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In-Vitro Near-Field Ablation of Biological Samples in the Mid-IR Wavelength Region
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As a first step towards the chemical analysis of biological samples with submicron resolution, we report our experiments on the sub-cellular ablation of biological samples in their native environment. This has the potential to combine the near-field IR ablation with mass spectrometry, thus facilitating the study of the spatial distribution of proteins in cellular samples, at sub-cellular length scales. We report the ablation of hard and soft materials: cellular acetate cover slips in water and myoblast cell samples in growth media, with spot sizes as small as 1.5um under 3um wavelength radiation. The ablation threshold and fluence in these processes have been measured. We found that there is a dramatic increase in the ablation threshold fluences when we go from far-field to the near-field region. We will also report on the difference in the ablation mechanism in air and water medium. This approach has the potential to identify the protein expressed in cells in a relatively non-destructive manner.

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Time-Resolved Single Molecule Microscopy Coupled with Atomic Force Microscopy
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The combination of atomic force microscopy (AFM) with single-molecule-sensitive confocal fluorescence microscopy enables a fascinating investigation into the structure, dynamics and interactions of single biomolecules or their assemblies. AFM reveals the structure of macromolecular complexes with nanometer resolution, while fluorescence can facilitate the identification of their constituent parts. In addition, nanophotonic effects, such as fluorescence quenching or enhancement due to the AFM tip, can be used to increase the optical resolution beyond the diffraction limit.
Here, we present a straight forward combination of a single-molecule sensitive, time-resolved confocal microscope with different commercially available atomic force microscopes (AFM). A sample scanning atomic force microscope is mounted onto an objective scanning confocal fluorescence lifetime microscope. The ability to move the sample and objective independently allows for precise alignment of AFM probe and laser focus with an accuracy down to a few nanometers. Time-correlated single photon counting (TCSPC) based confocal detection gives us the opportunity to measure simultaneously intensity fluctuations and fluorescence lifetimes down to the single molecule level. This enables studies of molecular complexes in the vicinity of an AFM probe on a level that has yet to be achieved. With these setups we obtained single molecule sensitivity in the AFM topography and fluorescence lifetime imaging of various samples. For instance, we show silicon tip induced single molecule quenching on organic fluorophores leading to imaging features far below the optical diffraction limit.

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Single Fibronectin Type III Module Desorption from Hydrophilic and Hydrophobic Surfaces: Study by Single Molecule Force and Raman Spectroscopy
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A central dogma in biology is that protein structure dictates function; hence structural changes upon adsorption to a surface should change protein function. This report tests the hypothesis that a fibronectin domain (FNIII10) is denatured by a hydrophobic surface. A single molecule mechanical unfolding method was developed wherein protein was tethered to a hydrophobic or hydrophilic surface at either its C- or its N-terminus and pulled from the other end by a soft cantilever probe. Force transitions that occur at the position corresponding to the end of beta-strands are identified with the secondary structure. Because sheering beta-strands gives the greatest resistance to pulling. The transitions may be associated at least half of the time with 2 characteristic protein conformations at the surface. Surface enhanced Raman spectrum of the protein domain on gold nanoparticles confirms the conformation changing of the protein on hydrophobic and hydrophilic surfaces. Identifying protein orientations and denaturation at surfaces should be useful in identifying the loss and retention of protein activity when surface associated, such as in biomolecular diagnostic tests, tissue engineering, as well as natural surface mediated processes, such as amyloid formation.

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Increased Adhesion of Glycated Proteins to Arteriolar Vascular Smooth Muscle Cells as Determined by Atomic Force Microscopy
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Modification of proteins by non-enzymatic glycation occurs in diabetes mellitus, aging and pathological states including renal failure. Glycation reactions span early reversible reactions to irreversibly modified species termed advanced glycation endproducts (AGEs). Long-lived extracellular matrix (ECM) proteins are particularly susceptible to AGE modification. As ECM proteins are known to regulate vascular function through binding with integrins, AGE formation may modify normal interactions between matrix and vascular smooth muscle cells (VSMCs) ultimately impacting vasoregulation. The purpose of this study was to selectively investigate the adhesion between either non-glycated or glycated fibronectin (FN) with VSMCs using Atomic Force Microscopy (AFM). AFM probes were functionalized with FN or glycated FN and cyclically interacted with isolated VSMCs using a force mode nanoindentation protocol. Adhesion was quantified as the rupture force required to disrupt adhesion bonds the formed during contact of the AFM probe with the cell and also as the probability of an adhesive interaction occurring. AFM probes coated with FN alone required 78.6 $\pm$ 19.9 pN (N=5) to disrupt adhesive bonds whereas glycated FN required 49.9 $\pm$ 5.7 pN (N=5). Binding probability was, however, significantly increased for AFM probes coated with glycated FN vs FN alone, 72.9 $\pm$ 3.5 % vs 63.0 $\pm$ 1.6 %, (N=5) respectively. Selectivity for binding to alpha5 integrin was investigated by adding a solubilized function-blocking antibody to the cell bath. Anti-alpha5 antibody significantly reduced binding to FN (14.27%) but had no effect on glycated FN. These results establish that glycation of ECM proteins can significantly affect their adhesive interaction with VSM and integrins and this could in turn lead to altered cell-integrin based regulation of VSM function. (Supp by P01 HL095486 and RO1 HL92241)

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A Method to Resolve Atomic Force Microscopy Feature Definition Issues for Cells Cultured on Nanofibrillar Scaffolds
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Atomic force microscopy is emerging as a new standard technique for nanobiomedical investigation. Even so, AFM remains an under-utilized technique and more importantly an under-developed enabler of significant new biomedical discoveries due to a general problem with inconsistent feature definition. In this work, astrocyte neural cells cultured on nanofibrillar tissue scaffolds that have shown promise for brain and spinal cord injury repair were investigated by AFM. It was inherently not possible to distinguish potentially important cell-scaffold interactions within the dynamic range of conventional AFM height, deflection, amplitude or phase images because the cell processes and edges were on the same spatial order as the background nanofibers, ~100 to 200 nm. We developed a diagnostic method based on analysis of standard AFM section measurements that provided clear guidance for the selection of a combination of image processing techniques to extract boundary information actually contained within the AFM images all along. The diagnostic conclusions were that the combination of dynamic range enhancement with low frequency component suppression would enhance feature definition of cellular edges and process relative to nanofibrillar tissue scaffolds. Implementation of this combination resulted in clear images of cellular processes and edges on the nanofibrillar surfaces. The clear images revealed previously unrecognized cell-cell interactions and provided new information for ongoing investigations of why cells cultured on nanofibrillar surfaces seem more biomimetic. The same diagnostic method was successfully applied to cerebellar granular neurons cultured on nanofibrillar surfaces, and would be useful in similar investigations, e.g., cardiomyocytes on nanofibrillar surfaces. The methods developed here can therefore extend the usefulness of AFM nanoscale imaging in regenerative medicine.

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The Effects of Contact Time on the Adhesive Properties of an Established Bacterial Biofilm
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Biofilms are complex aggregations of microbes formed at interfaces, and once established can be difficult to eradicate. Often, biofilms come into contact with clean surfaces and transfer cells causing contamination. We use the atomic force microscope (AFM) to study the effects of contact time on the adhesive interactions between an established biofilm and clean surfaces. AFM cantilevers are modified with living, Gram-negative bacteria biofilms, allowing us to leave the biofilm in prolonged contact with a substrate. As the cantilever retracts from the surface,