atomic force microscopy (AFM) and single molecule force spectroscopy (SMFS) were combined with electrophysiology, immunofluorescence, western-blot and molecular biology experiments.

The AFM was first introduced for imaging surface but over the course of several years its applications have expanded to the quantitative measurement of local force on a molecular level offering a very good high resolution in force measurement applications and for this reason is a useful tool to measure the force required to stretch and unfold polymeric molecules. This kind of application is named "force-spectroscopy". SMFS operates on the principle that the AFM cantilever can be treated as a spring whose force constant is determined by its resonant frequency and can be derived from the measurement of its thermal oscillation. Thus, the AFM cantilever deflection versus position can be transformed into force versus extension. The experiment involves the approach of the tip, withdrawal of the tip, stretching of the molecule and possibly one or

more rupture events. Approach and withdrawal takes only a few seconds. The isolation of a single 101 macromolecule for measurement, however, is nontrivial given the size of the AFM tip (μm) compared to the size of a single macromolecule (tens of nm). Normally more than thousand force-distance curves are collected but many of these curves contain no useful data, either because the tip failed to "catch" a molecule or because multiple interactions resulted in a complex force curve lacking the clean "fingerprint" that indicates a single protein extension. This is very typical of these experiments because the proteins can be randomly distributed or cannot uniformely distibuted, or the attachment to the tip and subsequent extension of the protein is not specific. Moreover, the tip can attach to the protein anywhere along its length, so there are not always enough domains between the substrate and the tip to give a definitive fingerprint. It is not uncommon that only a small fraction of the total force-distance curves is used for the final data analysis (Oesterhelt et al., 2000; Sapra et al., 2006). One of the theoretical common equations used to analyze the force-elongation curve is the worm-like-chain (WLC) model. This model describes a polymer as a continuous string with a characteristic length scale called its persistence length (polymer as a series of N segments of equal length l connected by joints that may point in any direction with equal probability), the contour length of the polymer is simply L=N xl, and the mean end-to-end distance.

CNG channels are transmembrane proteins expressed in vertebrate photoreceptors, in the olfactory sensory neurons but also in brain and other tissues. Their role in phototransduction and in olfaction is well established, but their role in other tissues has not yet been completely clarified (Seifert and Kaupp, 2002). However, CNG channels could be involved in synaptic transmission and in axonal pathfinding (Togashi et al., 2008). In their native forms, CNG channels are heterotetramers and different cell types have CNG channels with different subunit stoichiometry; native rod photoreceptors, for example, are composed of CNGA1:CNGB1 subunits with 3:1 stoichiometry. A-type subunits can form functional homomeric channels in heterologous expression systems with properties similar but not identical to native CNG channels; B-type subunits do not form homomeric functional channels, but coassemble with A-type subunits modulating their properties. The CNGA1 subunit is 690 AA long (about 70 kDa) and is composed by a transmembrane domain containing six helices that pass through the membrane, facing N- and C-termini of the protein to the cytoplasm. In the transmembrane domain, between S5 and S6, there is the pore region with the selectivity filter; moreover, between the S6 transmembrane domain and the cyclic nucleotide binding domain (CNBD) there is the C-linker region. After cyclic nucleotide (cGMP or cAMP) binding, a conformational change is transmitted from CNBD through C-linker to S6, opening the pore.

Belong to the super-family of voltage-gated ion channels these channels have a little voltage dependence, are activated by a direct binding of cyclic nucleotide, do not desensitize in the presence of a steady concentration of CNs and are non selective.

The objectives of this task were to obtain structural information and to characterize the conformational changes of CNG channels during gating using oocyte membranes over- expressing CNG channels. Our big question was how to find CNGA1 using the force- elongation curves. In order to analyse pulling experiments on CNG channels, we needed to understand and classify the force-elongation traces and choose those likely been associated to our proteins to obtain a sort of fingerprint and also to

demonstrate that they were obtained from the molecules under investigation. To reach our goal we tried different plasma membrane preparations and different kind of constructs. Working on this deliverable we were able to obtain pure plasma membrane from oocytes containing CNG constructs suitable to perform pulling experiments. At this point we are working on the construction of a molecule containing a marker necessary to rule out the possibility to be wrong during the force-spectroscopy analysis. Our idea is also to continue on this research using, non only the membrane extract from oocytes, but also using the HEK cells with the appropriate CNG molecule.

For the analysis of the engineered CNGA1 channel using SMFS to obtain a quantitative description about the folding of the protein as introduced in the previous deliverable we started with the results obtained from the membrane imaging and the construction of a suitable molecule to perform force- spectroscopy we have improved our system in order to collect new and reproducible results. To facilitate mechanically strong and specific interactions between the molecule, the cantilever tip and the surface for stretching measurements all the components were chemically modified using hexahistidine/Ni2+-NTA (C-term/cantilever tip) and gold/SH group (surface/Cys mutant). In order to increase the pulling efficiency by using a NTA functionalized cantilever tip we have first introduced a Histag at the C- terminal of the WT and the P366C channels obtaining a functional proteins (CNGA1_Histag and P366C_Histag). Starting to the concept that the length of the force traces should fit with the expected length from the Histag to the N-terminal (approximately 270 nm considering 0.4 nm/aa, Ainavarapu et al., Bioph. J., 2007) or to the position 366 (approximately 130 nm considering 0.4 nm/aa, Ainavarapu et al., Bioph. J., 2007) we performed some pullings experiments (see results section). However, it is not possible to assure that the channel binds covalently to the surface and the cantilever tip stretch the protein from the Histag, the total contour length of the force trace is a weak feature to use as single molecule signature. To solve the problem regarding the aspecificity of the binding of a functionalized tip we performed blocking experiment. Moreover, the preparation of a new construct fused to single molecule markers is in progress.

The problem of identifying single molecule force recordings from the protein of our interest instead of other molecules that can be present in a native plasma membrane can be addressed in several ways, but perhaps the most direct method is the use of single molecule markers. In particular, the protein of interest is serially fused to a tandem of well characterized Titin I27 domain (Rief et al., 1997). Stretching the I27 attached to the molecule of interest should show its characteristic force peaks, ensuring that our protein problem is being stretched (or unfolded). The last version of construct consists in P366C_(I27)2_Histag which has two I27 modules. The I27 is a protein model in force spectroscopy and widely used as SMFS marker (Valbuena et al., 2009; Oroz et al. 2010). In this case, when we pull the protein, if we observe two characteristic I27 force peaks plus the right contour length, we can conclude that we stretched the channel under study. We will perform also electrophysiology experiments to check its functionality. Preliminary results with HEK cells indicates that a GFP variant fused at the N-termini of the CNGA1 subunit shows high fluorescence signal, which could overcome the problem with the high background fluorescence signal in oocytes. Hence, to improve the previous construct, we will insert a GFP at the N-terminus of the CNGA1 (GFP_CNGA1_(I27)2_Histag and GFP_P366C_(I27)2_Histag), allowing its localization with fluorescence

microscopy of the protein on the peace of membrane. Working on this deliverable we were able to obtain force-elongation curves from specific CNG constructs. Because we are working on the construction of a molecule containing a marker our intention is to continue on this research analyzing these new construct in order to obtain an to validate the unfolding model of CNG channels. Because at the moment is unknown the 3D crystal structure, these measurements will represent a strong progress into the analysis of these ion channels in order to obtain structural information and to characterize the conformational changes during gating.

As per the analysis and characterization of odorant receptors in situ, we needed of a good method to obtain suitable samples useful to harvest known endogenous odorant receptors from olfactory sensory neurons. The olfactory epithelium contains cells able to detect the presence of low concentrations of odorant molecules and discriminate even very small differences among molecules with very similar chemical structure.

In particular, the olfactory receptor neurons are bipolar cells containing a single axon that synapses in the olfactory bulb of the brain and a dendrite that projects into the mucus and terminates in a knob or vesicle. From each knob there are fine cilia expressing odorant receptors. In the cilia the first olfaction event takes place: the binding of odorant molecules initiates a transduction cascade that leads, via G-protein and adenylyl cyclase activation, to an increase in the concentration of cAMP, which directly gates ion channels (Lagostena and Menini, 2003).

The odorant receptors are type of G-protein coupled receptor; these proteins are integral membrane proteins that possess seven alpha transmembrane helices. The extracellular parts of the receptor can be glycosylated and the loops contain two highly-conserved cysteine residues that form disulfide bonds to stabilize the receptor structure.

The olfactory neurons of lower vertebrates typically have about 6 motile cilia that can be as long as 200 µm while in mammals, the cilia are shorter (15–50 µm), thinner (0.11 µm), more numerous (greater than6 per neuron) and nonmotile.

SMFS results using P366C_Histag molecule. From CNGA1 unfolding the best experiments were performed by using direct incubation protocol of P366C_Histag injected oocytes in combination with functionalized surface (3MPTS-bMEtOH) and cantilever tip (Dithiobis-C2-NTA). In any case, we found some problems during this experiment, so we must to confirm these results by comparing them with results from other constructs (work in progress). Also, proper AFM imaging, blocking experiments and single molecule markers are required to be confident about the conclusions we can infer from these results.

Functionalization protocols As mentioned above, we decided to use two types of functionalization, one for surfaces and the other for cantilever tips. We thought that the interaction between the protein under study and the surface should be of higher mechanical stability than the interaction between protein and cantilever tip. In this way, after stretching the molecule, because the mechanical stability of the interaction sample/surface is larger than the interaction sample/tip, the protein should remain attached to the surface and not to the tip, minimizing tip contamination during the experiment. For that, we decided to produce the mutant P366C in order to increase the probability of covalent binding

between cysteine mutated residue 366 and a thiolated surface. In the case of the tip, we decided to use non covalent but highly specific and strong interaction to increase the pulling efficiency from the C-termini of the protein, where we inserted an hexahistidine tag, i.e., Ni2+-NTA/Histag.

Glass surface functionalization with 3MPTS. To functionalize glass surfaces with exposed thiol groups, we modified a protocol described elsewhere (Hossain et al., Bioph. J., 2006). Summarizing, we first dissolve 1 g of KOH in 30 ml of 80 % EtOH solution, then we sonicate coverslips in this solution for 15 min. A second sonication step was performed in 30 % EtOH for another 15 min. Then, a final sonication step of 15 min was carried out in MilliQ water. After cleaning, we dried the coverslips under N2 flow and subsequently immersed in 2 % (v/v) of 3-mercapto-propyl-trimethoxy-silane (3MPTS) in pure EtOH for 2 h under a hood equipped with a UV lamp. Then, we removed the excess of 3MPTS by rinsing thoroughly the coverslips with EtOH and sonicating them for 1 min. Curing of 3MPTS layer was performed after drying coverslips under N2 flow and placing them over night in an oven at 100 oC. After curing, in order to minimize non-specific interactions, a second layer of b-mercaptoethanol (b-MEtOH) was added by incubating coverslips for 2 h in 1, 10 or 100 mM of this chemical dissolved in pure EtOH. Finally, coverslips were rinsed with EtOH and placed in clean EtOH for storing or dried under N2 flow for using them.

Cantilever tip functionalization with NTA terminated molecules (Murata et al., 2001, Yokota et al., 2008). Gold coated cantilevers are required for functionalization because the molecule selected has a thiol group at one end and a NTA group at the other Before start with the functionalization we cleaned the cantilevers by immersing them into pure EtOH for 15 min. Then, were dried under N2 flow before place them under a UV lamp for another 15 min. Cantilevers were transferred in chloroform for another 15 min and dried under N2 flow. We repeat these three cleaning steps. After cantilevers cleaning, we immerse them in an aqueous solution containing 100 mM Dithiobis-C2-NTA for 1h. At this moment, we load the NTA with Ni2+ cations by immersing cantilevers in a 0.1 mM NiSO4 aqueous solution.

Functionalization controls. We need to test that the functionalization was performed properly, for that we have to acknowledge to Dr. Carrion-Vázquez (Cajal Institue, CSIC, Madrid, Spain) for kindly provide us a sample of (I27)12 polyprotein, model system in SMFS (Rief et al., 1997; Oberhauser and Carrion-Vazquez, 2008). This system is ideal for checking our functionalization protocols for three reasons: this construct has two cysteine residues at one end (covalent binding to surface), an Histag at the other end (tip binding) and its mechanical fingerprint is very well known (FU approximately 200 pN and DLC approximately 28 nm; Rief et al., 1997). First, we imaged the clean glass surface, then protein free 3-MPTS/100 mM b-MEtOH functionalized surface and finally after (I27)12 polyprotein incubation for 30 min.

It is clear that the protein was bound to the surface, so we proceeded with SMFS experiments which consisted in performing 2000 trials in each condition, without and with 300 mM Imidazole. Imidazole has the property of blocking Ni2+-NTA/His-tag interaction by displacing the His-tag from the complex. Nevertheless, in SMFS is very difficult to obtain large amount of force traces and with the functionalized tip the pulling efficiency did not increase dramatically. We obtained approximately the same number of traces with a non-functionalized gold tip and with the functionalized one under blocking conditions (approximately 0.9 %). In

the case of the functionalized tip (with no Imidazole) the pulling efficiency was slightly higher (approximately 2.5 %).

SMFS results using CNGA1_Histag molecule. Other traces were obtained with the WT CNGA1 channel modified with the Histag. The experiment shows a force-elongation curve that can be characteristic for CNGA1 channels. Now our goal will be to repeat this experiment with a marker, as previous described, to confirm our results.

Working on this deliverable we were able to obtain force-elongation curves from specific CNG constructs. Because we are working on the construction of a molecule containing a marker our intention is to continue on this research analyzing these new construct in order to obtain an to validate the unfolding model of CNG channels. Because at the moment is unknown the 3D crystal structure, these measurements will represent a strong progress into the analysis of these ion channels in order to obtain structural information and to characterize the conformational changes during gating.

With regards to the analysis and characterization of 7TM-GPCR, we used as model the rhodopsin, a molecules present in the intact discs isolated from retina. In this report, will be illustrated our method to prepare suitable samples focusing the attention to our experimental results. We decided to repeat these experiments to test our system and our analysis method. The rhodopsin, a 7TM-GPCR, is the predominant protein (95%) of disc membranes that are localized in the external segment of rod and cone cells. These cells are specialized neurons that detect photons into the retina and are subdivided into four parts that correspond to the synaptic terminal, the cell body, the inner segment and the outer segment containing the discs. Rod disc membranes are staked and surrounded by a plasma membrane and for every external segment - in mice - the number of discs corresponds to about 800. The 3D crystal structure of rhodopsin was discovered 10 years ago (Palczewski et al., 2000) showing a homo-dimeric structure containing seven transmembrane domain 348 amino acids long. In 2003 Engel and collaborators (Fotiadis et al., 2003; Liang et al., 2003) published the first high- resolution image of disc extracted from retinal mouse using an AFM microscope and up to now this is the only group able to obtain this quality of images opening a new era regarding the methods to analyze membrane proteins.

The rhodopsin – a 7TM-GPCR - is composed by a peptide and a chromophore: the polypeptide (named opsin) is composed by 7 transmembrane a-helices and consists of 348 aminoacids and the stability of the helical segments are increased by the disulphide bridge between two cysteines [Cys(110)-Cys(187)]. The chromophore (named 11-cis-retinal) is covalently bound via a Schiff-base linkage to a conserved lysine residue (Lys296). Using the SMFS it is possible to otain the representative force-elongation curve reflecting the unfolding of a single rhodopsin molecule. The results show that the length of unfolded rhodopsin is either 65 or 95 nm and that the spectra of the short length has three major peaks at about 15, 35 and 65 nm while for the long length the peaks are four at about 15, 35, 60 and 95 nm representing a good signature of unfolding (Sapra et al., 2006).

This method is modified from Nickell et al., 2007: C57BL/6 mice (4–12 weeks old) were maintained in complete darkness (greater than 2h) than they were sacrificed using eutanasia with CO2 and cervical dislocation. The eyes were removed and the retinas isolated in complete darkness with a dim red light.

Twelve mouse retinas were placed in a tube with 120 µl of 8% OptiPrep (Nycomed) plus protease inhibitor in Ringer buffer solution (130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl2, 1.2 mM CaCl2, 10 mM Hepes, pH 7.4, containing 0.02 mM EDTA) and vortexed for 1 min. The samples were centrifuged at 200 g for 1 min, and the supernatant containing the ROS was removed gently and placet on ice. The pellet was dissolved in 120 µl of 8% OptiPrep, vortexed, and centrifuged again. The vortexing and sedimentation sequence was repeated five-six times. The collected ROS supernatants were loaded on a 10–30% continuous gradient of OptiPrep in Ringer buffer and centrifuged for 50 min at 26,500 g using a swinging-bucket rotor (Beckman). ROS were harvested as a band, diluted three times with Ringer buffer solution and centrifuged for 3 min at 500 g to remove the nuclei. The supernatant containing ROS was transferred to a new tube and centrifuged for 60 min at 26,500 g. The pelleted material contained pure and osmotically intact ROS.

ROS were suspended in 2 ml of Lysis buffer solution (2 mM Tris-HCl, pH 7.4) and burst at 4°C for 15 h. Discs were loaded on a 15–40% continuous gradient of OptiPrep in Ringer buffer solution. The sample was centrifuged for 50 min at 26,500 g and the discs were collected from a faint band. The harvested intact discs were then diluted three times with Ringer solution and pelleted for 60 min at 26,500 g.

Disc membranes were adsorbed on mica in Lysis buffer solution for 15–20 min and kindly washed with recording buffer (20 mM Tris-HCl, pH 7.8, 150 mM KCl, 25 mM MgCl2) for three-five times. AFM experiments were performed using a JPK microscope and a calibrated oxide- sharpened silicon nitride cantilevers (OMCL-TR400PSA, Olympus). Topographs were acquired in liquid contact and/or non contact mode at minimal forces (100 piconewtons). To catch an individual protein we kept more that 1000 contacts - between cantilever tip and the membrane surface - for about 1 s with a force of 1 nN to give the protein the chance to adsorb on the stylus. The stylus and the membrane were separated at a velocity of 40 nm/s, while the force spectrum was recorded. The interaction between tip and surface, which is expressed in the marked discontinuous changes in the force, indicates a molecular bridge between tip and sample. This bridge corresponds to the length of one totally unfolded protein.

The preparation and the AFM experiments were performed under physiological conditions that means, in dark, in buffer solution, at room temperature and under normal pressure, without the application of any condition that can modify and affect the rhodopsin native oligomeric state.

After absorption of discs on mica surface, tapping mode AFM in recording solution was done using a short but soft cantilever. In our samples we observe double layer discs with height of 20 nm and diameter of 500 nm and also single layer discs with the same morfology described in literature, in fact four different surface types are evident: the regions numbered 1 and 2 corresponds to rhodopsin packed areas, the region number 3 is a lipid bilayer and the number 4 is the flat mica surface. Also the dimensions are the same, as you can see in the table below .We tried to perform SMFS measurements repeating the published experiments (Oesterhelt et al., 2000; Sapra et al., 2006). The reason is that we needed of these experiments to be sure that our interpretation of molecular force, interactions and unfolding of peptides is correct.

Working on this deliverable we was able to obtain force-elongation curves from Rhodopsin and we can conclude that our system is useful to study new transmembrane proteins "in situ" using SMFS.

Before to start the experiments using an unknown sample like the CNG channels we have used a more useful sample.

TERS on insulin amyloid fibrils with an upright AFM-Raman setup

The aim of this work is the characterization of a biological sample by Tip Enhanced Raman Spectroscopy (TERS) with a new up-right setup. The main goals of this deliverable are Evaluation of the AFM-Raman setup; Estimation of tip and tip/surface field enhancement by numerical simulation; Application of TERS probe for upright signal detection on insulin amyloid fibrils.

The chemical properties of single biomolecules are very difficult to investigate without perturbing their original state. Raman Spectroscopy combined with AFM instrument can provide chemical characterization of single molecules while the AFM probe is performing its topography. This technique is called Tip Enhanced Raman Spectroscopy (TERS) and has been applied to the study of biological samples since the last two decades. The progress is very little due to several reasons, one of which is the low Raman cross section of biological molecules. In this experiment the study of insulin amyloid fibrils (IAF)by TERS was facilitated by their drop-casting on a gold coated substrate [1]. The TERS probes were employed in a special TERS configuration in which both the illumination and the collection of the signal are located in an up-right objective.

TERS setup and sample preparation

A custom setup provided by NT-MDT (Zelenograd, Russia) was used. The setup is a combination of NT-MDT AFM and RenishawMicroRaman spectrometer. The apparatus is conceived for laser illumination of the probe from the top and for signal collection both in reflection mode and in transmission mode. Here we used exclusively the backscattered signal collection mode (upright configuration). A He-Ne source provides a linearly polarized 632.8nm laser light (12 mW) illuminating a long working distance objective (Mitutoyo Japan 100x, NA=0.7) mounted within a properly fabricated AFM head. Light is focused within a spot whose diameter is about 600 nm. An adjustable cantilever holder hosting the TERS probe is placed close to the focal plane of the objective. Scattered and/or reflected light is collected by the same objective and sent to the dispersive spectrometer (grating 1800 lines/mm) after being spectrally filtered through an edge filter (633 nm plus Rayleigh scattering).

AFM control is performed by means of a standard beam deflection method. A laser beam probing the cantilever bending is focused onto the cantilever by means of the top objective used for probe illumination. The AFM control beam is an 830 nm wavelength diode laser light. Topographic and spectral measurements are performed by raster-scanning the sample beneath the illuminated tip. In order to align the Raman laser with the tip apex, the instrument was equipped with mirrors moved by a closed-loop piezo-controlled scanner that allows an accurate three-dimensional positioning of the laser spot onto the tip.

For sample preparation gold coated silicon wafer was used as the substrate for all TERS experiments. A 50 nm gold layer was sputtered on

the substrate. Deposition of IAF on gold coated substrate was made without additional preparation. Mature amyloid fibrils were obtained from bovine insulin (5 mg/ml) (Sigma-Aldrich). Protein solution was incubated in HCl water solution (MilliQ filtered water) at pH 2.00 for 3 weeks at 60 °C. A drop of the preparation as such was pipetted directly on silicon substrate for acquisition AFM image. For TERS measurements, a drop of 20 ml of a 1:10 diluted solution was deposited onto the substrate. After 1 minute the sample was rinsed with 5 ml of MilliQ water, air-dried and put on the AFM sample holder as such. TERS tips were evaporated with a 25 nm Au layer while orthogonally rotating under the metal source.

We have performed a fully 3-dimensional calculation of a nose-type gold coated AFM tip. In particular, the chosen structure is 16 mm long with a tip radius of curvature of 11 nm.The gold optical properties were implemented according to a full Drude-Lorentz description. The chosen linear polarization was mainly transversal to the tip axis to match the experimental conditions (in our coordinate system almost parallel to y-axis).

A set of simulations was performed in order to determine the electric field enhancement provided by the tip when applied on insulin amyloid fibrils. To take into account the presence of the fibrils, we have modified the refractive index of the volume around the tip to 1.4. In particular, a gold-coated flat substrate was approached to the tip within a gap ranging from 1 nm to 10 nm. The choice of an ideally flat substrate was to remove any SERS (Surface Enhanced Raman Scattering) contribution to the field enhancement originating from the substrate itself. A second geometrical configuration was also implemented. In this case the TERS tip was approached to a smooth spherical surface, which simulates the local roughness of the surface. Also in this case was possible to avoid any SERS contribution to the field enhancement. It shows that only for distances far below 10 nm a strong field enhancement is obtained.

In fact, considering the polarization condition, the tip-end will behave like an isolated spherical object by creating a dipole-like field profile along the polarization direction [2]. The effect of the substrate starts emerging only for gaps below 2 nm in case of a flat substrate (blue/red-square lines), and 4 nm if the local roughness is considered (yellow-triangle line). In fact, below these values of the gap, the field enhancement is at least equal to 10, meaning a Raman signal of the order of 104, which well approach our experimental results. We have then modified the surface of the substrate to take into account the local roughness. In fact, SEM images of the gold substrate (not shown) suggest a surface with local roughness associated to island aggregated with an average diameter of 30 nm. We have then implemented this feature in the simulations, as shown in the first step is to acquire a topography image to locate the IAF that are mono-disperse on the substrate.

For TERS measurements, the oscillation amplitude of the tip is set at less than 10 nm. Once the suitable fibril is found, the tip is kept at a fixed position and a laser scanning collecting full- spectrum Raman data is performed to find the enhancement spot at the tip apex. To clarify this point, Upon finding notable points, the corresponding TERS spectra are collected at 1s time/point and 0.12 mW laser power moving the sample stage to the desired position. Offset for Raman shift spectra is set at the 520.1 cm-1 corresponding to the peak of the first order Si-Si phonon band [3]. Additionally, a reference microRaman spectrum on sputtered gold substrate of bulk IAF was acquired for peak comparison.

Combined AFM-TERS measurements were performed on gold-coated silicon substrates, covered with a monolayer of IAF.

Various characteristic Raman bands, distributed in the range of 800-1800 cm-1, were observed, each associated to different amino acids and to the conformational structure of the amyloid protein. In particular, the far-field spectrum of IAF (black line) shows various peaks, centered at 1003 (phenylalanine), 1035 (phenylalanine), 1675 (secondary structures) cm-1. In the blue TERS profile, a small contribution of a peak at around 1690 cm-1, related to the secondary structures is visible. Furthermore, the Raman bands related to single aminoacid features such as at 1610 (tyrosine aromatic side chain), 1574 (phenylalanine), 1546 (tryptophan), 1499 (CHx asymmetric bending), 961 (ring stretching), 885 (tyrosin side chain) and 846 cm-1 (tyrosin) were found to be very much enhanced in the TERS spectra [4, 5]. Particularly, the spectrum corresponding to the purple hot spot shows a very neat and clear profile. Hence, the combination of the TERS spectra provides both higher intensity and number of bands than the far-field microRaman analysis, namely better chemical description of IAF samples.

The far-field Raman signal, collected from the same measurement area but with the tip retracted, did not show any appreciable peak, with counts comparable to background noise of the instrument. We therefore estimate the field enhancement by supposing the far- field Raman signal level to be equal to the instrument noise level, and then applying the formula [6]: where STERS is the TERS signal level, SFFis the far-field Raman signal level (tip retracted), ATERSand AFF are the illuminated areas in TERS and far-field configuration, respectively. Then, by considering ATERS@ 120 nm2, AFF@ 104 nm2, we obtain a minimum field enhancement factor (g) in the order of 20.

As per the part regarding tumourgenesis: large amount of preclinical and clinical studies has been carried out towards this direction and has produced important achievements. To this end, a great contribution has been provided by the use of molecular profiling technologies such as transcriptomics and proteomics. In particular, proteome analysis appears as a very promising approach to dissect the dynamic process of tumorigenesis, where post-translational modifications (protein phosphorylation, trafficking, and protein-protein interactions with secondary effectors) play a critical role [7]. However, in all proteomics investigations the major difficulty is the detection of low-abundance proteins that represent the vast majority of the species in a biological extract [8, 9]. Several approaches have been taken to overcome this limit, in particular the use of pre-fractionation tools (such as chromatography, electrophoresis and similar methods) to remove high-abundance proteins that mask the signal of rare species. The large dynamic range of protein concentration in biological fluids/cells (that can reach up to 12 orders of magnitude) still remains a major challenge and has prompted integrated, multidisciplinary approaches aimed at developing miniaturized probes able to detect, visualize, characterize and identify a single or a small number of molecules [10-12].

Single molecule detection/identification/characterization appears indeed the as one of the most important goals in cancer research. To this end, the coherent focus of the "omics", the imaging and the nanotechnology approaches holds great potentials and is likely to improve our understanding how cancer develops at a molecular level, enabling

researchers to locate tumors, to assess their biological activity and to provide "ad hoc" treatments.

Mass spectrometry, combined with Raman spectroscopy and nanofabrication has been demonstrated as powerful tools for the study of biological samples at a single molecule level. This strategy, originally developed and presently used in our facilities, coupled with the recent achievements obtained by the coordinator of this proposal on optical trapping and plasmonics (i.e. a miniaturized single-fiber optical trapping system able to create a 3D trap and to provide manipulation capabilities) represents an important improvement and appears to be a promising and innovative approach in the challenge against cancer.

Two different techniques, i.e. electron-beam lithography and site-selective electroless deposition, were combined to achieve an active SERS (surface enhanced Raman scattering) substrate. It allows in obtaining a well-defined metal structure, with a considerable advantage in Raman signal enhancement and in device reproducibility. SERS substrates were based on silver and gold metal with different nanoparticles characteristics, obtained by varying metal deposition time. Two different peptides (W1837, wild and mutated type), sequential part of BRCT domain, were employed as probe molecules for SERS experiments in order to distinguish them.

The aim of this study is the realization of an active SERS substrate, where site selective silver nanoparticles utilizing electroless technique are assembled on nano-patterned Si substrate with different shapes and sizes. The nano-pattern based substrate, obtained by electron beam lithography, has the function of controlling the diffusion of the metal particles on the surface. On the other hand, the deposition of metallic (silver or gold) nanospheres, permits plasmonic enhancement. The electroless deposition on a substrate is based on an autocatalytic or a chemical reduction of aqueous metal ions. This process consists of an electron exchange between metal ions and a reducing agent [1.]. In some cases the substrate is the catalyst for the reaction, instead, in this work, Si substrate is itself the reducing agent. Recently a simple silver deposition method was developed, based on a fluoridric acid (HF) solution containing metal salt (AgNO3/Au2Cl3) [2,3,4,5.], where metal ion is reduced to metal form by the Si substrate oxidation.

A reproducible pattern of an array of nanostructures is written using an ultra-high-resolution EBL (CRESTEC CABL-9000C 50 KeV acceleration voltage) onto a clean Si wafer (100) spin-coated by a ZEP layer 50 nm thick. Substrates of different shapes are prepared with a maximum dimension size in the range of 50–1000 nm. Silver/gold nano-spheres are deposited on the substrate by means of electroless method, in which the patterned Si wafer is dipped in a 0.15 M HF (hydrofluoric acid) solution containing 1 mM metal salt (AgNO3/Au2Cl3) for different times (10–60 sec). After the etching process the Si wafer is rinsed with water and dried under nitrogen flux. The samples with 60 sec deposition time are used for Raman analysis as these samples are showing better Raman response. The driving force in this process is the difference between redox potentials of the two half-reactions, which depends on solution temperature, concentration and pH. Consequently, these parameters influence the particles size and density.

Micro-probed Raman spectra were acquired by using Renishaw inVia Raman microscope at room temperature through 50X objective of a Leica

microscope. The Raman spectra were excited by 514.0 nm line of an Ar+ ion laser in backscattering geometry. The laser power was fixed to 0.018 mW and an integration time 60 s. All the Raman spectra were baseline corrected using three-order polynomial function.

A scanning electron microscope (SEM) was used to observe the morphologies of the samples. 1(b-d) are silver-based SERS devices. Once the device is ready, the aqueous drop of molecular solution is deposited over device. After the excess water evaporation, SERS measurements were carried out.

In this work, two different synthetic wild and mutated types of W1837R and M1775R peptides with 16 amino acids were chosen. These peptides are the sequential parts of BRCT protein domain of BRCA1 tumor. In former, the amino acid 'W' (tryptophan) is replaced by 'R' (arganine) instead in latter 'M' (methionine) is substituted by 'R'. In this work, we shall discuss one by one regarding the analysis for distinction of tumorigenesis peptides using SERS technique. Various vibrational bands are discussed for both the peptides and in the last the comparison between these two wild and mutated peptides are also performed, in order to establish this technique as a distinguished and non-conventional.

SERS measurements were performed for this peptide, excited by 514 nm laser, in two different range; low frequency range which is also called the finger-print region from 350-2000 cm-1, and high frequency range from 2400-3700 cm-1. In the low frequency range, different vibrational bands, such as 1673, 1566, 1450, 1345, 1240, 1125 and 436 cm-1 are attributed to the stretching vibration of –C=O band from carboxyl group (-COOH), stretching of ring (pyrimidine) from proline, bending vibration from C-Hx band, stretching vibration of C-N band, out of phase N-H, stretching vibration of C-C, and rocking vibration of the same, respectively (6,7,8,9,10). In the inset, for high frequency range, also various SERS bands centred at 2736, 2875, 2940 and 2971 cm-1 are related to the C-Hx stretching vibrations of aliphatic group (11,12). One small but clearly visible band, centred at 3065 cm-1, is attributed to the aromatic ring from tryptophan amino acid. In addition, a broad band at around 3280 cm-1 is observed which are related to the N-Hx stretching vibration from arginine molecule.

SERS measurements for M1775R peptide, following the same procedure, were also performed. The in-circled peaks are those peaks which are different when performing the SERS measurements for wild type and mutated M1775R peptides. Difference Raman spectrum in low frequency range shows the negative band at around 1165 cm-1 which is typically from arginine amino acid. However, in case of higher frequency range, a positive band in the difference Raman spectrum at around 2140 cm-1 is observed, which is attributed to the -S-CH3 vibration, a contribution from methionine.

Conclusions

In this work, we have fabricated gold/silver bases nanostructure SERS devices by means of two techniques EBL and site-selective electroless metal deposition. Two sets of peptides, which are the sequential part of tumorigenesis BRCT domain, are taken into consideration as probing molecules. Using the SERS, a non-conventional technique, is used to distinguish the wild type peptides from mutated peptides. In order to make this technique in common use, a detailed research is further needed for all kinds of mutated peptides.

Potential Impact:
The scouting of unknown molecular entities in living biological systems by optical and mechanical (spectroscopic) characterization of single molecules is a major technological challenge of SMD. The objectives of this project cannot be achieved at a national level and that its success would bring a significant contribution to science and technology at European level. The project has a wide scope challenging scientific objectives, a genuine European perspective and real socio-economical implications.

The expected results are primarily new technological and scientific methods for fast, reliable and specific detection of a few molecules (or even a single molecule) in a complex, non amplified and unlabelled biological sample. Therefore the project contributes to the development of new scientific and technological knowledge and introduces substantial innovation in the European diagnostic.

The opportunity offered by the developed tools consisting in the analysis of single molecules and their related events in situ, will radically increase the ability of researchers to investigate cellular processes such as the discovery of membrane and channels properties, as it is expected to be demonstrated in WP7 and WP8. It is clearly auspicable that these new investigation techniques will become a routine in drug discovery and validation in the near future.

A Business Package has been realized with the intent to identify all the results of the project which are potentially exploitable in the market and to study, for each of them, the needed steps to make them commercially available. This analysis includes considering patent filing, assessment of the production costs and target market identification.

Besides the tip/cantilever developed at the project, other WP have achieved devices, products and concepts that have been included in the study.

Along the period of a scientific project with very ambitious goals and excellent partners dedicated to development, a huge quantity of results of different level of interest are produced. Besides those which were expected there are always coproducts of the activity which have to be considered individually.

During the time of the project and for all the partners this has been an extra point of attention besides the purely scientific research.

WP number Result code Result potentially exploitable Responsible Partner/s
1 1.a Nanocone Tips for AFM UMG, IIT
1 1.b Tripods (asymmetrical beads for optical tweezers) UMG, IIT
1 1.c Plasmonic antennas on optical tweezers beads UMG, IIT
1 1.d Fiber optical tweezers with microprisms fabricated onto optical fibers UMG, IIT
2 2.a Raman – Nanoscope CNR-IOM-TASC
2 2.b AFM new design NANOTEC
2 2.c Software tools for AFM 1: Force clamp acquisition tool NANOTEC
2 2.d Software tools for AFM 2: Raman Analysis and acquisition tool NANOTEC
5 5.a Nanotag metallic cores STRATH-AC

6 6.a Gold-silver-DNA structure TUDO

In order to analyze the interest of each of the results, a detailed questionnaire has been elaborated with critical questions regarding the essential points for the transfer of technology from the laboratory to the market.

The questionnaire is divided in three main parts and it is shown below.

SMD. WP9. Questionnaire for Business Plan

Part 1. Target Market Identification.

Partner name:
Date:
(please, fill one template for each device that can be put in the market)

Describe the device/result you think that can be put in the market:
- Name of this device/result
- Describe the potential users
- Describe what the device/result is for
- main characteristics
- main applications
- usability and compatibility
- does the device/result can be used on its own?
- if not,
- which technologies does it need to be used?
- is it compatible with the technologies on the market?
- Describe the device/result in terms of competitiveness
  why is it competitive in terms of technical characteristics, feasibility, durability and cost (compared to other similar devices on the market)?
- how long do you expect it to be competitive?
- Who are the main competitors?
- Provide the technical description including:
- device/result properties
- functional description
- application description
- suggested operating conditions and application recommendations
- schema describing their actual dimensions
- Describe what kind of company could put this device/result in the market? (pharmaceutical, instrumentation, chemical products, cantilever, …)
- Other information
- Describe the level of maturity of development for the device/results
- (1) far from market - scientifically demonstrated
- (5) very near to market – commercial prototype ready
- Do you foresee future developments in terms of:
- Automatization
- Serial Manufacturing
- On line process monitoring
- Scalability production processes
  Do you foresee any new collaboration for these developments? If yes, what kind

Part 2. Assessment of the production costs.

Production

- Describe the process of production for the device/result
- Describe what kind of manufacturer/s will be required to produce this device/result (electronics / microfabrication / mechanical workshop / chemical lab / …)
- Describe what kind of infrastructures or facilities are required to manufacture the device/result (clean room / chemical lab / ...)
- Describe the scalability of the manufacturing process
- Is it possible to apply a mass production process for manufacturing?
- Reproducibility of the device/result is very important for commercial exploitation
- Describe the current level of reproducibility of the device/result in itself and about its main properties
- Describe the process to improve the level of reproducibility
- Describe the Quality Tests that would guarantee a reasonable level of reproducibility of the device/result
- Estimate time of life of device/result and describe stockage possibilities or needs

Cost
- Estimate the cost for final development of the device/result into a commercial prototype
- Describe the breakdown of the development cost
- Estimate the time for final development of the device/result into a commercial prototype
- Describe the breakdown of the development time
- Estimate the production cost
- Describe the breakdown of the production cost
- Estimate the production time
- Describe the breakdown of the production time

Risk assessment
- Name and difficulty of the risks that can appear (very critical=1; not critical=3; very likely to happen=A; not likely to happen=C)
- at final development into a commercial prototype
- at production process

Part 3. Patent filling.

- Do you foresee patents for your devices/results (or have any in process)?
- If so,
- what is the preliminary title of the patent
- describe what in particular will be patented
- when is the patent expected?
- what kind of patents will be applied for: national, EU or international?
- Is the patenting process following the Consortium Agreement regarding the IP issues?

From the information gathered after circulating this questionnaire. Main reasons to consider some of these results as not interesting for commercialization have been for example that the fabrication process is not scalable to mass production (results 1.c and 1.d) or that the processes for fabrication are already covered by one or more patents out of SMD consortium (i.e. result 5.a).

WP number Result code Result potentially exploitable Responsible Partner/s

1 1.b Tripods (asymmetrical beads for optical tweezers) UMG, IIT
2 2.b AFM new design NANOTEC
2 2.c Software tools for AFM 1: Force clamp acquisition tool NANOTEC
2 2.d Software tools for AFM 2: Raman Analysis and acquisition tool NANOTEC

Selected results can be classified into 2 different categories. Result 1.b "Tripods" is a consumable for specific experiments with Optical Tweezers and the profile of the company potentially interested in putting them into the market would be the companies already providing these kind of consumables (Fused Silica Beads); for example and just to mention some:
http://www.fusedsilicawafers.com; http://www.micropore.co.uk/; http://www.spherotech.com/ .
These companies are already experts on the market sectors and could be able to develop the business successfully.
Other kind of company potentially interested in putting these "Tripods" into the market could be those companies already providing the Optical Tweezers Set Up (for example: http://www.aresis.com/ ; http://www.elliotscientific.com/; http://www.jpk.com; http://www.thorlabs.de/). This kind of companies could play as distributors of the Tripods, however the manufacturing process would be far from its current business and therefore the interest for complete exploitation of the idea would be limited.

Results 2.b, 2.c and 2.d are related to instrumentation, in particular the Scanning Probe Microscopy field. Nanotec company is expert in the sector and therefore has a very appropriate profile to exploit the results. For these results all the developments have been market oriented since the beginning of the project. As it will be explained along the document they, or some of their phases, are being implemented into commercial prototypes at Nanotec and will be ready for the market in the next years.

In the following sections the selected results are described from a commercial point of view.

1.b Tripods (asymmetrical beads for optical tweezers)

The main advantage of the new design of beads for Optical Tweezers is the fabrication of the plasmonic antenna on the optical tweezer beads in order to combine the optical trapping capabilities, including force measurements, with Raman spectroscopy.

These newly designed beads, with three different trapping points and a protruding tip, allow the user true full 3D spatial orientation control of the plasmonic antenna carried by the fabricated bead.

The geometry is optimized to guarantee both full 3D orientation control and spatial separation between trapping points and Raman excitation points. Another interesting feature of this structure is the fact that suffers less Brownian motion- related displacement noise with respect to smaller structures such as the standard spherical beads for Optical Tweezers.

Regarding the developed manufacturing process, the tripod structures are fabricated by using the two-photon lithography (TPL). The advantages of this technique, which is intrinsically a 3D structuring process, since

the photo-ploymerizableresist is exposed only in the focus spot of an intense laser beam, rely on the straightforward process that allows creating structures that would be very complex or even impossible to fabricate with conventional lithographic methods. Moreover, as recently demonstrated, the spatial resolution attainable using TPL is sub-100 nm using laser wavelengths around 800 nm, which is well below the diffraction limit.

Fabrication time for each tripod up to now is 3 minutes, which is very reasonable for a laboratory prototype, but still too high from an exploitable point of view. This process however is scalable and compatible with mass production. The reproducibility of the tripod fabrication is good.

The cost of the material for fabrication process, the polymerizable resin, is 30 EUROS/50 ml.

This fabrication method, the two-photon lithography (TPL), is briefly described as follows. A 100 femtoseconds pulsewidth, 80 MHz Ti:Sapphire laser oscillator (Tsunami, Spectra-Physics) is used as the excitation source for two-photon photopolymerization, process and its central wavelength is tuned to around 720 nm. The output laser power at the back focal plane of the microscope objective is controlled by using a variable attenuator made by an achromatic half-waveplate and a polarizer. The exposure time for each pixel is controlled through a computer-driven mechanical shutter (Oriel Electronic Fast Shutter, Newport). The beam is expanded and collimated by a telescope in order to obtain overfilling of the focusing microscope objective. Further, laser beam is reflected by a 45° dichroic mirror directly onto the objective back aperture. The dichroic mirror reflects most of the NIR laser beam and transmits part of the visible spectrum (400 – 550 nm) enabling real-time monitoring of the sample surface with a CCD camera. We choose to use a dry semi-apochromatic microscope objective (Olympus, LUCPlanFLN 60x, N.A.=0.70) equipped with a spherical aberration correction collar to demonstrate the achievement of good results even without using more expensive oil immersion objectives.

A suitable coverslip holder is mounted on a xyz piezo-stage (Tritor 101, Piezosystem Jena) for positioning in horizontal and vertical directions. The travel range of the piezo-stage is 80 µm in each of the x, y, and z directions. A dedicated software, developed by IIT group within the SMD project, translates the structure-points to piezo stage positions and controls the synchronization of the movements with the mechanical shutter, in order to achieve the desired local dose.

A tube lens images the sample onto the CCD, which is used to check the position of the beam focus and for real-time monitoring of the photopolymerization process.

The photopolymer chosen for fabrication is a commercial UV curing adhesive (NOA 63, Norland) with optimum sensitivity in the 350-400 nm range.

A small amount of of NOA 63 is dropped onto a coverglass and let to achieve a mechanical equilibrium. A pre-exposure with a UV lamp is done for a few seconds in order to increase the resin viscosity. The laser power (measured before the objective) is set to approximately 6.5 mW with a pixel exposure time of 20 ms.

As it has been mentioned above, the profile of the companies potentially interested in putting the tripods into the market would be the companies already providing beads for Optical Tweezer applications (Fused Silica Beads); for example and just to mention some: http://www.fusedsilicawafers.com; http://www.micropore.co.uk/; http://www.spherotech.com/).
These companies are already experts on the market sector (Optical Tweezer users) and could be able to develop the business successfully as a new product line in their catalogue.

Other kind of companies potentially interested in putting these "Tripods" into the market could be those companies already providing the Optical Tweezers Set Up (for example: http://www.aresis.com/ ; http://www.elliotscientific.com/; http://www.jpk.com; http://www.thorlabs.de/). This kind of companies could play as distributors of the Tripods, however the manufacturing process would be far from its current business and therefore the interest for complete exploitation of the idea would be limited.

Regarding the profile of the potential customer, in principle the market is very specific and focused on researchers using Optical Tweezers, and among them, those interested in combining the optical trapping capabilities, including force measurements, with Raman spectroscopy. This combined experiment is still not an extended technique and is at research level, being under development in itself. However, the potential interest of the combination of force measurements and Raman characterization is very high. Since the optical tweezers is a technique that traps, images, and manipulates small objects with biological interest such as biomolecules, supramolecular assemblies and dendrimers in three dimensional space, the interest of the technique from the biotechnological point of view is clear, this sector can potentially include the sectors of Health, Pharmaceutical industry and Food.

As it has been said before, the tripods are a consumable for applications of Optical Tweezers in these fields and there is nothing available in the market with similar characteristics.

2.b AFM new design
The newly designed AFM covers the main goals of the project, being compatible with the developed tips as well as making it possible the combination of AFM with other techniques such as Raman and Tip Enhanced Raman (TERS). The market interest of these capabilities has to be considered separately.

The first one, compatibility with the SMD tips, is a clear advantage in the case that these tips become commercially available for the potential users of the microscopy technique. Given the case that the tips can be manufactured and offered to potential customers, the newly designed AFM will have the advantage of being ready for the application, while the more standard designs will need to wait for specific adaptations.

Combination of AFM with Raman and TERS characterization has an increasing interest in the market, both at fundamental or applied research labs and at R&D industry departments. The sectors of application are even wider as those for just AFM. Some of the commercial AFM manufacturers already have some product covering the combination with Raman, mainly for application on biological samples on the cell scale, but they present some drawbacks

- in particular for Single Molecule applications - as pointed out by the experts at the SMD project. These points have been considered all along the development at WP2, mainly at the specifications definition in order to have a final design more competitive than what is already available. The design produced within the SMD project has considered very carefully issues as mechanical stability of the AFM, which is essential for Single Molecule experiments, and optimization of the optical paths and distances to maximize the optical sensitivity for the Raman signals, which is also essential when studying Single Molecule events.

The market sector for this product is in various fields, from Materials science and engineering to Health or Food research labs. Potential customers are researchers of either fundamental or applied labs.

The ideal profile of the company to exploit the result is an AFM developer, manufacturer and vendor with partner/s expert at Raman instruments commercialization and application. Nanotec is already on the way of putting this result into the market. During the SMD project, Nanotec has established contacts with one of the main manufacturers of Raman instruments (Horiba – IvonJobin) for the combination of the newly designed AFM with their instruments. The open design of the new AFM makes also possible the option of the customers preparing their own optical set-up or using the one that already exists in their lab, increasing in this way the market potential for the new AFM.

Regarding the competitors in the market, some of the other AFM vendors offer combination with Raman (Bruker; JPK; NT-MDT; Nanonics; Witec). To be competitive against these options, SMD design has been focused on the highest stability for the maximum AFM resolution and on the optimization of the optical paths and distances to maximize the Raman sensitivity. Being also prepared for large scale samples such as biological cells, the new AFM has been optimized for small range samples and Single Molecule experiments. This is an important strength and differential point against the competitors design. Compatibility with Dulcinea control unit and the well known WSxM software is an important advantage against the competitors products.

The pathway for exploitation starts from the current situation with a laboratory prototype built. Then several steps need to be taken before successfully putting the product in the market. These steps include cost analysis for manufacturing, which is currently in process at Nanotec R&D department; modification of the design to transfer from the laboratory prototype to a commercial prototype, to guarantee robustness, reproducibility and friendliness of use both for the customer as well as for the technical support and optimize manufacturing costs. Then collaboration with first customers will be established to increase the number and type of applications demonstration and generation of application notes. Meanwhile training of technical and sales human resources is needed, as well as price definition to launch the product at the commercial catalog.

2.c Software tools for AFM 1: Force clamp acquisition tool

One of the main interests of the SMD project is the combination of Force Spectroscopy and Raman Spectroscopy. For this kind of measurements it is needed to have complete control of the parameters for the Force Spectroscopy experiment. During the developments at WP2 a specific software module has been created based on the input given by the expert partners in this kind of experiments.

This software module has been integrated into the Develop Version of the commercial software for AFM-STM-SNOM created and distributed by Nanotec (WSxM) to be combined with the Control Unit for AFM-STM-SNOM commercialized by Nanotec (Dulcinea). The combination of WSxM+Dulcinea is used for the commercial AFM and STM developed by Nanotec as well as to control other party or home-made AFM/STM/SNOM. It is also used for the control of the newly designed AFM described at the previous section. The integration of the developed software module in the WSxM will improve the performance of the control unit offering a new mode of operation for the Force Spectroscopy application.

WSxM is already one of the best known and more powerful control software for AFM/STM/SNOM. It is world-wide used and is upgraded regularly being free to download and use by the research community (not for commercial purposes). The newly developed module for Force Clamp experiments will be available for all the users of the WSxM software.

The market sector for this result corresponds mainly to the research labs interested in Single Molecule Pulling. This sector is small and very specialized but represents one of the forefronts of the research applications of the AFM and therefore, demonstrating success in this field is an interesting reference for an AFM manufacturer, both for commercial reasons as well as for the technical challenge involved.

Some of other vendors have specific modules for the pulling of molecules (JPK, Asylum) and they are compatible only with their AFMs and are either integrated into their control software or offered separately as an extra module to be bought. Including the newly developed software into the WSxM widens the number of applications of Nanotec control unit for AFM-STM-SNOM and offering this new module without extra cost integrated into the already well-known WSxM guarantees a very wide diffusion among AFM laboratories.

2.d Software tools for AFM 2: Raman Analysis and acquisition tool

One of the main interests of the SMD project is the combination of Force Spectroscopy and Raman Spectroscopy. For this combination it is necessary to have complete control of the synchronization of the two instruments measuring both signals, the Force and the Raman signals. During the developments at WP2 a specific software module has been created to prepare the control unit of the AFM for this synchronization. An extra advantage for the interest of this result is that it can be used for the synchronization of the AFM/STM/SNOM with Raman spectrometer or with most data acquisition systems (provided that fits the specifications). The possibility of synchronized data acquisition from different set-ups widens the application fields and therefore the market for the AFM control system. Besides the synchronization software module, a specific tool for the analysis of the combined AFM+Raman data has been developed.

These two software modules (synchronization and data analysis) have been integrated into the Develop Version of the commercial software for AFM-STM-SNOM created and distributed by Nanotec (WSxM) to be combined with the Control Unit for AFM-STM-SNOM also commercialized by Nanotec (Dulcinea). The combination of WSxM+Dulcinea is used for the commercial AFM and STM developed by Nanotec as well as to control other party or home-made AFM/STM/SNOM. It is also used for the control of the newly designed AFM described in section 2b. The integration of the developed software module in the WSxM will improve the performance of the control

unit offering new options for the user: Analysis tool for combined AFM+Raman data and the possibility of acquiring synchronized data from the AFM and other measurement set-up such as Raman spectrometer.

WSxM is already one of the best known and more powerful control software for AFM/STM/SNOM. It is world-wide used and is upgraded regularly being free to download and use by the research community. The newly developed modules for Raman Analysis and acquisition experiments will be available for all the users of the WSxM software.

The market sector for the synchronization module result is wide in the sense that allows the synchronization of the AFM with other measurement instruments. In particular for the synchronization with Raman spectrometers, the sectors of applications are several including for example Materials science and engineering and Health. Being such different sectors of applications, there are different profiles for potential users and the commercial exploitation of the modules have to be addressed in specific ways with the development of user-friendly modules that allows this combination. For those advanced users that develop the combination set-up by themselves, the software module for synchronization will be available at the Develop WSxM version.

The market sector for the analysis of combined AFM+Raman data covers different sectors as well. The use of the analysis tool is available for all of them in a more direct way than the acquisition tool and therefore the potential number of users is much wider.

On the side of the dissemination: the dissemination activities have been conducted in a perspective of a public engagement in science and technology, as the European Commission recommended in the domain of science communication in the last years. Therefore the dissemination of the scientific results of the project has been meant in terms of sharing knowledge and opening dialogue among different social actors, so that both SMD researchers, other experts and the lay public and have been considered. The activities were of three kind: on one hand, researchers were involved in communication through training activities and making them thinking about the importance of science communication in modern society; on the other hand, researchers became communicators and communication producers; and finally a public science communication event was organized. The activities have been performed in Italy, in the Genoa area, with the Italian Institute of Technology researchers working on SMD project.

Regarding the training activities, SMD researchers attended two seminars about science communication – one dedicated to the present role of scientists in society and the theory of science communication (from the public understanding of science to the public engagement in science), the other one focused on the basic elements of an effective communication. They also responded to a questionnaire where they showed their own attitude and experience in public communication; results were discussed in group, and common points were underlined.

Personal interviews and the publishing of a public blog on the internet were the two main activities which involved researchers as communicators. Questions about their professional life, their aspiration as scientists and their work in the project were asked and it made the SMD researchers the main- characters of communication – in the sense that they decided what to tell and what not to – and also the main object of a blog on the

internet (see http://www.singlemoleculedetection.blogspot.com online). The blog was published in October 2010 and after only 4 months it has reached around 30 readers (SMD researchers excluded). The main aspects of the research project are there described.

A video describing the activities of SMD has been realized, with the collaboration of all the partners of the project 33 publications published and the partners attended conferences and workshop where as invited speakers they discussed the items of the SMD project.

List of Websites:
http://www.singlemoleculedetection.eu