NPL Report COAM 2

Review of Techniques for Single Molecule Detection in Biological Applications

by
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Executive Summary

This report reviews various experimental techniques that have the potential to detect single molecules and highlights their possible application to the detection of biomolecules. These have been grouped into two categories within the report; Scanning Probe Microscopies (SPMs), those methods yielding force and current data, and Applied Optical Spectroscopies, those techniques providing optical and spectroscopic information. The theory of each technique is described along with relevant current research and the requirements that each technique might generate for further measurement research.

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Introduction

In the early 1980's scanning probe microscopes (SPMs) produced the first real-space images of the surface of silicon. Now, SPMs are used in a wide variety of disciplines, including fundamental surface science, routine surface roughness analysis, and spectacular three-dimensional imaging - from atoms to micron-sized protrusions on the surface of a living cell. In some cases, scanning probe microscopes can measure physical properties such as surface conductivity, static charge distribution, localized friction, magnetic fields, and elastic moduli. As a result, applications of SPMs are now very diverse.

Applied optical spectroscopies are a set of techniques that provide optical and spectroscopic detail at very high resolution. In practical terms, the wavelength used itself defines the minimum information limit, despite the efforts of lens makers. Only by lowering the wavelength can the resolution be improved below a micron or so, as the recent (and costly) trends towards deep ultra-violet laser optics in semiconductor fabrication illustrate. These techniques provide spectroscopic data and detection in principle down to the single molecule level. There is even the possibility of imaging on the single molecule scale.

Many of the initial applications of single molecule spectroscopy in biotechnology have been in the realm of extremely sensitive imaging and analyte detection. More recently a number of studies that are truly aimed at studying the spectroscopy and dynamics of single molecules and their reactions and interactions with the molecules and complexes that surround them have emerged. There are major advantages to studying dynamics and spectroscopy at the level of single biological molecules which are reviewed in some detail in this report.

The Appendix contains the recent US National Institute of Health call for basic research in the areas of single molecule detection and manipulation. This illustrates some of the research areas that represent the 'state of the art' for these techniques.

probe. Because of the constant and small radius of the tip (2.5-10 nm) and the high aspect ratio (1:100) of the carbon nanotube, the lateral resolution has been much improved judging from the apparent widths of DNA and nucleosomes. The carbon nanotube probes also possessed a higher durability than the conventional probes[3].

- Additionally biomolecules have been ruptured using AFM to measure bond strengths[4]. This is usually done by biochemically modifying the AFM tip and introducing into a tissue binding site. The tip is then removed and the force taken to break the binding can be measured.
- Covalent Functionalised Nano-Probes: Scanned probe tips may be appropriately functionalised to discriminate between a variety of chemically or biologically significant single molecules adsorbed onto a suitable surface. For example carboxyl groups (-COOH) are readily derivatised by for example attaching an amine group[5]. If a nanotip is terminated by a pendant carboxyl group to which an amine (RNH_2) is attached the probe may then be brought into proximity with a surface which has been prepared with a suitable density of self assembled monolayer molecules. Thus the covalent modification of nanotube tips enables the straightforward creation of well defined probes which are sensitive to specific intermolecular interactions that define the properties of many chemical and biological systems. In the biological field for example a nanotube tip has been covalently modified with a biotin ligand. This tip then interacts with streptavidin receptors which are linked to the surface by biotin groups.
- A method has been demonstrated for multiplexed detection of polymorphic sites and direct determination of haplotypes in 10-kilobase-size DNA fragments using single-walled carbon nanotube (SWNT) atomic force microscopy (AFM) probes[6]. Labelled oligonucleotides are hybridized specifically to complementary target sequences in template DNA, and the positions of the tagged sequences are detected by direct SWNT tip imaging.

Detectability, measurement and calibration possibilities

- Ultra fine AFM tips can be used to map the morphology and orientation of single, large biomolecules. More realistically the examination of tissue morphology is relatively easily achievable. The most interesting applications of the AFM lie in its combination with the SECM or as a local EM amplifier in SERS experiments.
- For morphological applications the AFM would be used in a non-contact mode looking at the forces between the tip (or modified tip) and substrate while the tip is scanned in two dimensions.
- Opportunities for measurement and calibration possibilities lie in the determination of protein or tissue orientation and morphology. There is also opportunity for the measurement and calibration of the force constant between two biomolecules if one modifies the AFM tip with a protein molecule and introduces this modified tip into a tissue sample[7].

By scanning the sample with respect to the tip in a three-dimensional raster pattern an image of the atomic structure of the molecule can be obtained. Such a technique may allow observation of individual molecules within cells and show their full three-dimensional structure with atomic scale spatial resolution in a non-destructive observation process. Researchers could observe previously hidden structures. It would be possible to observe individual macromolecules in their native form embedded in their natural environments. This is a capability with no current technology affords at present. MRFM with single-nucleus sensitivity is still beyond present-day experimental capability because the magnetic force generated by an individual nucleus is so small. The goal of single electron spin detection and imaging is a challenging one. This is because the forces involved are so small and because the behaviour of individual spins in the presence of a nearby ferromagnetic tip is not well understood.

Recent Applications

Up until recently this process has been sufficiently insensitive to require $>10^8$ identical spins in an ensemble to be readily detectable. What has happened over the past few years is that a number of novel methods for reading out the resonance condition have been proposed which can improve the detection sensitivity in small samples by many orders of magnitude so that one can begin to consider the possibility of detecting a spin flip (and hence of carrying out sensitive high resolution spectroscopy) in a single molecule.

Recent work has moved towards detection single spins. Attention has focussed on theoretical analysis of the problem[9] as well as analysis of the optimal tip configuration for MRFM of microscale buried features[10].

- Ultrahigh frequency MRFM has also allowed the detection of hyperfine structure and an indication of the influence of relaxation time on the technique.
- More recently IBM in collaboration with workers at Stanford University have developed ultrasensitive micromechanical cantilevers which are 200 microns long but only 65 nm thick. It is thought that at liquid helium temperatures the sensitivity available would be sufficient to detect individual electron spins.

  A spatial resolution of < 1μm in three dimensions should be possible. Another magnetic resonance force microscope has achieved detection of 3×10^{11} electron spins at room temperature in an applied field of 0.025T[11]. The paper discusses future potential improvements in sensitivity. The same group has reported the detection of slice-selective electron spin resonance with an external magnetic field gradient comparable to local interatomic gradients, using the techniques of magnetic resonance force microscopy[12]. An applied microwave field modulated the spin-gradient force between a paramagnetic DPPH sample and a micrometer-scale ferromagnetic tip on a force microscope cantilever. A sensitivity equivalent to 184 polarized electron moments in a one-Hertz detection bandwidth was attained. The authors mapped the tip magnetic field with a resonant slice thickness of order one nanometer, thereby demonstrating magnetic resonance on length scales comparable to molecular dimensions[13].
- There are few actual applications of NMFR of the microscale biological systems published to date. An interesting initial step in this direction has been reported[14]. Calculations of the MRFM spectra obtained from a 2,2-diphenyl-1-picrylhydrazyl particle have been performed for various conditions. The results

characteristics between the UME and the sample and the electrochemical reactions occurring at the sample surface. Although not as spatially resolved as for an AFM or STM, because of the relatively long range of the tip-electrochemical-substrate interactions making resolution of nano-islands, for instance, more problematic, the SECM can probe both the topography and reactivity of the surface with a resolution governed by the tip dimensions.

Recent Applications

- Bard[17] has shown that the detection of single ferrocenyl molecule in solution is possible using a tip of nanometer proportions. Bard also used a phenomenon very important in low current probe applications known as feedback diffusion. As a tip is moved towards a surface, currents increase as transport times decrease until the tip is close enough to hinder the UME's semi-infinite hemispherical diffusion layer after which time the current drops. If the tip is brought even closer to the substrate the current experiences a massive amplification as a feedback diffusion regime is established, as shown in . In theory the same single molecule can be thought of as circulating between the tip and substrate undergoing successive oxidation and reduction reactions. Bard[18] has also used this method to look at single ruthenium bipyridyl complexes in solution.

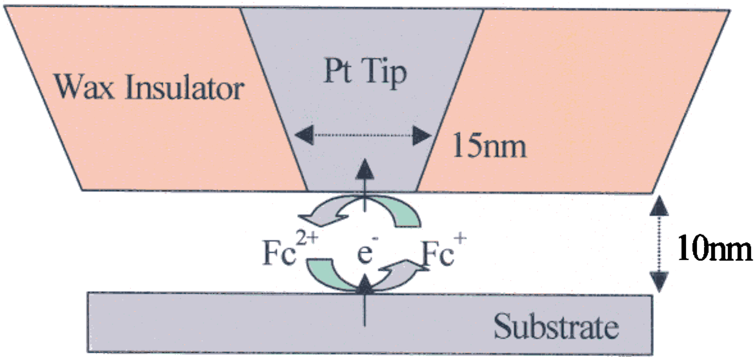

Figure 3 Idealised schematic illustration of the tip geometry and conditions for feedback diffusion during electrochemical SMD. After reference 8.

- SECM has been used for biological applications such as the transport of species through membranes, tissues and pores. It can be used to examine the rate of transport across a tissue or membrane and whether transport is uniform or localised and in addition what factors are governing the transport.
- Specific applications have included the quantitative imaging of transport through dentine[19] and imaging the localised transport and permeability in cartilage[20].

Detectability, measurement and calibration possibilities

- As Bard has shown the ultimate detection limit of the single molecule has already been achieved. It should be possible to achieve a similar level of detection using biomolecules or biomodified electrodes and probe redox couples in solution.
- One would determine current densities at various electrode-substrate separations and use these to calculate diffusion coefficients and electron transfer rates for the

- Traditionally STM has been used to examine semiconductors and binary semiconductors where resolution has surpassed SMD and is pushing the quantum dot envelope[22] and is regularly used in nanofabrication[23] and electrochemical nanostructuring[24].
- STM also provides the capability to perform Inelastic Electron Tunnelling Spectroscopy (IETS). IETS may be used to measure the spectra of single biomolecules adhered to substrates using a cryogenic STM.
- Bottom-up fabrication methods aim to assemble and integrate molecular components with specific functions into electronic devices that are orders of magnitude smaller than conventional lithographically produced electronic circuits. Organic molecules containing redox centres support resonant tunnelling and display promising functional behaviour when sandwiched as molecular levels between electrical contacts. For example a redox switchable group forms the backbone of a bifunctional ligand that attaches a gold nanoparticle to a gold substrate. Electron or hole injection into the redox centre is controlled by the potential applied between the substrate and a counter electrode and the resulting change in barrier transparency can be used to control electron tunnelling between nanoparticle and substrate. The tip of an STM is positioned above the nanoparticle and is used to monitor the electron tunnelling. Such a nanostructure can be prepared by immersing a (111) Au film on glass in a 1mM solution of N,N-di-(10-mercaptodecyl)-4,4'-bipyridinium dibromide for 18 hours, before immersing in a toluene solution of gold nanoparticles, to attach well separated particles to the surface.
- An area of particular interest for bio-STM research would be the investigation of the conductivity of the central metal ion in porphyrin-based metallo-proteins. Little work has been carried out in this area and there is certainly room for advancement of knowledge on this subject. In particular the copper/zinc or manganese central ions within superoxide dismutase would warrant investigation. STM investigations of copper proteins appear in the literature[25] but do not focus on the conductivity of the central metal ion, whilst other general studies have focussed on the feasibility of studying metallo-proteins using STM[26,27].
- Superoxide dismutase is an essential enzyme which catalyses the conversion of two superoxide anions (a highly destructive by-product of oxidative metabolism) into hydrogen peroxide and oxygen. The active site contains two metal ions, copper and zinc for the cytosolic form and two manganese ions for the mitochondrial form, and it has been proposed that, in the cytosolic form, the Cu ion accepts an electron from the superoxide anion to catalyse the reaction. The reaction mechanism for the mitochondrial enzyme is unclear.
- STM images of DNA molecules embedded in metal films have also been recorded[28]. In addition electrochemical techniques have been used to characterise proteins[29,30].
- There has been considerable debate in the literature as to the electrical properties of DNA. It seems that the electronic properties have been intimately linked to the exact base pair sequence of the DNA. It now appears that that charge carriers can hop along the DNA over distances of at least a few nanometres. However, the situation among investigators who are measuring electronic transport through DNA molecules over larger distances is much less clear. Much of this work on the electronic properties of DNA has involved the use of STMs or STM technology. This would represent a novel and exciting application of the STM.

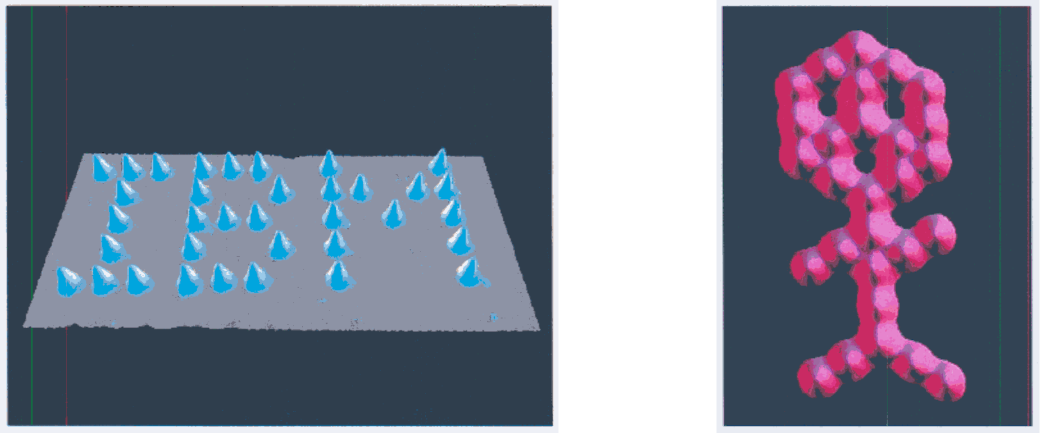

Figure 5 Atomically and molecularly resolved STM images. Xenon on Nickel (110) (left) and carbon Monoxide on Platinum (111) (right). From reference 84.

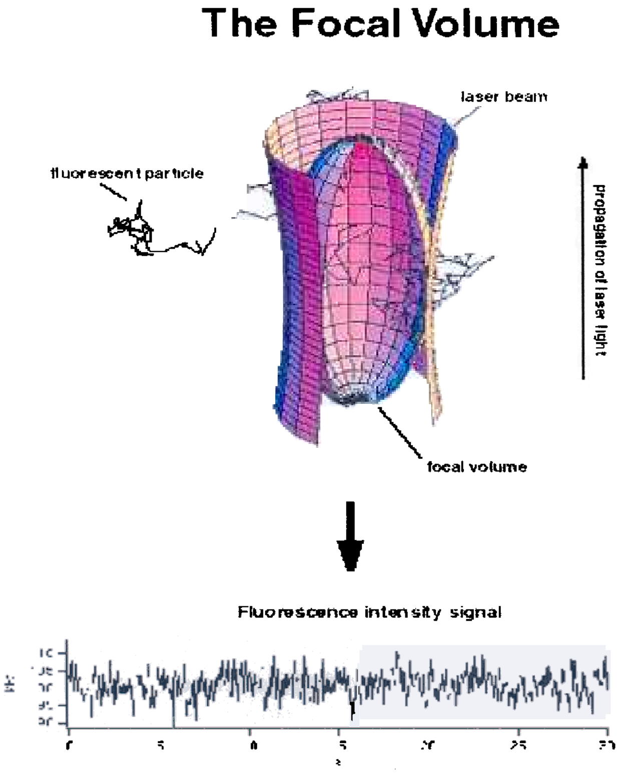

Figure 7 A representation of the confocal volume in a FCS experiment. From reference 42.

One important application of FCS in the biochemical sciences is the determination of thermodynamic and kinetic parameters such as association and dissociation constants of intermolecular reactions in solution. The measurement principle for nearly all FCS applications is based on the change in diffusion characteristics when a small labelled reaction partner (e.g. a short nucleic acid probe) associates to a larger, unlabeled one (target DNA/RNA). The average diffusion time of the labelled molecules through the illuminated focal volume element that is inversely related to the diffusion coefficient, increases during the association process. By calibrating the diffusion characteristics of free and bound fluorescent partner, the binding fraction can easily be evaluated from the correlation curve for any time of the reaction. A dual-colour instrumental extension of the standard confocal FCS set-up enables two different fluorescent species to be monitored in parallel and opens up the possibility for dual-colour cross-correlation analysis.

Recent Applications

The most recent FCS work has centred on the determination of chemical rate constants[43].

Moves towards more FCS use at the single molecule level[44,45]. The push to characterise, mechanistically, the behaviour of single molecule is a general goal in molecular biophysics. Recent advances in single-molecule detection at room temperature by laser induced fluorescence has meant that individual macromolecules are now detectable using FCS[46].

- Biotechnological systems are also becoming an increasingly popular target for FCS with studies even probing inside the cell nucleus[47]. In addition, optical techniques are used to observe the behaviour of single DNA fragments[48]. DNA is a suitable biomolecular target due to its immense size and robustness. Advances have also been made in the area of protein folding dynamics and DNA transcription[49].

qualitative analysis of interfacial systems in this way, the very nature of the interface limits the measurement procedure. An interface provides a heterogeneous environment for molecular species, so emission characteristics (e.g. molecular fluorescence quantum efficiencies) are dependent on the precise location of individual fluorophores within this environment. Consequently, time-integrated measurements are limited, since even a 'simple' interface will almost certainly contain more than one type of fluorescent centre. Additional selectivity is required to obtain a more complete understanding of the interface. We have achieved this through time-resolution. Variations in the photophysical properties of interfacial species can be detected through changes in fluorescence decay profiles, and the spatial distribution of interfacial species can be mapped more closely.

Time-resolved evanescent wave induced fluorescence spectroscopy (TREWIFS) has previously been used to probe interfacial photophysics. For example, TREWIF studies of molecular and polymeric species have demonstrated dramatic changes in fluorescence quantum efficiencies at dielectric interfaces. Although these gross variations are readily measurable, the true nature of the modification is not fully understood. In particular, it is observed that decay profiles describing interfacial fluorescence are almost always far more complex than those resulting from species in the bulk phase. The use of lifetime distribution analysis (LDA) methods in conjunction with conventional sum-of-exponentials (SOE) analysis methods have recently been used to expose this complexity. LDA methods allow differentiation in systems containing up to 4 discrete component populations, and systems comprising a continuous distribution of emitting species.

Recent Applications

- Depth profiling work on dielectric surfaces using time-resolved and time-integrated EWIFS[50].
- The use of time-correlated single photon counting (TCSPC), both simulated and experimental and lifetime distribution analysis (LDA) allowing differentiation of a system containing up to four component populations.
- Movement towards single molecule detection capability allied with integration of the technique to a 'lab-on-a-chip' environment.

Detectability, measurement and calibration possibilities

Potentially an upper detection limit of single molecules is achievable. Similar low probe volume fluorescence studies have resolved single molecules possessing fluorophores. More than 10,000 photons may be emitted from each single molecule as it passes through the probe volume – well above the detectable limit.

Measurement parameters would mostly be the intensity of the fluorescent light from the probe volume. This could be measured from a variety of substrates and biomolecules. Intensity of EWIFS with a changing surface concentration of

Recent Applications

- Transmission SNOM is capable of a far higher optical resolution than conventional optical microscopy. ***Figure 8*** shows a portion of plasmid DNA consisting of approximately 10000 base pairs. The sample was prepared on a mica substrate. The transmission SNOM image (left) reveals a strong contrast. The simultaneously acquired topography (right) shows the width of the same molecule as just below 180 nm. The apparent width is larger than the expected 100 nm due to a convolution with the shape of the probe tip.

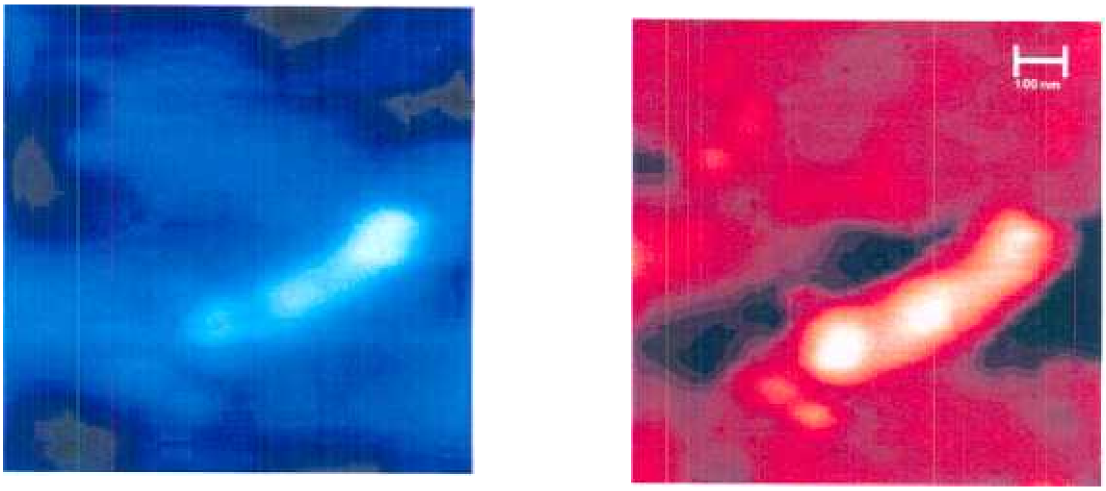

Figure 8 Comparison of transmission SNOM image of plasmid DNA (left) with topographical image of the same sample area (right). Due to light scattering, the plasmid DNA appears bright in transmission mode. From reference 53.

- Single molecules have often been observed in the literature using SNOM. This has included the imaging of biological samples[54] and the detection of single dye molecules[55,56].

  Individual carbocyanine dye molecules in a sub-monolayer spread have been imaged with near-field scanning optical microscopy. Molecules can be repeatedly detected and spatially localized (to approximately λ/50 where λ is the wavelength of light) and the orientation of each molecular dipole can be determined. This information is exploited to map the electric field distribution in the near-field aperture with molecular spatial resolution.[57].

Detectability, measurement and calibration possibilities

- Potentially an upper detection limit of the single molecule is achievable. It is clear that the single molecules most often detected and characterised in the literature have been dye molecules and DNA fragments.

off silver or gold. Often large SERS enhancements are related to the co-location of two nano-islands at a critical distance. The explanation for this phenomenon, and the relationship between the island size and separation are still not fully understood.

The second mode of enhancement is chemical in origin. The formation of a charge transfer complex between the surface and the analyte molecule is the cause of enhancement and with many charge transfer complexes having electronic transitions in the visible region of the EM spectrum, resonance enhancement is possible. Molecules with lone pair electrons or π-clouds show the strongest SERS. The effect was first seen with pyridine.

The extremely small cross sections of Raman scattering would preclude its use at single molecule level. However, this situation can be dramatically altered for surface-enhanced Raman scattering. Large enhancement factors on the order of 10^{14} corresponding to effective Raman cross sections of $\sim 10^{-16}\mathrm{cm}^2$ per molecule in non-resonant surface-enhanced Raman scattering (SERS) at near infrared excitation[59]. Such Raman cross-sections comparable with or higher than effective fluorescence cross sections are sufficient for single molecule Raman spectroscopy. To achieve effective Raman cross sections on the order of $10^{-16}\mathrm{cm}^2$ per molecule, molecules have to be attached to aggregated colloidal silver particles[60] in aqueous solution (surface-enhanced Raman scattering). A diagrammatic representation of a Raman microscope used for SMD is shown in ***Figure 10***.

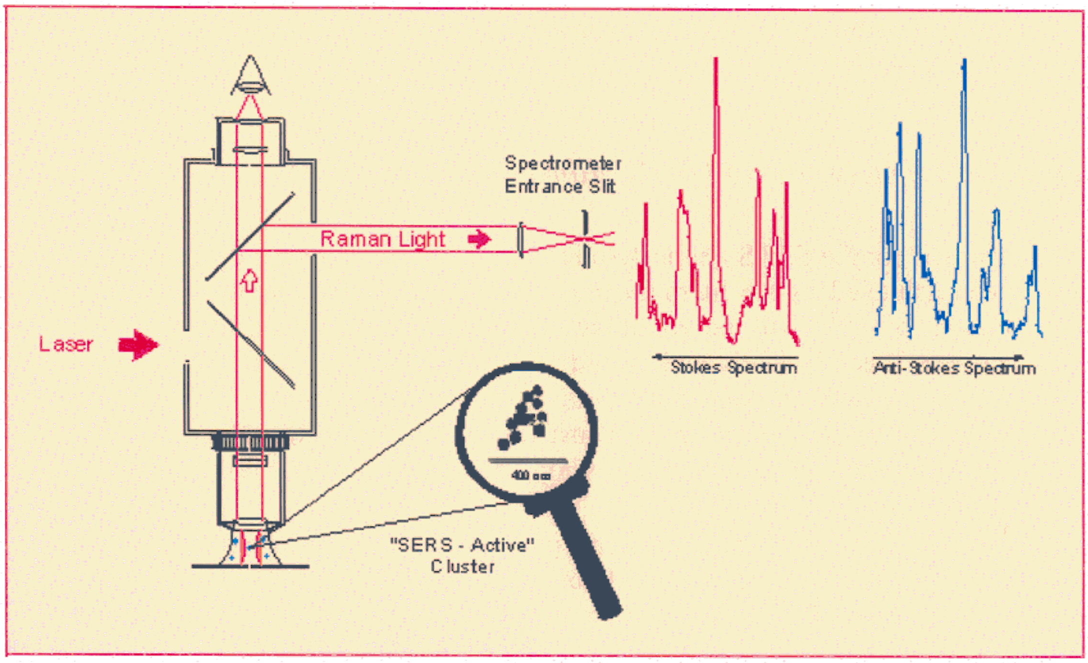

Figure 10 A diagrammatic representation of a Raman microscope used for SMD. From reference 61.

SERS work in the field of biomedical applications has centred around examining small numbers of molecules adsorbed on colloidal particles of silver and gold. Near-infrared

Surface Plasmon Resonance (SPR)

A surface plasmon is a collective surface excitation of conduction electrons in a metallic conductor. The controlled excitation of such a quasi particle can be effected by means of photon irradiation or current injection. Surface Plasmon Resonance (SPR) imaging usually refers to the former and as a technique in its own right it is unlikely to be of great use for single molecule detection but has great relevance to the mechanism of SERS enhancement on surfaces and is analogous to EWIFS (see below) effects. At an interface between two transparent media of different refractive index (glass and water), light coming from the side of higher refractive index is partly reflected and partly refracted. Above a certain critical angle of incidence, no light is refracted across the interface, and total internal reflection is observed. While incident light is totally reflected the electromagnetic field component penetrates a short (tens of nanometers) distance into a medium of a lower refractive index creating an exponentially detenuating evanescent wave. If the interface between the media is coated with a thin layer of metal (gold), and light is monochromatic and p-polarized, the intensity of the reflected light is reduced at a specific incident angle producing a sharp shadow (called surface plasmon resonance) due to the resonance energy transfer between evanescent wave and surface plasmons. The resonance conditions are influenced by the material adsorbed onto the thin metal film. A satisfactory linear relationship is found between resonance energy and mass concentration of biochemically relevant molecules such as proteins, sugars and DNA. The SPR signal, which is expressed in resonance units, is therefore a measure of mass concentration at the sensor chip surface. This means that the analyte and ligand association and dissociation can be observed and ultimately rate constants as well as equilibrium constants can be calculated.

A fraction of the light energy incident at a sharply defined angle can interact with the delocalised electrons in the metal film (plasmon) thus reducing the reflected light intensity. The precise angle of incidence at which this occurs is determined by a number of factors, in practice the principal determinant becomes the refractive index close to the backside of the metal film, to which target molecules are immobilised and addressed by ligands in a mobile phase running along a flow cell. If binding occurs to the immobilised target the local refractive index changes, leading to a change in SPR angle, which can be monitored in real-time by detecting changes in the intensity of the reflected light, producing a sensorgram. The rates of change of the SPR signal can be analysed to yield apparent rate constants for the association and dissociation phases of the reaction. The ratio of these values gives the apparent equilibrium constant (affinity). The size of the change in SPR signal is directly proportional to the mass being immobilised and can thus be interpreted crudely in terms of the stoichiometry of the interaction. Signals are easily obtained from sub-microgram quantities of material. Since the SPR signal depends only on binding to the immobilised template, it is also possible to study binding events from molecules in extracts, i.e. it is not necessary to have highly purified components. A diagrammatic representation of a SPR experimental arrangement is shown in *Figure 15*.

also been used to demonstrate the optical emission arising from the surface polarons. Enhancement factors of 10^8 have been obtained in this way[88].

Recent applications

SPR imaging has been used as a surface sensitive spectroscopic technique for measuring interactions between unlabelled biological molecules with arrays of surface bound species[89].

- Study of a variety of DNA/DNA[90], DNA/protein[91] and protein/protein[92] interactions.
- Scanning of guanine-guanine mismatches in DNA by synthetic ligands using surface plasmon resonance[93].
- Study of monolayer DNA hybridisation events by SPR[94].

Detectability, measurement and calibration possibilities

- Potentially an upper detection limit of multiple molecule detection with large biomolecules is possible. Even with current state of the art SPR set-up this level of detection is probably still out of reach. A more realistic target is the detection of sub-microgram amounts of material. It would be a reasonable aim to push this boundary down towards 100 ng detection and lower.
- Measurement parameters would include the angle of incidence of the laser light and the incident power of the laser light (including its wavelength). The SPR signal (reflected light) would then need to be measured in real time for intensity, rate of signal change and SPR angle. Amount and type of bio-species in a flow cell arrangement would also require variation to gauge their affect on the SPR phenomenon.
- The opportunities for calibration and measurement standards lie in the measurement of time constants for association and dissociation of biomolecules with receptors on the gold surface. In addition the force constants of the protein-receptor interactions are measurable. These quantities would require measurement for different species and at different concentrations.

Appendix

US NIH call for basic research on the detection and manipulation of single molecules.

(Release Date: February 12, 2001)

PURPOSE

The purpose of this program announcement is to encourage basic research on the detection and manipulation of single molecules. Recent advances in optical imaging and biomechanical techniques have demonstrated that it is possible to make observations on the dynamic behaviour of single molecules, to determine mechanisms of action at the level of an individual molecule, and to explore heterogeneity among different molecules within a population. These studies have the potential to provide fundamentally new information about biological processes and are critical for a better understanding of cellular function. Current high-resolution methods, such as x-ray crystallography and NMR, have provided a vast array of structural detail for biological molecules, yet the output of these methods is limited by its static molecular view and ensemble averaging. Single molecule methods provide an alternative set of approaches that will lead to a more direct view of the action of individual molecules without the need to infer process or function from static structures. Real-time measurements on the spatial and temporal fluctuations of single molecules in living cells, which are not possible using other methods, are a major goal of this initiative. Despite the promise of single molecule methods, there are a number of technical challenges that must be met to optimise these studies. Development of the collateral chemistry and instrumentation required to carry out single molecule studies is essential for progress. New tools and strategies, as well as refinement of current methods, are also needed. Single molecule methods are likely to lead to significant advances in understanding molecular movement, dynamics, and function.

HEALTHY PEOPLE 2010

The Public Health Service (PHS) is committed to achieving the health promotion and disease prevention objectives of "Healthy People 2010," a PHS led national activity for setting priority areas. This Program Announcement (PA), Single Molecule Detection and Manipulation, is related to one or more of the priority areas. Potential applicants may obtain a copy of "Healthy People 2010" at http://www.health.gov/healthypeople/.

RESEARCH OBJECTIVES

Background

Recent advances in the detection and manipulation of single molecules offer great promise for enhancing our understanding of the behaviour of individual biological macromolecules in the living cell. Scanning probe techniques allow imaging of single molecules on surfaces, and specialized optical techniques enable their characterization in complex environments. Single molecule biomechanical studies have been used to

Targets for study

Potentially any biological molecule is a target for study. Typical molecules are members of multi-component systems that change in response to environmental cues or specific cellular signals. Examples of experimental systems currently under study at the single molecule level include but are not limited to:

- Protein folding: pathways, existence of intermediates, kinetics, heterogeneity
- Enzyme catalysis: mechanism of catalysis, conformational changes
- Ion channels: local structural changes, kinetics
- Signalling: formation of multimers, kinetics of cascades, phosphorylation dynamics
- DNA, DNA binding proteins, RNA: binding constants, regulation of gene expression
- Membrane structure: restricted diffusion, phase changes
- Molecular motors: motility, processivity, directionality
- Complex cellular structures (e.g., transcription complexes): assembly, dynamics

The specific objectives of this program announcement are:

(1) To encourage investigators to develop and extend existing single molecule technologies to examine molecular motion, behaviour, heterogeneity, and fluctuations over time and space;

(2) To devise new tools and strategies for studying single molecules;

(3) To validate the methodology used to study single molecules to establish the reliability of the observations. Differences between ensemble and single molecule measurements need to be clarified so that the contributions of the single molecule to the ensemble behaviour are understood.

(4) To encourage studies on the 3-D visualization of cellular processes in real-time, in the living cell, at high resolution. Many complex cellular processes, such as signalling or translation, are amenable to analysis using single molecule methods.

(5) To develop the collateral chemistry to facilitate the detection and handling of target molecules. The categories of greatest need in chemistry are to:

- Improve the photophysical properties of fluorophores and other labels used for single molecule spectroscopy, including the synthesis of probes with improved luminescent characteristics that are compatible with intracellular conditions; optimisation of quantum dots, plasmon and Raman probes, and G/C/Y/R-fluorescent proteins;

- Develop new classes of probes or new strategies for labelling single molecules, particularly those that can be used for in vivo studies;

This program announcement addresses the need for an expansion in basic research in single molecule studies. It has become increasingly clear that state-of-the-art single molecule methods offer a powerful new approach to understanding subcellular structure and function. These methods have significant advantages over more static methods since they are designed to make observations on molecules as they move in time and space. Goals of this initiative are to observe the dynamic behaviour of individual molecules, to explore heterogeneity between molecules, and determine mechanisms of action. A long-range goal of these studies is to extend the measurements to the intracellular environment where individual molecules will be viewed as they move inside the cell, carry out specific functions, or behave as components of larger systems. In order to fully realize the potential of these tools, there are technical barriers that must be overcome. Development of the chemistry and instrumentation that support single molecule studies is emphasized as well as innovative new methods and tools that will facilitate single molecule approaches.

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o Develop better methods for insertion of site-specific labels for detection of single molecules, and better mechanical handles for their manipulation;

o Design better surface attachment protocols to immobilize single molecules or cells for in vitro measurements.

In order to achieve these goals it will be necessary to create strong collaborations with chemists with the goal of testing new chemistry on single molecule problems.

(6) To develop improved instruments to optimise high-resolution single molecule measurements. The goals are to:

o Refine currently used techniques such as high resolution laser microscopy, near-field scanning optical microscopy, confocal microscopy, wide-field microscopies such as TIR (total internal reflection microscopy) or epifluorescence, optical tweezers and AFM;

o Develop instruments with the capability to carry out higher resolution measurements, such as time-resolved/time-gated CCDs for faster, more sensitive detection; higher resolution AFM; optical traps to measure forces in the femtoNewton range; multiphoton spectroscopy optimised in the 50 nm range; flow chambers designed for 0.01 msec measurements;

o Design instrumentation using principles to enable future commercialisation so that more investigators will have access to it and will not be required to build their own.

Applicants are encouraged to include physicists, engineers and computational scientists in the strategies to solve instrumentation problems related to single molecule studies.

Scope

This PA emphasizes the need to encourage the participation, in addition to biologists and biophysicists, of chemists, engineers and physicists in single molecule research. Because of the level of experience and skill required, support may include career track, senior postdoctoral scientists with expertise in chemistry, physics, and instrument development.

State-of-the-art instruments that are optimised for high-resolution studies on single molecules often require several years to build and are not commercially available. Instrument development is essential for growth in this field and should therefore be recognized as a legitimate research activity on a grant application. As such, this type of research does not have to be "hypothesis-driven" to be considered worthy of support.

The funding Institutes may provide a substantial contribution for the acquisition or development of instruments, when the instrument is justified as part of the supported research effort. In all cases, the cost of the instrument and associated operating support must be consistent with the scope of the research project(s) with which it is associated.

Summary

manipulate individual molecules and to measure the force generated by molecular motors or covalent bonds. The development of new probe technologies, such as quantum dots and high-resolution laser fluorescence microscopy, allow real-time observations of molecular interactions and trafficking within living cells. These tools enable individual members of a population to be examined, identified, and quantitatively compared within cellular sub-populations and substructures.

Single molecule studies have the potential to provide spatial and temporal information that is impossible to obtain using other, more static techniques. X-ray crystallography, nuclear magnetic resonance, and electron microscopy have provided a wealth of information on molecular structure, yet none of these methods can be used to make measurements on the in vivo dynamic movements of single molecules in intracellular space or to observe the behaviour of single molecules over extended periods of time. Using single molecule methods, it should be possible to study time trajectories and reaction pathways of individual members in a cellular assembly without averaging across populations. Cellular processes, such as exocytosis, flux through channels, or the assembly of transcription complexes, could be visualized. Individual differences in structure or function generated by allelic polymorphisms should be detectable at the level of the single molecule. Monitoring the coordinated expression of a gene or group of genes in specific tissues, or at certain developmental stages, is within reach using these technologies. Thus, single molecule methods are recognized as an important new set of tools that can be applied to high resolution studies in many areas of biology.

On April 17-18, 2000, the National Institute of General Medical Sciences (NIGMS) sponsored a workshop to explore the progress and potential for targeted research in single molecule detection and manipulation. Topics that were discussed included single molecule fluorescence studies, imaging and manipulation of single molecules with Atomic Force Microscopy (AFM), studies of single channels, biomechanical studies on single molecules using optical tweezers, and computational studies based on biological machines. In addition to making presentations on their most recent work, the participants were asked to discuss how to develop further the technologies to facilitate progress in this field. A summary of the workshop can be found at http://www.nigms.nih.gov/news/reports/single_molecules.html.

This program announcement is issued in response to the findings and recommendations of the workshop, as endorsed by the National Advisory General Medical Sciences Council at its May, 2000, meeting. It recognizes the powerful impact that single molecule research may have on research in biology, and it addresses the needs of potential research programs in this area.

Scientific Objectives:

The goals of single molecule research are to observe the dynamic behaviour of individual molecules, to explore heterogeneity among molecules, and to determine mechanisms of action. Single molecule studies are uniquely designed to yield information about molecular motion, behaviour and fluctuations over time and space. An important aspect of the research will be to measure features of individual molecules that are masked by ensemble measurements. Real-time observation of single molecules in live cells, relative to in vitro studies, is an important goal.

Summary

The detection of single molecules is one of the ultimate goals of analytical chemistry. Specific challenges include the characterisation and identification of individual molecules and furthermore their detection in a matrix of similar chemical entities. Many of the first applications of "single molecule spectroscopy" to biotechnology lie in the realm of sensitive imaging of ensembles containing hundreds or thousands of molecules. Eventually studies that are truly aimed at studying the spectroscopy and dynamics of single molecules and their reactions and interactions with the molecules and complexes that surround them will be a major goal.

There are major advantages to studying dynamics and spectroscopy at the level of single biological molecules. An example is the study of sequential dynamics where a population of molecules undergoes a series of events, such as a complex assembly process or linear polymerisation, which become out of phase. Consequently the detailed dynamics of individual steps are lost when the ensemble is studied as a whole. When observed at the single molecule level, the evolution of the phase of each molecule through the course of events is not lost. Another benefit of single molecule spectroscopy is that single molecules exist at any given time in particular conformational states within a particular solvent environment. Single molecule studies can also be carried out by scanning probe microscopy which allows the measurement of mechanical or electrical properties of single molecules, such as binding forces, bond strength and conductivity.

This report reviews various experimental techniques that have the potential to detect single molecules and highlights their possible application to the detection of biomolecules. These have been grouped into two types; Scanning Probe Microscopies (SPMs), those methods yielding force and current data, and Applied Optical Spectroscopies, those techniques providing optical and spectroscopic information. It is clear that both of these types of method have enormous potential to be used for research into the fundamental biological and chemical processes underpinning biotechnology.

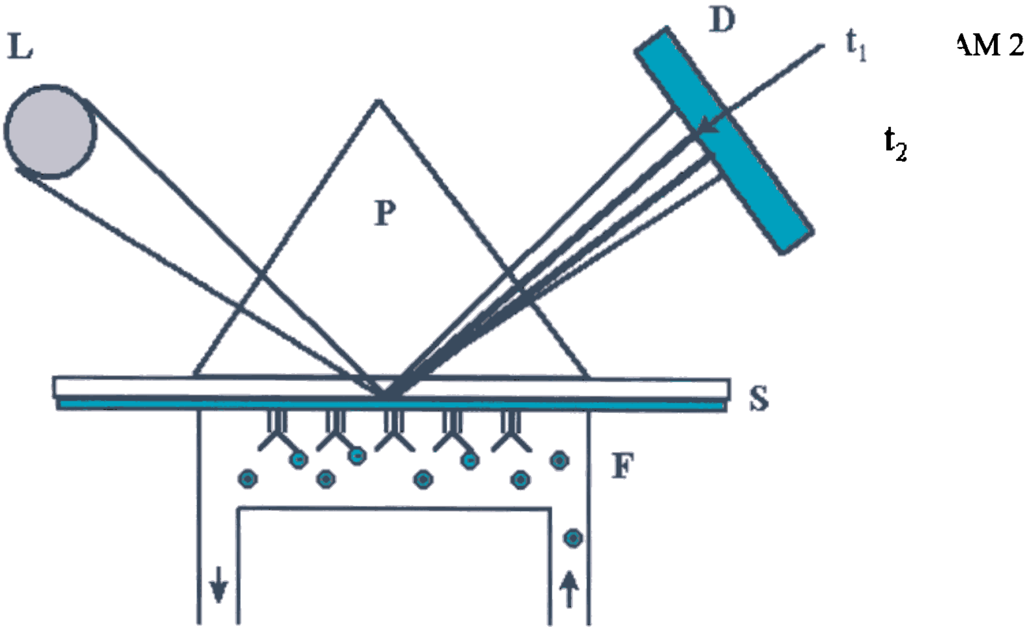

Figure 15 Schematic of a surface plasmon resonance detection unit. L: light source, D: photodiode array, P: prism, S: sensor surface, F: flow cell. The two dark lines in the reflected beam projected on to the detector symbolise the light intensity drop following the resonance phenomenon at time = t_1 and t_2. The line projected at t_1 corresponds to the situation before binding of antigens to the antibodies on the surface and t_2 is the position of resonance after binding. From reference 85.

The light source for SPR is a high efficiency near-infrared light emitting diode that has a fixed range of incident angles. The SPR response is monitored by a fixed array of light sensitive diodes covering the whole wedge of reflected light. The angle at which minimum reflection occurs is detected and converted to the resonance units. The SPR angle depends on several factors, most notably the refractive index into which the evanescent wave propagates on the non-illuminated side of the surface. In addition the other parameters are the wavelength of the incident light and the properties of the metal film. Biomolecular binding events cause changes in the refractive index at the surface layer, which are detected as changes in the SPR signal. In general, the refractive index change for a given change of mass concentration at the surface layer, is practically the same for all proteins and peptides, and is similar for glycoproteins, lipids and nucleic acids.

More recently there has been a great deal of excitement generated by the observation of optical transmission through an array of sub wavelength sized holes[86]. Recent explanations of this anomalously high transmission centres on the excitation of surface plasmons by the incident wave, these plasmons propagating through the metallic conductor, to re-radiate electromagnetic radiation on the opposite side. Such a structure has obvious potential for biological molecular applications.

Surface plasmon excitation by an injected current has not been seriously applied to biological molecules as far as is known, but has already demonstrated the ability for sub-atomic electron-orbital analysis[87]. Typically a scanned probe conducting tip (STM or AFM) is positioned close to a surface feature and current is injected into the surface from the tip. Optical emission is observed, the spectral distribution of which characterises the electronic environment of tip. Arrays of silver filled nanotubes have

SERS (the wavelengths at which silver and gold show very strong enhancements) could be applied to the study of low-concentration compounds in body fluids, blood and tissue and to the SMD of selected dye molecules used for the labelling of biomedically interesting molecules[61].

Recent applications

- Recently[62] it has been shown that the tip of an atomic force microscope (AFM) can be used to selectively produce surface enhanced Raman scattering for localised Raman spectroscopy. The work demonstrates that a conventional AFM tip can provide spatially selective enhancement of a Raman signal using SERS. The AFM tip is acting as a locally introduced nanometer sized 'cluster' from which enhancement mechanisms may operate. Clearly we would be interested in applying this novel technology to biomolecular applications. We are also interested in using AFM as a biotechnology / SMD technique in its own right (see section 3). It seems clear that the even greater tip curvature of a STM tip should enable higher enhancement levels although this has yet to be tested and biological applications have yet to be attempted.

  Recent SERS applications have increasingly involved the use of nanoparticles of gold or silver suspended in solution with target molecules attached[63].
- Attention has also been turned to environmental applications of thiol coated SERS substrates[64] and studies of chemicals in seawater[65].

  The application of SERRS as an analytical tool in forensic science has even been considered[66].

Detectability, measurement and calibration possibilities.

- The aim for the SERS application is towards single molecule detection. This has been achieved in the literature but rarely and not consistently. Enhancement factors of 10^{15} will be routinely required for regular SERS-SMD. At the moment it is not clearly understood what constitutes a good SERS substrates and the production of good substrates is seldom reproducible. The basis of a successful project would be to consistently achieve SERS enhancements of 10^7 and upward. Stimulated emission Raman involving novel substrates is additionally an area of interest.
- Clearly the output measurement parameter is the intensity of the Raman spectrum as a function of the Raman response, measured against wavelength. The variation of input wavelength, intensity and polarisation state may also help to understand the SERS processes.

The clearest opportunities for measurement and calibration standards involve the reproducible manufacture and characterisation of Raman substrates[67] able to enhance signals by a factor of 10^7. Beyond this goal the characterisation of enhanced Raman spectra for bio-entities and eventually single molecules should be an objective.

Measurement parameters would consist of the reflected light intensity (or the transmitted intensity for transmission SNOM) with position as the probe is scanned across the substrate. Measurements may be performed with different wavelengths of incident light.

- The opportunities for measurement and calibration standards would lie in orientational parameters of biomolecules on surfaces and possibly the local absorption and emissivity of dye molecules and characterisation of aperture diameters.

Surface Enhanced Raman (SERS) and Surface Enhanced Resonant Raman (SERRS) Spectroscopy

Surface enhanced Raman scattering (SERS) is a useful technique resulting in strongly increased Raman signals from molecules that have been attached to nanometer (or equivalent) sized structures. Generally, it is agreed that more than one effect contributes to the enhancement effect, referred to as a large effective SERS cross-section.

The enhancement mechanisms are roughly divided into electromagnetic and chemical effects. The electromagnetic enhancement results from enhanced optical fields due to excitation of electromagnetic resonances in the metallic structures (often referred to as surface plasmon resonance (SPR)). An example of the experimental set-up for surface enhanced resonance Raman spectroscopy (SERRS) is shown in *Figure 9*. SERRS occurs when the molecule being probed, for instance a dye molecule, undergoes an electronic transition at the wavelength of the incident radiation. It has advantages over surface enhanced Raman spectroscopy (SERS) in that it does not need a specially prepared rough surface. Molecules adsorbed in close proximity to the surface experience an exceptionally large electromagnetic field. Vibrational modes normal to the surface are most strongly enhanced.

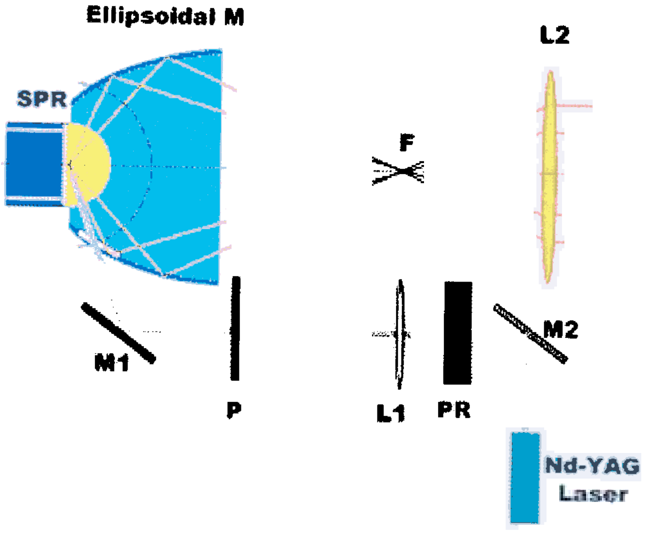

*Figure 9 Surface Plasmons are excited using a hemispherical prism. Raman scattered light is collected with the ellipsoidal mirror (**M**). A Nd-Yag laser is used to excite SP Raman. The laser beam enters the SPR cell through a small rectangular hole in the body of the ellipsoidal mirror. From reference 58.*

The best morphology for surface plasmon resonance excitation is a small (< 100 nm) particle or an atomically rough surface. The greatest enhancements are normally seen biomolecules would also represent an important measurement. Time correlated measurements are known to be awkward[51] and time resolved data may not be obtainable.

The opportunities for calibration and measurement standards lie in the measurement of fluorescence intensities for a variety of biomolecules on the gold surface and the variation of these intensities with surface coverage. These quantities would require measurement for different species and at different concentrations.

Scanning Near-Field Optical Microscopy (SNOM)

Scanning Near-Filed Optical Microscopy (SNOM in Europe and NSOM in North America) is high resolution optical microscopy realised by scanning a small spot of "light" over the specimen and detecting the reflected (or transmitted) light for image formation. This is the only similarity to confocal microscopy, where the focal point is scanned. It is a method for observing local optical properties of a surface that can be smaller than the wavelength of the light used. The SNOM principle relies on optical tunnelling which is a quantum mechanical phenomena resulting from photon delocalisation that allows light to cross propagation barriers such as an interface. Sir Isaac Newton was perhaps the first to experimentally observe and document optical tunnelling[52]. More recently the principle has been used to surpass two theoretical limits of light, namely the "speed limit" and the "far-field diffraction limit."

The resolution of the SNOM image is defined by the size of the aperture. SNOM utilises tiny apertures of a diameter in the range from typically 50 - 100 nm, *i.e.* smaller than half the wavelength of visible light. Typically such apertures are prepared in the metal coating at the apex of an optically transparent, sharp tip. Light cannot pass through such an aperture, however an evanescent field, the optical near-field, protrudes from it. The optical near-field decays exponentially with distance, and is thus only detectable in the immediate vicinity of the tip. The optical resolution limit for SNOM is governed by the light intensity passing through the aperture. A practical limit is usually found with aperture diameters between 80 nm and 200 nm, but in ideal cases even down to < 20 nm. If the aperture is brought in close proximity to a sample surface, the presence of the sample causes a disturbance of the optical near-field, which leads to the emission of light from the location opposite to the aperture. Scanning the aperture at a distance of typically about 10 nm from the sample, and simultaneously detecting either emitted light either in reflection or transmission mode produces a high resolution optical image. In contrast, conventional optical microscopy relies on observation in the so-called far-field and the achievable resolution is therefore limited by diffraction.

SNOM instruments are technically closely related to Scanning Force and Tunnelling Microscopes (SFM and STM), since the probing involves scanning of either the probe tip or the sample, together with a tip/sample distance regulation. This makes SNOM a true Scanning Probe Microscope, which is easily combined with SFM and STM in a single instrument.

Detectability, measurement and calibration possibilities

An upper limit of single molecule detection may be possible. To achieve this will require good equipment design, a very low focal volume and a high quantum yield biomolecule-fluorophore system. A more realistic limit may be the detection and molecular characterisation of the order of 10^3 molecules in the confocal volume.

Measurement parameters would include the concentration of the solutions used and the size of the confocal volume, giving a measure of the number of molecules probed instantaneously. Incident wavelengths and powers are easily varied input parameters and the wavelengths and intensity of the emitted fluorescence as a function of time are important resulting measurements. By measuring the time-correlation of the fluorescence and the input and output polarisation states of the optical radiation, structural and orientational information is available.

The opportunities for calibration and measurement standards lie in the determination of optical quantum yields, fluorescence lifetimes, interaction strengths, bonding lifetimes and preferred orientation configuration for a variety of biomolecules. The determination of fluorescence intensity as a function of concentration of solution is also of importance with respect to biomolecular fluorescence standards.

Evanescent Wave Induced Fluorescence Spectroscopy (EWIFS)

Photon-based surface analysis techniques have become popular in recent years. These spectroscopic methods do not require highly specialised conditions, are generally non-destructive, and are well suited to the study of solid-liquid interfaces under ambient conditions. Of notable merit is evanescent wave induced fluorescence spectroscopy (EWIFS). EWIFS uses total internal reflection of light to generate a surface-specific, electromagnetic disturbance (*an evanescent wave*) at the interface between two dielectric media. The evanescent wave forms in the optically less dense medium, and its electric field amplitude decays exponentially along the axis normal to the interface. The evanescent wave is used to selectively excite fluorophores in the interfacial region (within ~500 nm of the geometric boundary).

Fluorescence spectroscopy is an technique applied to the study of atomic and molecular phenomena. It is used routinely to investigate molecular structure and conformation, molecular motion, energy transfer and solvent rearrangements; due primarily to its unrivalled sensitivity and high information content. Consequently, the coupling of the evanescent wave with the method of fluorescence spectroscopy yields a highly sensitive probe of interfacial environments.

The conventional EWIFS approach relates time-integrated fluorescence information (emission intensities and spectral profiles) to the concentration and conformation of species in the evanescent volume. Even though EWIFS has proved useful in the

APPLIED OPTICAL SPECTROSCOPIES

Fluorescence Correlation Spectroscopy (FCS)

Since the initial experiments in the early 1970s Fluorescence Correlation Spectroscopy (FCS) has been an increasingly used technique, gaining much influence especially in the biological sector. FCS is used in a wide range of experiments involving the temporal dynamics of the fluorescence from molecules in solution. FCS is a particular example of fluctuation correlation techniques, where the laser-induced fluorescence from a very small probe volume is autocorrelated in time. The volume element is defined by the focus of the laser beam, approximately 10^{-15} litres. In terms of single molecule detection the concentration of fluorescent molecule in the sample volume is normally of the order of nano- or picolitres. If no other optical processes influence the fluorescence characteristics the number of photons emitted will depend only on the number of molecules in the sample volume as defined by random particle motion. Under these conditions, occupation numbers will be a few, one or no molecules. A diagrammatic representation of the experimental set-up for confocal FCS is shown below (*Figure 6*).

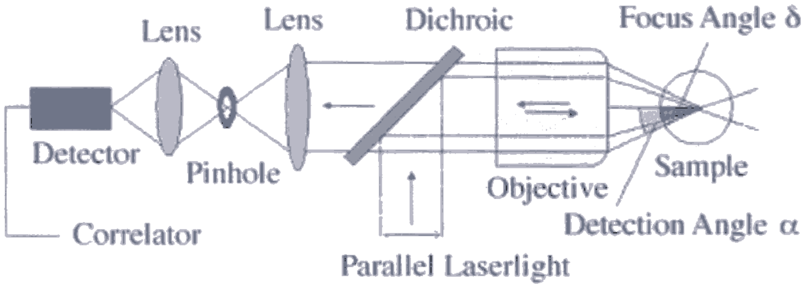

Figure 6 A diagrammatic representation of the experimental set-up for confocal FCS. From reference 41.

Within the confocal volume (which is displayed in ***Figure*** 7), produced by the focussed laser beam, fluorophores will be excited leading to a burst of photons that can be detected by the photomultiplier. Within the system, the fluorophores will move through a small open region and/or undergo transitions between different states with different fluorescent yields. Smaller molecules will move more quickly through the confocal volume than larger molecules. The fluorescence is measured as a function of time and temporal fluctuations in the fluorescence from the volume can be measured. The average duration of a fluorescence fluctuation will decay with time, giving rise to a temporal autocorrelation function. The mechanisms and rates of the processes that cause the decay can be derived from the shape and rate of the decay of the autocorrelation function. The number density of the fluorophores in solution can be calculated from the magnitude of the autocorrelation.

Early experiments in the field led to a variety of results for the electronic properties of DNA. This is now understood to be due to the great variety of DNA sequences, layouts and conditions[31].

- In order to understand the DNA sequence-dependence of the electronic properties the relative energies of the base-pair combination have to be compared[32,33]. This has been done using, computational, photoemission and electrochemical methods.

  Several mechanisms for the transport of electrons through DNA have been proposed[34]. There is experimental evidence for charge-tunnelling and thermal-hopping mechanisms. However there is less experimental evidence to confirm theories involving widely delocalised band structure.

  There has been fundamental work investigating the distance dependence of electron transport within DNA[35] and superconductive behaviour has also been reported recently[36].

  Interest in the electronic properties and reagent specificity of DNA has led to studies of DNA array detection using site specific nanoparticles probes[37], DNA-templated assembly and electrode attachment of conducting silver wires[38] and the self-assembly of two-dimensional DNA crystal[39].

Detectability, measurement and calibration possibilities

- Detection of individual atoms and molecules regularly reported in the literature[40] (see). Imaging is indirect via molecular orbitals. The bonding orbitals are observed if the tip has a negative bias whereas the anti-bonding orbitals are observed if the tip bias is positive. Observation of single biomolecules on suitable surfaces should be achievable. The technique may be carried out in air or moderate vacuum though for the highest *sub-atomic* resolution requires ultra-high vacuum conditions, to avoid rapid build-up of adsorbed molecules.
- One would attempt to measure the position and orientation of molecules on the surface and with a variable bias analysis map out the HOMO and LUMO molecular orbitals of the adsorbed biomolecule.
- Measurement and calibration opportunities appropriate to biological situations would be limited since the technique is, by definition, ex-situ.

  But note an important area which has not yet been widely addressed and which plays to NPL's strengths is the morphological characterisation of biomolecules in well defined in vitro situations. To understand the molecular functionality such studies are necessary before in vivo operation can be properly understood.

species in solution. Analysis of these quantities with position over a biomodified electrode by scanning the modified AFM tip would provide a good indication of the morphology of the substrate in terms of electroactive and non-electroactive areas.

- Opportunities for calibration and measurement standards would lie in the determination of concentrations, diffusion coefficients and electron transfer rates for electro-active biomolecules in solution and a morphological current map of biosamples attached to modified electrodes

Scanning Tunnelling Microscopy

The operating principle of the Scanning Tunnelling Microscope (STM) takes advantage of an electrical current that flows between two conductors (the tip and the substrate) that are separated by very small distances. This is known as the tunnelling current and depends exponentially on the distance between the tip and the substrate. The technique is much like AFM but measures changes in current not force and must be operated *in vacuo*. The STM usually has a higher resolution than AFM techniques. Similarly to the AFM, STM operation has two modes. These modes are constant current (where the tip is moved up and down to maintain a fixed tunnelling current as it is scanned across the surface) and constant height (where the tip is kept at a constant height and changes in tunnelling current are measured). These methods of operation are shown diagrammatically in .

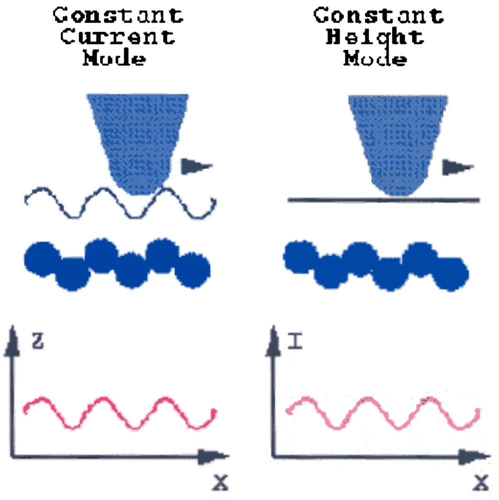

Figure 4 Diagrammatic representations of constant current and constant height modes in STM operation. From reference 21.

Constant height mode is less often used since one runs the risk of crashing into large artefacts as the tip is scanning across the surface. The STM is not restricted just to conducting materials.

Recent Applications

Recently it has been applied to organic systems, ordered monolayers, polypeptides, conducting polymers and biological materials.

are compared with experimental data and excellent agreement is found. The systematic variation of the signal intensity as a function of H~1 and H~m provides a powerful tool to characterize the MRFM apparatus.
Another paper reports the first NMR microimaging and analysis of cell sorting in a three-dimensional aggregate of dissociated hydra cells by using the stimulated echo method together with temperature scheduling of measurement and regeneration phases[15]. The density correlation analysis, transforming the intensity profile to a cell density profile, showed that cell sorting in a natural three-dimensional process proceeds logarithmically with time. Several features leading to regeneration, special for three-dimensional aggregates, were also observed.

Detectability, measurement and calibration possibilities

- The ultimate goal of atomic resolution would seem to be possible using MRFM. In additional total internal characterisation of biomolecules would be available with this technique. Indeed it has long been a dream of the scientific community to have a general purpose imaging technique that can image the three-dimensional structure of a single protein at the atomic level.
- Typical results would comprise 3-D images in space of biomolecules or more likely a chemical shift map of three-dimensional space over the area mapped which could then be interpreted in terms of molecular entities.
  The fluorescence of a single molecule exhibits light and dark periods which reveal the jumping back and forth between the singlet and triplet manifold (quantum jumps). It has been demonstrated[16] that microwave-induced changes to the distribution of the triplet-residence times can be used to detect magnetic-resonance transitions between triplet sublevels and to determine their kinetics. By synchronizing resonant microwave pulses with the quantum jumps of a terylene molecule it proved possible to record transient-nutation signals with a high contrast. This represents another measurement possibility.
- Opportunities for calibration would involve the absolute characterisation of protein form and structure and the more challenging area of the characterisation, interpretation and distribution of the chemical shift of single atoms within molecules.

Scanning Electrochemical Microscopy (SECM)

A common conversion of the AFM in recent times has been to a Scanning Electrochemical Microscope (SECM). This change of usage requires insulating the AFM tip almost completely, leaving the minimum amount possible exposed at the end. This then forms the working electrode in the electrochemical cell. This ultra-microelectrode (UME) may then be used to scan across a surface and the current measured as a function of position to acquire an electrochemically topographic map of the substrate. Obviously the response of the SECM is governed by the mass transport

- The ability to locate and identify a ligand site on a biomolecule attached with a known orientation to a surface represents one of the potentially most significant applications of AFM in this general area.

Magnetic Resonance Force Microscopy (MRFM)

An extension of AFM and one of the most prominent of the emerging nanoelectromechanical systems (NEMS) technologies is magnetic resonance force microscopy (MRFM). The energy separation of useful magnetic sub-levels within a molecule may range from μeV to meV and be detectable using either nuclear or electron spin resonance. In the generic spin resonance technique a quantisation axis is established by an applied external magnetic field (or in the case of a nuclear quadrupole moment by the application of an electric field) followed by an additional oscillatory field orientated orthogonally to the static one, at an angular frequency ω chosen to satisfy the resonance condition

$$\omega = \Delta E / \hbar$$

Where ΔE is the energy separation of the levels (differing by z-component of spin quantum number $|\Delta m_z|=1$). The equalisation of the level populations under this condition changes the static magnetisation (which may be detected by a sensitive magnetometer) or leads to absorption and dissipation of energy (which may be detected by reduction of oscillation level in an r.f circuit or distributed cavity resonator).Like conventional magnetic resonance MRFM uses a uniform radio-frequency field to excite the molecular or atomic magnetic moments or 'spins' into resonance. A nano-magnet provides a magnetic field that varies so strongly in space that the nuclear-resonance condition is satisfied only within a small volume, which can be as small as the size of an atom. This magnet also interacts with the resonant nuclear spins to generate a small back action force that causes the cantilever on which the nanomagnet is mounted to vibrate. For a single resonant nucleus the size of this force is a few attonewtons. A typical MRFM apparatus for molecular imaging is shown below (*Figure 2*).

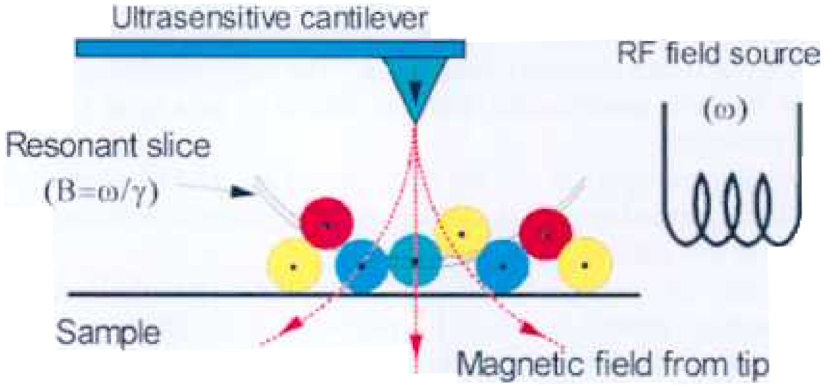

Figure 2 A typical MRFM apparatus for molecular imaging. From reference 8.

SCANNING PROBE MICROSCOPY

Atomic Force Microscopy (AFM)

The fundamental operation of the AFM is displayed in

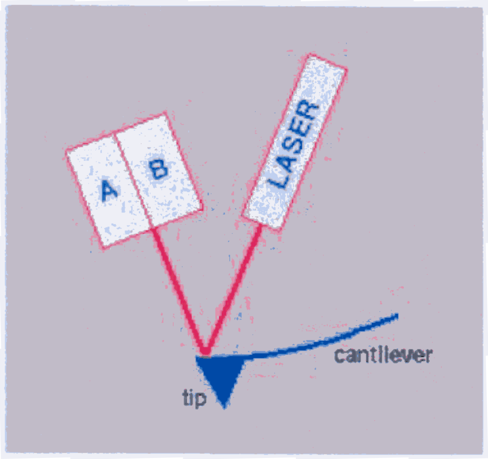

Figure 1 Diagrammatic operation of an AFM. From reference 1.

Basically, the cantilever bends in response to forces between the tip and the sample. This causes changes in the angle of reflection of a probe laser beam off the back of the cantilever and thus a change in the position where the laser beam strikes the detector. In this way the bending of the cantilever may be monitored. The movement of the tip around the sample is performed with an extremely precise positioning mechanism based on piezo-electric motors. The scanner is capable of sub-angstrom resolution in the x-, y- and z- directions. The AFM can be used to examine complex surface morphologies with resolution defined only by the sharpness of the tip. There are two modes of operation commonly employed in AFM studies, constant height and constant force (much like constant height and constant current regimes in STM). In constant force operation the tip always feeds back a constant force and the morphology is derived from the changes in height caused by the z-piezo drive to keep the force constant. Constant height operation requires the tip to be kept still with no z-piezoelectric scanning and the surface morphology is determined from the variation in the bending of the cantilever.

Recent Applications

Biological applications of AFM have been have included examining biomolecules adhered to surfaces to obtain an idea of their orientation and structure. Alternatively small biomolecules may be attached to the AFM tip and then as the tip is brought into contact with another biological surface, for instance a tissue or cell, the force felt by the tip could be measured as a function of distance leading to an idea of the biomolecular interaction.

Recently AFM techniques have been used to examine structural biology of tissues using carbon nanotube AFM probes[2]. To overcome problems of limited spatial resolution a carbon nanotube can be attached to a conventional scanning

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