Single Molecule Detection (SMD)

Single Molecule Incentive

The ultimate limit to analytical sensitivity is the reliable detection of single molecules. Recent technical advances in optical detection and manipulation have made the detection of isolated, light emitting probe molecules a reality. Thus we are witnessing a burgeoning interest in the imaging and spectroscopy of single molecules, particularly within the fields of cell biology and drug discovery.

Of particular importance to biology is the possibility of direct, real-time visualization of single biological macromolecules and their assemblies under native physiological conditions, offering great promise for enhancing our understanding of the behaviour, interactions and trafficking of individual biological macromolecules within the living cell. Such studies have increased medical and pharmaceutical significance within the developing post-genomic era of proteomics, providing the means to track the behaviour and mechanistic roles of biochemically relevant single proteins.

It is reasonable to ask why it is interesting to observe the emission from a single fluorophore, since numerous fluorophore labels can be detected within a diffraction-limited spot. There was a time when the scientific community was content with detecting the combined signal of a vast number of fluorescent (or Raman active) molecules from a point of interest within the sample. There is a rapidly growing realization however, particularly within the life science and materials science fields, that the information content afforded by imaging the single fluorophore markedly exceeds that offered by the "bulk" ensemble measurement, yielding invaluable insight into individual molecular properties and their micro-environment.

Experiments on single molecules have attracted vivid interest in many branches of fundamental research because they allow for the study of molecular properties normally disguised in inhomogeneous distributions of an ensemble. In contrast to observing the position of several labeled molecules at a given position, single molecule detection provides additional information, such as:

- Polarization or image spot shapes indicate orientation and reorientation.
- Time traces of intensity, emission spectrum, or fluorescence lifetime provide information on local dynamics and diffusion.
- Fluorescence Resonance Energy Transfer (FRET) provides information on the proximity of specific labeled sites less than 10nm apart.
- Position sensitivity allows an investigator to locate a molecule and follow simultaneously the translational motion, re-orientational motion, and the internal dynamics of the individual molecules.

Single molecule studies are uniquely poised to yield information about molecular motion, behavior, and fluctuations over time and space. There are many biological molecules that can avail from examination at this level, typical subjects being key members of a system that are receptive to specific cellular signals, environmental perturbations or drug intervention. Cellular mechanisms that have been examined include ion channel activity, protein folding, enzyme activity, membrane structure, molecular motors/motility and vesicle transport. Single molecule detection is a way to study detailed physical and chemical properties that allows for scrutiny of fundamental principles and mechanisms, and may lead to technological and methodological developments.

Single molecule techniques also have key potential in material development. The single molecule is an excellent probe of local (nanoscale) properties since it is a quantum light source with spectrum and lifetime that is sensitive to its chemical and physical environment. The rotational and translational motion of single molecules can be measured and used to understand the local mechanical properties of the material in which the molecules are embedded. Thus groups are seeking to understand and catalogue the great variety of behaviours of single molecules in technologically relevant environments to better use these probes to study the small-scale structure.

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Parallel Detection by Camera

One of the compelling aspects of single molecule studies is the ability to directly observe distributions of individual properties and molecular behaviour. Obtaining distributions of such parameters requires studying a large number of individual molecules. Single molecule microscopy using camera detection has the capability to effectively investigate many spatially separated individual molecules in a parallel fashion.

Such an approach can make for more efficient experiments and ensure that the molecules are imaged under identical conditions. Specific statistical distributions of properties can be determined which contain more detailed information than the ensemble-averaged mean values obtainable by bulk measurements

From an analytical perspective, single molecule measurements have ultimate sensitivity, opening a new era of femtomolar chemistry, by which biological reactions and high-throughput screening can be carried out with minute samples, which are normally insufficient for measurement purposes.

One area of particular commercial vibrancy is the drive towards extremely efficient whole genome sequencing using single molecule approaches. The ultimate goal here is to make available the capability to screen the entire 3 billion bases of the human genome within 24 hours, for less than $1000!

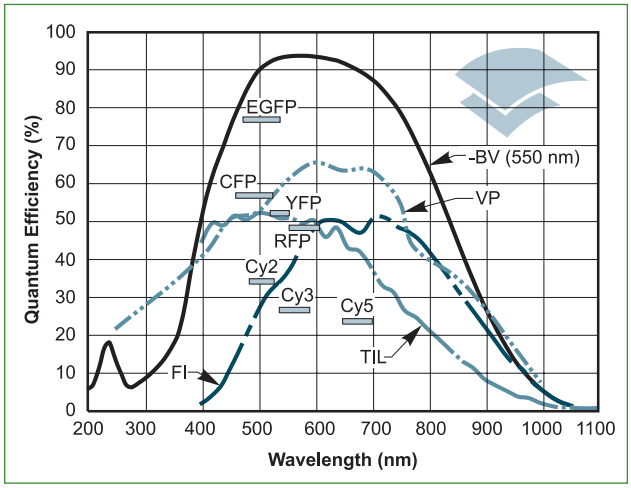


Figure 1 - Quantum Efficiency and Fluorescent Dyes relevant to Single Molecule Detection

Camera-Based SMD Techniques

The continually improving technologies of modern lasers, ultrasensitive detectors, analysis software and novel optical configurations, permit detection and investigation of single molecule fluorescence images, trajectories, spectra, and lifetimes.

Some of the camera-based techniques adaptable to single molecule detection are listed below, their suitability depending more on the specific system under study:

- Confocal Raman Microscopy
- HyperSpectral Imaging
- Spinning Disk Confocal Laser Scanning Microscopy
- Total Internal Reflectance Fluorescence Microscopy (TIRFM)
- Widefield Epifluorescence Microscopy

Background Photon Noise - The Need to Minimize

Background photon noise level must be low compared to the signal level in order to allow discrimination of single molecules. This is usually the most serious limitation to sensitive detection. Just as stars can be seen at night but are obscured by sunlight scattered from our atmosphere during the day, the key to SMD is to minimize background, which includes fluorescence or Rayleigh/Raman scattering of the bulk medium containing the molecule of interest.

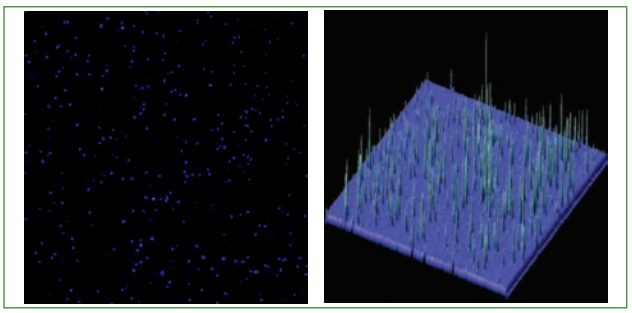


Figure 2 - Single Cy3 molecules immobilized on a glass surface, imaged by objective-type TIRFM using the Andor iXon^{EM}+ DU-897 back-illuminated EMCCD; exposure time 30ms.

This is normally realized by some combination of spectral, temporal or spatial filtering. Spatial discrimination, has the effect of increasing the apparent concentration of the molecule of interest - one molecule confined to a 1μL and a 1fL volume has effective concentrations of 10^{-18} M and 10^{-9}M respectively.

The proportion of the total signal that originates from the molecule can thus be increased significantly by miniaturization. Spatial discrimination also helps to isolate single molecules, as the probability of finding more than one molecule in the same volume element (for a given bulk concentration) decreases with a decreasing volume.

TIRFM

A relatively straightforward way to limit the observation volume is to excite the molecules by Total Internal Reflection (TIR) illumination. TIRFM makes use of an optical effect that can be adapted to observe fluorescent events occurring at the interface between two optical media of different refractive indices. Excitation light incident upon such a boundary, travelling at an angle greater than the critical angle, undergoes total reflection.

The electromagnetic field of the total internal reflected light extends into the sample beyond the interface, extending only a few hundred nanometres into the second medium of lower refractive index - essentially in the z direction. Furthermore, this Evanescent Field decreases exponentially in intensity along the z-axis of penetration. Only the section of the specimen located within the evanescent field undergoes fluorescence excitation.

TIRFM is limited to the area within a few hundred nanometres of the glass/sample interface, where total internal reflection is occurring. By imaging the surface onto a camera, individual molecules can be registered as each migrates to the proximity of the surface. This imaging method allows the observation of many distinct molecules simultaneously, yielding a high throughput and increased statistical viability.

TIRFM is an increasingly popular technique for visualizing, with high signal to background ratio, single molecule processes that occur in and around the membrane of living cells (partially due to availability of novel membrane-specific fluorophores). In contrast to the spinning disk confocal technique which can yield rapid optical sections of approx. 1μm thickness, the excitation volume of a TIRF evanescence field extends only approx. 100nm into the sample, the intensity of which decays exponentially across this distance. Since only a very thin sliver of excitation is being produced, we only detect photons that are created within that excitation volume, which has the effect of significantly improving signal to background.

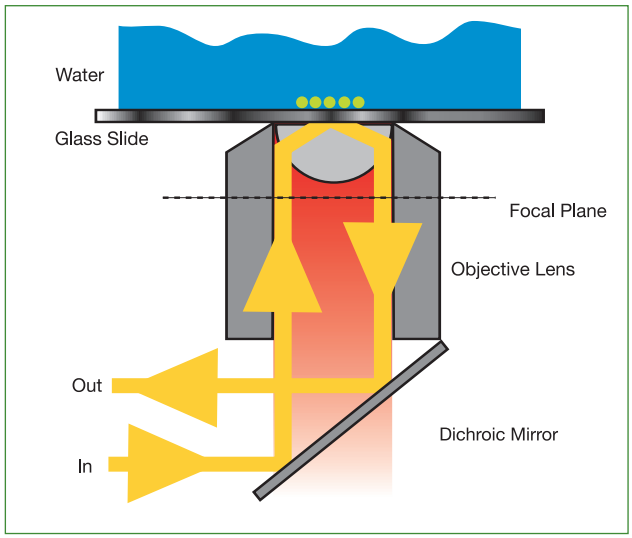


Figure 3 - Illustration of objective-type TIRFM for Single Molecule Detection

Whilst a thin excitation volume of the order of 100nm depth sounds very useful, it must be remembered that due to the nature of the TIRF effect, we cannot step this excitation plane through the volume of the cell. TIRFM is a surface interface phenomenon, and as such is restricted to studying sample that is within 100nm or so of the glass interface of the slide. For events within the bulk volume of the cell, we must rely on widefield epifluorescent or a rapid confocal scanning approach.

Multiple Probes and FRET

It is often of interest to dynamically image multiple different biomolecules, and their interactions within the sample volume. The TIRFM technique can readily be extended to image multiple fluorophores labels, through integration of a multi-line laser system, preferably a solid-state laser solution with Acousto-Optical Tunable Filter (AOTF) modulation.

This technique can be readily adapted for FRET analysis, preferably through integration of a suitable beam splitting device on the emission side.

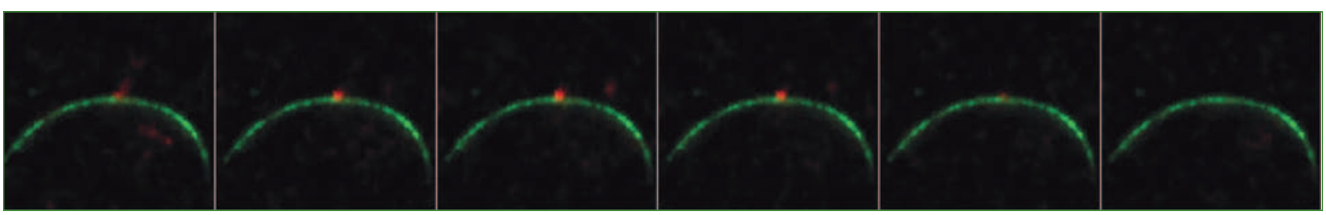


Figure 4 - Transport of a single NTF2-Alexa633 molecule across the nuclear envelope of a biological cell at high time resolution, recorded with the iXon^{EM} DV860 back-illuminated. An image of a HeLa cell nucleus stably expressing the GFP-conjugate of POM121 was acquired in the green channel. This image was overlaid by a movie sequence demonstrating the binding of a single NTF2-Alexa633 molecule to the nuclear envelope, the first step of nucleocytoplasmic transport (red channel). Integration time, 2.5 ms; 400 frame/sec; field size, 18.5x18.5 μm^2. The data were filtered with a 3x3x3 Gaussian kernel in x, y and t, background-subtracted and gamma-adjusted (1.5) before overlay with the green channel. Courtesy of Prof. Dr. Ulrich Kubitscheck, Institut für Physikalische und Theoretische Chemie, Bonn, Germany.

Electron Multiplying CCD (EMCCD) - the Last word in Ultra-Sensitivity

Andor's EMCCD technology is the ideal detector for dynamic single molecule imaging. The extraordinary Signal to Noise (S/N) offered is significantly greater than that afforded by conventional CCD cameras when operated at fast readout speeds. EMCCDs exhibit frame rates that are ideally suited to dynamic acquisition of transient single molecules and their interactions. Since EMCCDs are array detectors, multiple single molecules can be illuminated and imaged in parallel, greatly improving the experimental throughput and statistical viability of SM data.

Underlying all direct imaging studies of living cells or organisms, is the desire to preserve the living subject for as long as possible, through minimization of both phototoxic cell/tissue damage and also photobleaching of the incorporated fluorophores. As such, techniques for studying single molecules benefit markedly from using EMCCD detection technology, permitting the laser or lamp excitation light to be attenuated.

The spectral properties of single molecule systems can be accessed in a number of ways. For example, simply through fast switching between multiple fluorophores excitation/emission, or even splitting of the emission signal simultaneously onto different areas of the sensor. Through minimization of excitation powers, the rates of dye photobleaching and cell phototoxicity are significantly reduced.

iXon^{EM}+ imaging EMCCD platform displays single photon sensitivity combined with high Quantum Efficiency (QE) at multi-MHz rapid readout speeds.

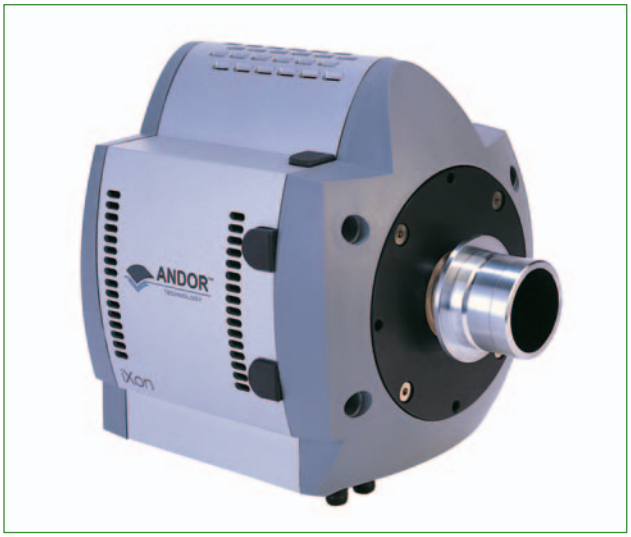
iXon^{EM}+

Andor's pioneering iXon^{EM}+ is a revolutionary range of cameras that provides single photon detection sensitivity, highest QE, and -100°C Thermoelectric (TE) cooling at rapid frame rates, utilizing Andor's award-winning EMCCD technology.

Features & Benefits of the iXon^{EM}+

- EMCCD - single photon sensitivity + high QE (> 90% available)
- RealGain™
- Use lower laser powers: significantly reduce photo-bleaching of single molecules
- Faster frame rates for tracking dynamic events and interactions
- Minimized darkcurrent from unparalleled –100°C TE cooling: and lowest clock - induced - charge

For further information on the iXon^{EM}+ range, please refer to pages 178 -183 of the Products section.



iXon^{EM}+

iXon^{EM}+ models

- iXon^{EM}+ DU897 back-illuminated: housing a 512 x 512 back-illuminated sensor offering single photon sensitivity, > 90% QE, 16 x 16µm pixel size and –100°C cooling @ >34 full frames/sec.
- iXon^{EM}+ DU885: housing a 1Mpixel sensor offering single photon sensitivity, > 60% QE, 8 x 8 µm pixel size and –100°C cooling @ > 30 full frames/sec.
- iXon^{EM}+ DU860 back-illuminated: housing a 128 x 128 back-illuminated sensor offering single photon sensitivity, > 90% QE, 24 x 24µm pixel size and –100°C cooling @ > 500 full frames/sec.
- iXon^{EM}+ DU888 back-illuminated: housing a 1024 x 1024 back-illuminated sensor, 13 x 13µm pixels, offering single photon sensitivity >90% cooling to -90°C and a large field of view.

Luca^{EM}

Luca^{EM}

Andor's Luca^{EM} is the latest EMCCD innovation, a highly cost-effective option making EMCCD available to every laboratory carrying out single molecule detection with widefield epifluorescence microscopy.

Operate gain off for conventional CCD operation under brighter conditions - turn on the EM gain when the photons become scarce! Luca^{EM} DL-658M provides single photon detection sensitivity and high QE at 30 full frames/sec, in a TE cooled, USB 2.0 camera platform.

Features & Benefits of the Luca^{EM}

- Single photon sensitivity + high QE (~ 52% max in Luca^{EM} DL658M, with extended red response)
- RealGain™
- Fast frame rates: ~ 30 frames/sec (faster with sub-array / binning)
- USB 2.0: easy interfacing to all computers
- Multiple camera support: separate FRET emission wavelengths to more than one camera!

For further information on the Luca^{EM}, please refer to pages 172 - 177 of the Products section.

Newton^{EM}

Newton^{EM}

Features & Benefits of the Newton^{EM}

- Single photon sensitivity + QE > 90%
- Spectrally overlapped single molecule signal, e.g. SM-FRET, can be collected with extremely high spectral resolution and S/N
- Fast spectral rates
- USB 2.0 simple interfacing: direct from camera to PC
- Minimized darkcurrent from unparalleled –90°C TE cooling
- EMCCD and conventional amplifiers: operate at fast frame rate with EM gain, or as classic slow readout CCD for longer exposures

For further information on the Newton^{EM} range, please refer to pages 194 - 197 of the Products section.

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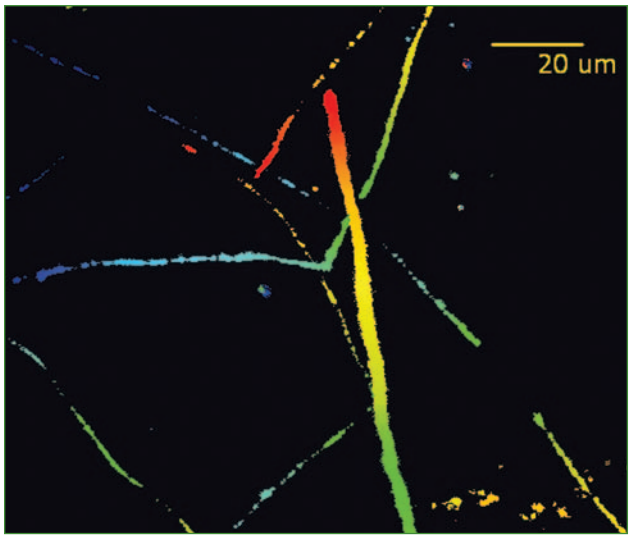

Figure 5 - Quantum dots are immuno-linked to dynein molecules, which carry their cargo along extra-cellular microtubules (not visible here). Using Andor iQ and iXon DV887 back-illuminated, 4000 frames were acquired with 30 ms exposure in frame transfer mode and simultaneously displayed and streamed to hard disk at 30 fps using iQ's ImageDisk functionality. The data has been processed by maximum intensity time encoding, in which each pixel is replaced by the time value when it was brightest and then pseudo-colored. Red pixels are the latest in time, while blue are the earliest, giving a map of the rate and direction of transport.
Courtesy Dr Stefan Diez, Max Planck Institute, Dresden, Germany.

Which EMCCD platform?

Either iXon^{EM}+, Luca^{EM} or Newton^{EM} cameras can be used to deliver enhanced EMCCD performance at high frame rate, the choice depending on a number of factors such as:

- Cooling requirement: dictated by photon background contribution of the optical configuration, eg. TIRFM vs Widefield
- Sensor pixel format required
- Frame rate: the absolute fastest pixel readout rates are from the iXon^{EM}+ DU-885
- PCI v USB 2.0 interface
- Number of cameras required: e.g. two lower-cost Luca^{EM} cameras could be used for detection of separated donor and acceptor emissions
- Spectral resolution required: Newton^{EM} offers spectral dispersion over 1600 channels

iQ Multi-Dimensional Imaging Software

iQ software interface

Features & Benefits of Andor iQ

- Optimized for Andor EMCCD: software and camera from the same company gives the most optimized combination in the market
- Tight synchronization of camera with other hardwares: maximum frame rates with minimal light loss
- Accessible dashboard and wizard structure: makes light work of even the most complex of protocols
- Comprehensive multi-dimensional processing, analysis and visualization
- Multi-dimensional rendering and analysis of multiple fluorescence images
- FRET and colocalization of multiple fluorescent labels
- Tracking of individual membrane proteins or vesicles
- Modular Structure: cost-effective and powerful solution to your application

For further information on Andor iQ, please refer to pages 246 - 253 of the Products section.

Andor Solis (i)

Features & Benefits of Andor Solis (i)

- Intuitive and comprehensive image acquisition
- Extensive range of image procession and analysis function
- Kinetic plots / ROI analysis

Other Components for SMD

- Optosplit II or Dual View signal splitters: simultaneous imaging of two emission wavelengths by one monochrome EMCCD, ideal for FRET. Please refer to pages 266-267 for further details.
- Piezo Z100 rapid z-stage: record up to 100 widefield or confocal z-sections per second, to dynamcally track single molecules through 3 spatial dimensions. Please refer to page 284 for further details.
- Polychrome V or OptoScan: fast (millisecond) switchable monochromator light sources for rapid and selective excitation of multiple fluorophores. Tuneable from ~320nm to 700nm. Please refer to page 278 for further details.
- Lambda 10-series filter wheels: fast and smooth filter changing for multi-wavelength protocols. Please refer to page 260 for further details.
- Andor Laser Combiner: up to four co-linear solid-state lasers, ideal for multi-fluorophore TIRF experiments and multi-user environments. AOTF control for rapid switching between wavelengths and attenuation of power. USB 2.0 interface and control through Andor iQ software.
- TIRF Illuminator: this prism-type TIRF illuminator takes inputs from one or two lasers (from Andor Laser Combiner) and provides independent computer controlled illumination angle(s) to optimize TIRF imaging. Through Andor iQ software, it is possible to modulate, on a millisecond timescale (via an inbuilt galvo mirror), the TIRF illumination depth in synchrony with Luca^{EM} or iXon^{EM}+ EMCCDs. This permits varying of optical beam penetration into the specimen and allowing dynamic events to be tracked almost simultaneously at different depths. Control of illumination depth using TIRF allows easy identification of fluorescent regions that may be moving away from the cell membrane. Please refer to page 276 for further details.
- Revolution XD spinning disk confocal system: readily applicable to dynamic confocal imaging of single molecules and single molecule FRET pairs, enabling elucidation of quantified molecular dynamics, such as protein-protein interactions, protein-DNA interactions, and protein conformational changes. High S/N multi-dimensional movies can be built through combination of CSU, iXon^{EM}+, iQ software and Andor Laser Combiner with matched solid-state laser lines and AOTF for laser balancing/rapid switching. Please refer to page 232-237 for further details.
- 3rd party software compatibility: all-in-one package that provides up-to-date, fully supported compatibility for iXon^{EM}+ and Luca^{EM} cameras within a range of 3rd party software, including Image J, LabView, IDL and Igor Pro. Please refer to page 243 for further details.

Case Study

iXon helps discover secrets behind protein unfolding

Dr. Atom Sarkar is a neurosurgery resident at the Mayo Clinic (Rochester, MN) but is carrying out his research goals in Professor Julio Fernandez's Single Molecule Force Spectroscopy lab at Columbia University (New York, NY). The role of nanotechnology in neurosurgery is central to his research.

Currently he is delving into the world of protein mechanics. Why? Because a knowledge of a protein's structural dynamics is central to the understanding of many neurological diseases ranging from brain tumors to neurodegenerative processes such as Alzheimer's Disease. Protein folding and unfolding happen on the nanometer scale. Therefore Atom needs a technique of nanometer or sub-nanometer resolution. Confocal won't cut it. He has been using a customized total internal reflection (TIRF)/atomic force microscope (AFM)/electron multiplying charge coupled device (EMCCD) to study protein unfolding, with the aim of developing a TIRF-only method to follow protein dynamics with sub-nanometer resolution. This will open protein unfolding experiments to many more scientists.

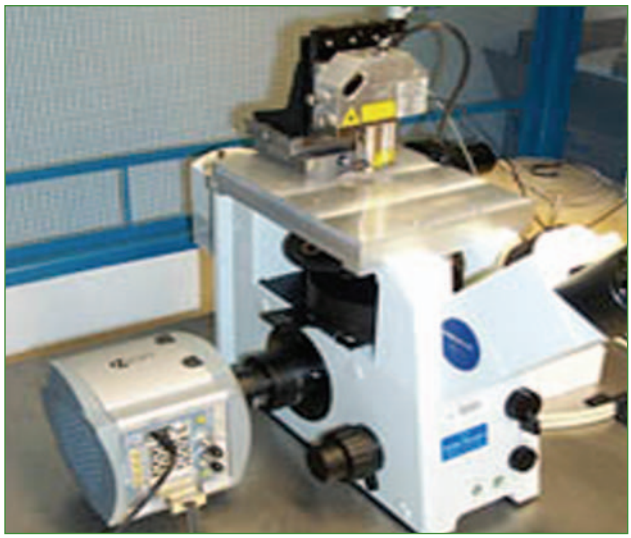

Figure 6 - Combined TIRFM and AFM

The key principle behind the methodology is the use of a nanometer-scale calibrated evanescent wave to measure the position of a fluorophore moving along the optical axis. By using a TIRFM generated evanescent wave that has an intensity which decays exponentially as a function of vertical distance, Atom can correlate changes from fluorescence intensity into displacements in the vertical axis.

In order to calibrate the evanescent wave, an AFM/TIRFM/EMCCD device was constructed consisting of an AFM head mounted on top of a TIRFM microscope equipped with an electron multiplying charge coupled device (EMCCD) (see Figure 6 above). The system exploits the distance dependent evanescent wave as a "ruler" to deconvolve fluorescent intensity into length (see Figure 7 below).

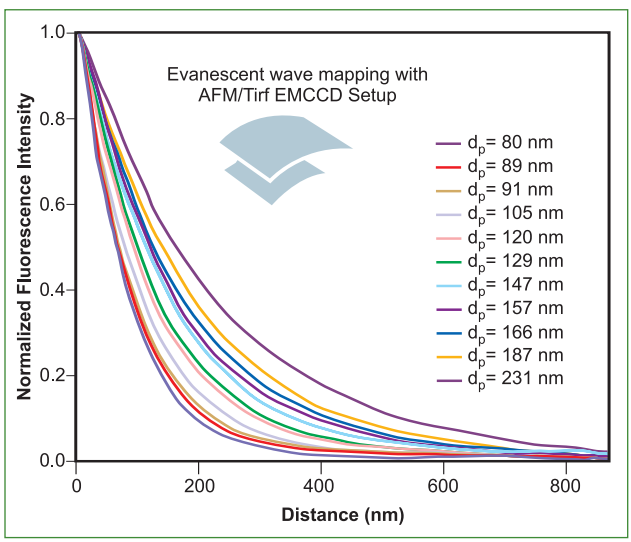

Figure 7 - Measurement of a series of evanescent penetration depths.

The six curves depicted in Figure 7 nicely illustrate the measured evanescent field decay and how by changing the TIRFM conditions, the evanescent wave penetration depth, d_p, can be adjusted to fit experimental needs. The ability to measure distance with the evanescent field has been dubbed Evanescent Nanometry.

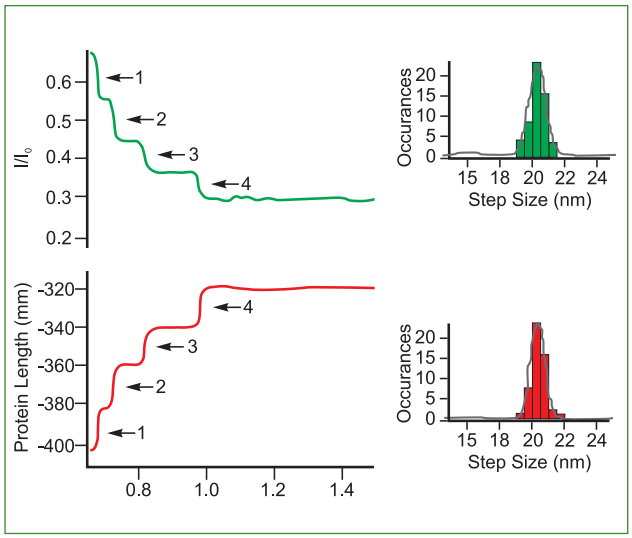

Figure 8 - Evanescent Nanometry has already been used to follow the forced mediated unfolding of the protein ubiquitin, shown below, and these results rival the precision and accuracy obtained by standard AFM only studies.

Current experiments involve replacing the AFM entirely and attaching magnetic fluorescent beads to the proteins. The magnetic beads allow the tethered proteins to be manipulated by an electromagnetic tweezer set-up, while the fluorescence of the bead enable nanometer precision monitoring of the proteins unfolding and folding dynamics, which are captured by the EMCCD.