The combined application of AFM and LSCM: Changing the way we look at innate immunity

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The paradigm for how we approach scientific research is rapidly changing and currently reflects not only the advances in equipment but also the interdisciplinary nature of the research itself. With a special focus on immunological research, we demonstrate how this evolution has impacted the life sciences. In particular, we describe the integration and application of two different imaging technologies, the Atomic Force Microscope (AFM) and Laser Scanning Confocal Microscope (LSCM). Interfacing AFM and confocal microscopy makes it possible to directly correlate high-resolution surface features with specific fluorescently tagged molecules thus, generating a better understanding of receptor-ligand interactions as well as surface feature analysis, measurements that can now be made in real-time. Advantages, disadvantages and technological challenges also will be described. We expect the combination of these complementary techniques will continue to advance and provide significant methods to investigate complex biological system in a non-invasive way.

Keywords Atomic Force Microscopy; Laser Scanning Confocal Microscopy; AFM; LSCM; innate immunity; mammalian cells

1. Introduction

The increasingly complex problems that are now being faced in the biological sciences and in particular immunology, have by necessity resulted in the development of new technologies and ideas. Specifically, analytical techniques such as AFM and LSCM have played a significant role in discovery as evidenced by their inclusion in an increasing number of scientific publications. Furthermore, the recent combined use of these techniques is now being applied to a diverse range of interdisciplinary scientific research areas, including biological engineering and biophysics. The combined application is contributing to a greater understanding of dynamic processes for example the heterogeneous nature of biological membranes under physiological conditions [1], organization of protein assemblies essential to protein signaling [2], antigen-antibody binding events [3] investigation of lipid bilayers [4], characterization of bacterial cells [5] and mammalian cells [6], to name just a few.

Innate immunology serves as an excellent example to highlight the potential of AFM/LSCM. It has been shown that the innate immune system is an evolutionally conserved system capable of providing the first line of defense against pathogens. Like many other systems, there is increasing evidence that immune cells are in constant communication with the surrounding environment via transmembrane receptors and/or sensors. These interactions with surrounding tissues, present in different organs, are thought to be able to pass messages from the outside of the cell to within and by doing so, shape the immune response making it specific to the site of activation [7-9]. This constant communication between innate immune cells and tissues, through the formation of stable receptor-ligand complexes, is also thought to play a role in shaping and regulating the adaptive immune response [10,11]. The conformation and stability of these complexes are thought to behave like a “molecular switch” and be important in the correct signalling into the cell [12]. Therefore this system must be tightly regulated to prevent inappropriate signalling, indicating that the innate immune response is more specialized than

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initially thought [13]. The mapping of the events essential in host defense is technically very challenging and requires sophisticated methods to decipher.

2. AFM applications in innate immune research:

Like many areas of the life sciences, there are a wide array of different methodologies applied to the investigation of immunology. As such, research ranges from clinical studies to using molecular and cell biology. For example, one area of focus includes the micro-environment surrounding immunologically significant cells which is thought to influence the outcome in response to a pathogen. Some of this effect is likely to be determined by mechanical influence on the cells, such as the strength of the receptor-ligand interaction between a cell and the surrounding tissue [14] as well as the role of membrane lipids in shaping the responses [15]. Therefore, the investigation into the mechanical properties is important and complements previous research techniques in providing a more complete picture of the state of an immune response to pathogens.

The AFM is rapidly emerging as a valuable research tool in the biological sciences. It operates by scanning a sharp tip across the sample surface (Fig. 1). The movement of the tip is generally tracked optically using a laser that is reflected onto a photomultiplier tube (PMT). The movement of the laser is then converted into a three-dimensional (3D) view of the sample topography which is an accurate representation of the sample in the z direction as well as the other axes. Using information generated from the tips movements across the surface, other features can be determined; such as regions of hardness and softness via phase imaging. An example of this application is observed in the paper by Savigny *et al* [16]. Their research highlights the ability of AFM to obtain topographical data of cell membranes and relate this information to the different mechanisms that occur during exocytosis in living cells [16]. AFM can also be used to map forces including mechanical, Van der Waals, capillary, electrostatic and magnetic. This method of mapping mechanical properties of cells has been used to investigate cell rheological properties of neutrophils. Research carried out by Yanai *et al* [17] and Roca-Cusachs *et al* [18] demonstrated possible mechanisms of neutrophil locomotion [17] and activation [18]. An interesting example where AFM has added to our current understanding of different disease states is its application in the investigation of protein misfolding [19]. This research illustrated that the formation of ion channel structures caused premature cell death as well as played an important role in physiological

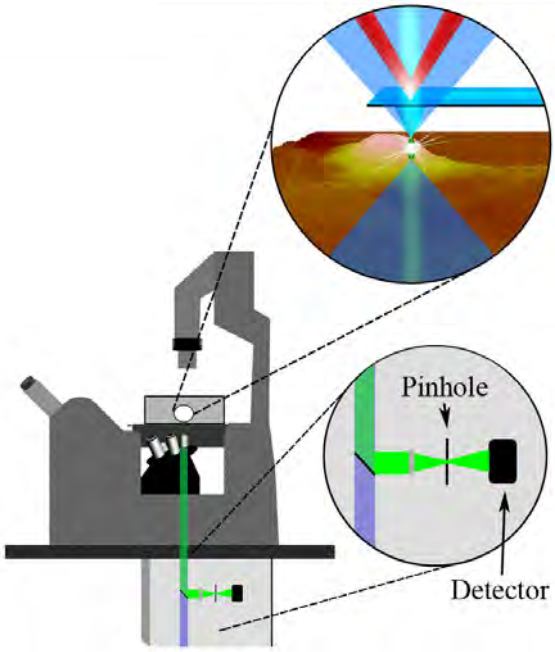

Fig. 1 Simplified schematic representation of a combined AFM and LSCM system. The AFM is made up of a scanner that is held stationary, whilst the sample (in this case a cell growing on a coverslip) is moved relative to the tip. The AFM is scanner located at the sample stage above the objective lens turret. The **top right inset** illustrates the blue laser beam focused to a diffraction-limited point aligned with the sharp AFM cantilever tip. Some fluorescence emitted from the sample as a result of confocal laser excitation will be collected by the objective lens and redirected back into the confocal scanhead located below the microscope. The **lower right inset** shows the green fluorescence signal (i.e. GFP or Q-dot) being refocused to a point at a pinhole aperture and subsequently collected by a PMT or APD detector.

states including cell volume and mechanics [19]. AFM has also been applied to the investigation of protein conformational states and the mapping of surface ion channels in bilayer membranes as well as the effects of electric potential on these channels [20].

The measurement of forces i.e. force mapping, can be further extended by the use of chemically functionalized tips, allowing the attachment of molecules to measure single molecular events [21], to the attachment of whole cells to measure more complex interactions [22,23]. The ongoing development of specialized AFM tips has resulted in the improved capability to more accurately measure tiny forces present between immunological receptors or molecules. AFM tips that are commonly used have spring constants ranging from approximately 0.02N/m to 50N/m and can be applied to investigate forces between molecules that can range from a few piconewtons to hundreds of micronewtons. Examples of important interactions that have been investigated using AFM are numerous and range from single molecule interactions e.g. DNA strands and peptides [24], receptor-ligand interactions [22], to those present in whole cells [23].

The most noticeable limitation of current AFM's is the time it takes to generate an image, making the capture of some dynamic biological processes presently unobtainable. In addition, and as a result of slower scan rates, thermal drift can be problematic which often requires specific software to filter this effect out. Efforts are underway to rectify these problems, involving both instrument modifications as well as specialized tip production. Resolution is generally considered a strength of AFM's, however, some of the resolution limits directly are attributed to cantilever fluctuations. To address this, the production of smaller cantilevers that dissipate less through liquids, are expected to result in fewer fluctuations [25]. Another important consideration is the shape of the tip and specifically its radius of curvature. It is a key component in the resolution of the image, something that can be overlooked when deciding on the best way to obtain the information in question. The tip geometry can also limit image resolution as well as introduce a number of other artifacts. Finally, hysteresis of the piezo material used to create the scanner as well as cross-talk between the x,y,z axes are important effects when imaging with an AFM. The development of piezo stacks or using alternative materials has reduced these problems. Imaging biological materials is complex as the forces, present at the surface due to repeat scanning, can physically disrupt the sample. The ability to take high-resolution images e.g. 1000 by 1000 pixels or greater and use the software to zoom into an area of interest means that the sample can be imaged once and multiple features can be analyzed from this one image. This ability to collect a high number of data points from either an image or a force curve naturally will allow a greater understanding of the sample being investigated.

3. Confocal applications in innate immune research:

Like AFM, the prospect for understanding biological phenomena at the cellular and subcellular level using confocal microscopy has never been better. Since its invention in 1955 by Marvin Minsky [26], confocal microscopy has transformed our ability to image the cellular mellieu within fixed or living organisms. This is in large part due to the confocal microscopes ability to reject out-of-focus light from the final image, resulting in a high resolution, high contrast, non-invasive optical slice from a specimen. There are many variations on how a confocal image can be obtained (i.e., spinning disk, line and point scanning), but the most common is a point scanning system which uses a laser beam focused by an objective lens to a small diffraction limited spot within a sample. Any fluorescence or reflected light that is generated at that spot returning through the objective lens is refocused to a conjugate focal plane with a pinhole aperture (Fig. 1). It is this aperture that imparts "confocality" to the image by blocking the out-of-focus light. Any in focus signal that makes it through the aperture is detected by an avalanche photodiode (APD) or photo-multiplier tube (PMT) detector. Linear mirror galvanometers are used to raster scan the laser spot point-by-point across the sample in the objective focal plane and the resulting data is displayed as a digital image on a computer screen. Since a comprehensive account of the theory and principals of confocal microscopy are beyond the scope of this review, readers are referred to the *Handbook of Biological Confocal Microscopy* [27] for more detail. This informative tome includes relevant topics of not only confocal microscopy, but also other optical sectioning methods such as

deconvolution and multiphoton microscopy, that can also potentially be used in conjunction with AFM as described herein.

The power of LSCM is not strictly limited to acquiring discrete optical sections. With motorized or piezo focus control, optical sections can be acquired along the z-axis to yield full 3D volumes of data. In addition, powerful techniques such as fluorescence recovery after photobleaching (FRAP)[28], photo-activation [29], uncaging [30], fluorescence resonance energy transfer (FRET) [31], fluorescence lifetime imaging microscopy (FLIM) [31], fluorescence correlation spectroscopy (FCS) [4] four-dimensional data acquisition [32] and spectral imaging [33] can all be utilized as informative tools for understanding bio-molecular and cellular behavior. In fact, the value of many of these techniques can be directly attributed to the development of a broad array of vital fluorescent probes which can be used to identify or track specific chemical information within fixed or living cells. The discovery and exploitation of fluorescent proteins [34], when expressed genetically as a fusion protein, permit visualization of any number of cellular targets and has been especially transformative in the field of fluorescence microscopy. Finally, and of no less significance, the rapid progression in computer, laser and other hardware technologies also have clearly played a critical role in the utility of confocal microscopy to the life sciences.

The universal nature of innate immunity as a generic mechanism of host defense against pathogens provides numerous opportunities for optical microscopy, including LSCM. Specifically, confocal microscopy has demonstrated its utility as a mainstream and essential tool for immunological research. One straightforward example illustrating this point, includes a binding and phagocytosis assays with lysosome and/or actin-labeled macrophage following incubation with GFP-tagged E. coli in fixed and living cells [35] LSCM also provided a valuable 3D perspective of human alveolar macrophage phagocytosis of Cy 3-tagged *Bacillus anthracis* spores [36]. Of course, confocal microscopy is not strictly limited to *in vitro* systems. For example, a recent study of plague-like disease caused by the pathogen *Francisella tularensis* showed that ASC and Caspase-1 play an important role in the innate immune response [37]. In this work, confocal microscopy was used to localize the pathogen in spleen sections as well as DNA damage via the TUNEL assay in cultured cells. Without doubt, the application of LSCM for immunological research is considerable and certainly not limited to the few examples provided above. In many respects the experimental potential is hindered simply by equipment access and the imagination.

Like any analytical tool, LSCM is not a panacea and has several noteworthy limitations. Axial and lateral resolution, albeit superior to conventional fluorescence microscopes, are low (a few hundred nanometers) when compared to AFM and electron microscopy. The depth within a sample an image can be obtained is limited by optical considerations such as spherical aberration, light scattering and adsorptive properties of the sample and the objective lens working distance. With living cells, photo- and cytotoxic effects, photobleaching and environmental control must be considered. The rate of image acquisition for a single unaveraged frame of a typical point scanning system with 512x512 pixel resolution is ~ 1 second, but one minute or more is not uncommon when signals are low and averaging required. This, of course, still compares favourably to most AFM image capture rates which can take at least several minutes and often much longer. Finally, high-end LSCM's can be very expensive, especially, if near-infrared or ultraviolet lasers are required.

4. Combined application of AFM and confocal systems:

The combined application of AFM and confocal systems presents the opportunity to investigate both topographical data from the AFM with the corresponding chemical and often internal details obtained when using the light microscopy [38, 39]. The ability to both image at high resolution, a strength of the AFM, combined with the ability to unambiguously identify structures, a strength of the confocal microscope, presents an improved way of investigating an array of different samples. The integration of these techniques presents the easiest way of creating a microscopy system with both functionalities. The challenges of integration range from positioning of the sample to laser light used for detection of tip movement by the confocal system. Figure 1 represents a generalized solution used for a combined AFM-confocal system which is readily suited for an inverted light microscope with the AFM being located

above the objective lens at the sample stage and the LSCM at either a side or base camera port. There are generally two types of AFM's that are used in conjunction with the confocal microscope: Those that scan the tip across the surface and those that move the sample beneath a stationary tip. Systems that move the tip relative to the stationary sample would require temporal and positional synchronization of the galvonometer directed LSCM excitation beam with the AFM tip, while scanning stage systems simply require the tip to be parked directly over the center of the objective at the focal point of the objective. Noy, 2003 [38] estimated the alignment, hence image mismatch, of his stage scanning system was within 150nm and could be improved through an iterative process. The continued development of both instrumentation and software for alignment of the AFM tip with the focused confocal laser beam will allow for greater correlation accuracy of the sample being imaged. Fortunately, until this is a standard AFM/LSCM feature a great deal of useful information can still be extracted (Figs. 2A and B) and when higher level image registration is required, other useful software tools are available (Fig. 2C).

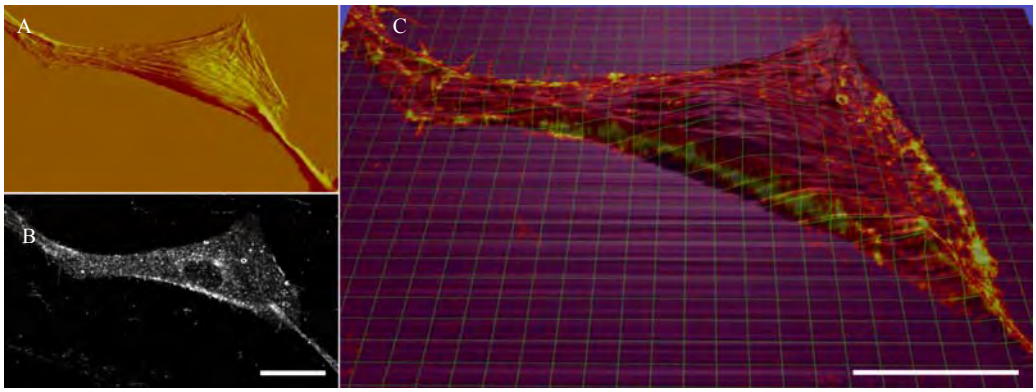

Fig. 2 High-resolution image generated by the combined AFM and LSCM of living MC3T3 osteoblast cells. **A.** Deflection error image generated by the AFM, **B.** The same cell as Figure 2A labeled with FM4-64X membrane dye imaged by LSCM. This dataset highlights the sites of staining of cell membranes as well as cell membrane topography. **C.** This correlated image of Figures 2A and B generated using the nanoManipulator software developed by the CISMM NIH NIBIB Resource at University of North Carolina at Chapel Hill (P41-EB002025-21A1). Figure C kindly created by Dr Russell M Taylor II from University of North Carolina at Chapel Hill. Figures 2A and B generated on a Zeiss LSM 510 NLO LSCM equipped with a Veeco Bioscope II AFM. Scale Bar = 30 microns.

The ability to relate topographical features to known chemical composition of surfaces, as illustrated above, means that the colocalization of the molecules can now be investigated to a greater extent. The combined use of AFM and LCSM systems has also meant that the mechanical loading of cells can be followed from the initial loading of a cell. The following images (Figs. 3A and B) represent an example if its application. This concept is important in many biological events from the immune response to the transmission of messages' between non-immune cells e.g. osteoblast cells as illustrated in Figure 2. Furthermore this method of measuring rheological responses of cells in the presence or absence of different stimuli can be extended to include the mapping of forces over the entire cell. By doing so it is possible to investigate specific topographical and rheological changes of the cell membrane in response to a stimuli. The combined used of AFM and LCSM allows for the use of specific fluorescent labels to different structures of the cell, so that the locationalization of specific responses both at the cell membrane and within the cell can be followed. As shown in Figure 3 multiple structures can be labeled at once. Two dyes were used in this case, these being, the membrane dye FM4-64X, a dye that inserts itself into the outer leaflet of the cell membrane used to investigate cell membranes structures as well as vesiculation events, and the nuclear dye DAPI, a nuclear and chromsome stain that binds to AT regions of DNA. The use of multiple dyes allows us to follow dynamic processes both outside and within a cell in response to a mechanical stimuli over time in live and fixed cells.

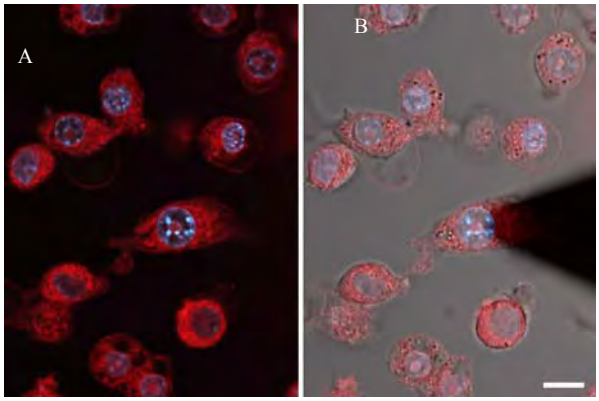

Fig. 3 Membrane and nuclear-labeled RAW 264 macrophage cells imaged by AFM and confocal. **A.** Confocal image of cells labeled with DAPI (blue) and FM4-64X (red). **B.** Same image as Fig. 3A with transmitted light overlay showing triangular cantilever positioned over cell. Figs. 2A and B generated on a Zeiss LSM 510 DUO LSCM equipped with a Veeco Bioscope II AFM. Scale Bar = 10 microns.

Furthermore, the ability to chemically modify the tip allows the addition of a fluorescently tagged molecules, such as the addition of Qdots as shown in Figure 5 to investigate fluorescently tagged receptor or ligands. The proximity for these molecules to membrane-expressed molecules can be determined using single molecule FRET analysis. This ability to bring two ligands together and determine the effect specifically is only made possible by the integration of these two systems. An example of this application is the investigation of macrophage responses to a number of different pathogens or pathogen associated molecular patterns (PAMPs). Macrophages play an important role in the innate immune response, so an important step in understanding these responses is to investigate how they respond to a range of different stimuli from both pathogens and surrounding tissue. As the tip is functionalised binding events between receptors and ligands can be related to specific regions of the cell membrane.

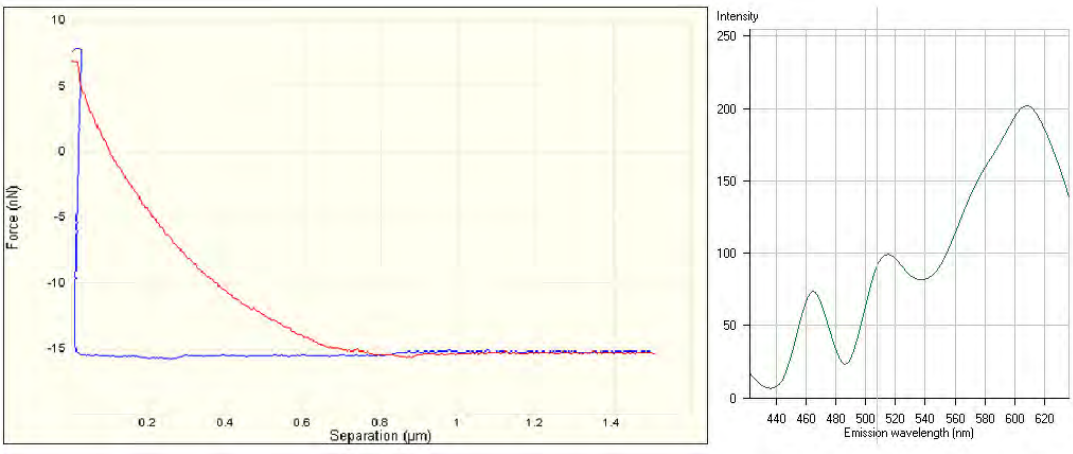

Fig. 4 Correlative Physical (force distance curve) and chemical (spectral) data can be acquired. **A.** Representative force curves showing force measured off a cell (red) and a control curve measured off the substrate (blue), used to determined rheology of the cell. **B.** Spectral fingerprint at tip region of same cell located in Figure 3B, This spectrum indicates, as expected, that at the location of the tip, FM4-64X with an emission maximum of approximately 610nm dominates the signal. Figure 4 data generated on a Zeiss LSM 510 DUO LSCM (with META spectral detector) equipped with a Veeco Bioscope II AFM.

This experiment presented in Figure 3 can also potentially be extended for precise force distance measurements as well as spectral information (Figs. 4A and B) and raman confocal [39] on a pixel by pixel basis. Investigation into biological pathways is an important area of immunological research. The ability of AFM to bring into contact a fluorescently labeled modified tip with a sample or even apply forces to a sample provide ways of determining the events that immediately follows these interactions. Events like calcium fluxes, important to many cell systems, can be followed using the high-speed

confocal system. The integration of images, speed the AFM scans and the speed the confocal scans are important considerations when planning these experiments.

Many reported configurations, whether built in an individual lab or commercially available use a similar footprint. Although there are still relatively few reports using integrated light microscopes in general with an AFM the diversity of the research projects explored is a testament to the advantages of this combination. Although not confocal, Madl *et al* [40] used conventional fluorescence and differential interference contrast (DIC) to position and correlate an antibody functionalized AFM tip on live and fixed cells expressing SRBI receptor clusters for force extension measurements. Sharma *et al* [6], performed live cell experiments with confocal and phase contrast imaging to monitor actin changes in the zebrafish stratified epithelium during wound healing. Another group [41] implemented a more sophisticated approach combining single molecule spectral and confocal capabilities with the topographic AFM features of *Rhodobacter* light-harvesting complexes. Single molecule detection via AFM with LSCM, FCS [42], total internal reflection fluorescence (TIRF) [4] and near field scanning optical microscopy (NSOM) [5] was also reported and reviewed [43] previously by several other groups. NSOM, in particular is a closely related technique to AFM. Briefly, the microscope operates using a sharpened optical fiber that is metal coated so that a small aperture (approximately 25nm diameter i.e. sub-wavelength aperture) at the tip of the fiber. The aperture is used to illuminate a smaller area than conventional diffraction limit, allowing for more than an order of magnitude improvement over conventional optical methods, including LCSM. The image is scanned under the tip to generate an image in a similar way to AFMs. NSOM presents an alternative method for investigating complex biological systems [44].

5. Future applications:

The combined approach of AFM and confocal microscopy systems is in its infancy, however early applications suggest it will have an important impact in a number of research areas ranging from biological sciences, biological engineering to material sciences. The ability to resolve and identify structures within a complex mixture of components is an important step in determining the precise role of different molecules within the system. The continued development of quantum dots that can be functionalized (Fig. 6) to investigate different components as well as a complex array of conjugated molecules [45, 46], can be used as beacons to identify specific and individual molecules within a complex environment for example like those present in cells. The combined approach has made it possible to start looking at both the size and conformation of optically active compounds, overcoming some of the limitations of the instruments as separate entities. As mentioned previously, one primary limitation of photon-based optical systems is resolution and this is one major motivation to implement correlative AFM and LSCM. Researchers have been able to lower the resolution barrier through techniques such as NSOM, stimulated emission depletion (STED) [47], saturated structured-illumination microscopy (SSIM) [48] and stochastic optical reconstruction microscopy (STORM) [49]. Although these techniques have pushed the optical resolution limits it remains to be seen whether these approaches will be broadly as applicable as AFM and confocal. Furthermore, the continued development of an AFM system capable of operating at physiologically significant rates to capture the early events in the immune response would be invaluable. For example a calcium flux into an activated T cell takes milliseconds. We also anticipate the increased use of carbon nanotubes either unfunctionalized or functionalized with a fluorescently tagged compound to investigate specific structures. Furthermore the development of mathematical models in biology at both a cellular and molecular level, will also add to our understanding of immunological events. We will also see the continued development of confocal pixel-AFM tip movement correlation as these systems continue to be applied to an increasingly diverse range of research areas.

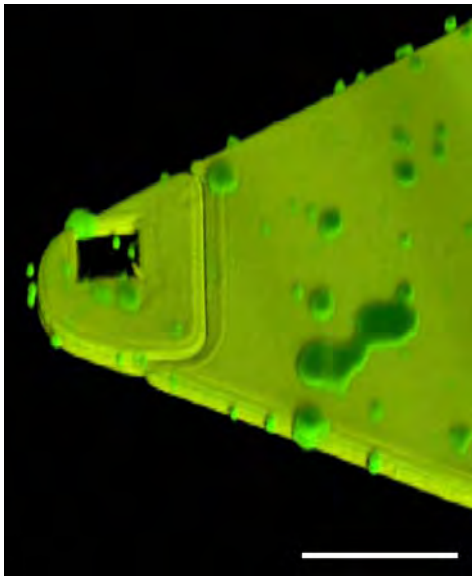

Fig. 5 AFM tip coated with 525nm Qdot nanocrystals. 3D shadow rendering of an AFM cantilever tip region in reflection mode (yellow) following functionalization with 525nm Qdots (green). Due to the steep angle of the AFM tip, the laser light is deflected away from the objective and hence the tip itself (small dark rectangle near end of cantilever) is not visible. However, small foci and larger clusters of green Qdots are observed attached to the cantilever and tip. Qdots have a number of suitable applications in the AFM/LSCM hybrid due to their brightness, and exceptional resistance to photobleaching. Individual functionalized Qdots on AFM tips are more easily visualized than organic dyes due to their unique properties. Fig. 5 generated on a Zeiss LSM 510 VIS LSCM. Scale Bar = 10 microns.

6. Summary:

The advantage of acquiring multiple measurements on a single sample has the distinct advantage of avoiding different sample preparation conditions often required for diverse analytical techniques. Like many biological processes, the immune response to pathogens is a complex integration of many components, as noted by Matzinger [7] we need "to measure as many of them as we can and to check those measurements in as many ways as possible". To expand our current understanding "we should not stick with simplistic combinations of measures we have tended to use thus far"[7].
Technological and sample specific problems are still present with these techniques, like any other available method. Rigorous evaluation of potential problems must be considered in order to minimize their effects. At the current pace of equipment development, we eagerly await the removal of barriers that will carry us to the next level of scientific exploration. Namely but not limited to high-speed AFM, lower noise systems improved LSCM/AFM integration, more sensitive scanning AFM's and development of computer models to better understand results from these experiments. Because we are looking at a single sample using several techniques at the same time, we can eliminate variability given by making measurements on different samples under different conditions.

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