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Invited Paper

The Fluorescence Apertureless Near Field Microscope...A Step Towards Imaging Information in DNA

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ABSTRACT

Single molecule imaging with optical methods has become an important tool in biophysical studies. However, when imaging molecules at room temperature using far field optics, one can only resolve molecules that are separated by a distance greater than the diffraction limit of the microscope, about 220 nanometers. Near field techniques have allowed researchers to image with resolutions on the order of 30-50 nanometers. However, there are numerous reasons to try to push the resolution limit further. One that particularly concerns our group is the notion to try to image information in DNA in order to measure sequence information. To that end, we have developed a new type of near field microscope, the fluorescence apertureless near field microscope (FANSOM).

Keywords: Near Field Microscopy, Single Molecule Imaging, DNA Analysis

1. Single Molecule Imaging and Other Background

The first optical images of single molecules were demonstrated in 1989, using laser induced fluorescence of cryogenically cooled samples. By 1994, several groups had demonstrated single molecule fluorescence imaging at room temperature with laser induced fluorescence (1,2). Recently, our group has shown that a minimal system for single molecule imaging exists and relies on using only a standard fluorescence microscope, a mercury lamp for excitation, and an inexpensive cooled CCD camera (3). These results should allow the proliferation of single molecule imaging techniques into groups that are not optics specialists.

There are a number of challenges to overcome in order to image single fluorophores. First, most molecules emit on the order of a million photons before irreversibly photobleaching. Thus, the detection system must be sensitive enough to collect and measure these photons. Typically this is accomplished by using a high numerical aperture objective for good collection efficiency and a sensitive detector. Cooled CCD cameras and photon counting avalanched photodiodes are generally the detectors of choice due to their low dark counts. Another obstacle that must be overcome is the removal of all unwanted sources of background fluorescence. In practice, this means working with ultra-pure, spectroscopic grade solvents. Also, if the molecules are deposited on a glass slide or cover slip it means that the surface must be cleaned meticulously.

Another challenge to be overcome for single molecule imaging is resolution. With far field optics, the diffraction limit is ~ 225 nanometers, much larger than the size of a single molecule. The reason that single molecules can be resolved in figure 1 is that the molecules are extremely dilute: their spatial separation is on the order of micrometers. This point can also be seen dramatically in figure 2, which shows images of DNA molecules with single fluorescently labeled nucleotides incorporated onto each end. While the nucleotides can be visualized, one must bear in mind that there are 50,000 nucleotides from one end of the molecule to the other and thus it would be impossible to image the sequence information in DNA with any far field optics.

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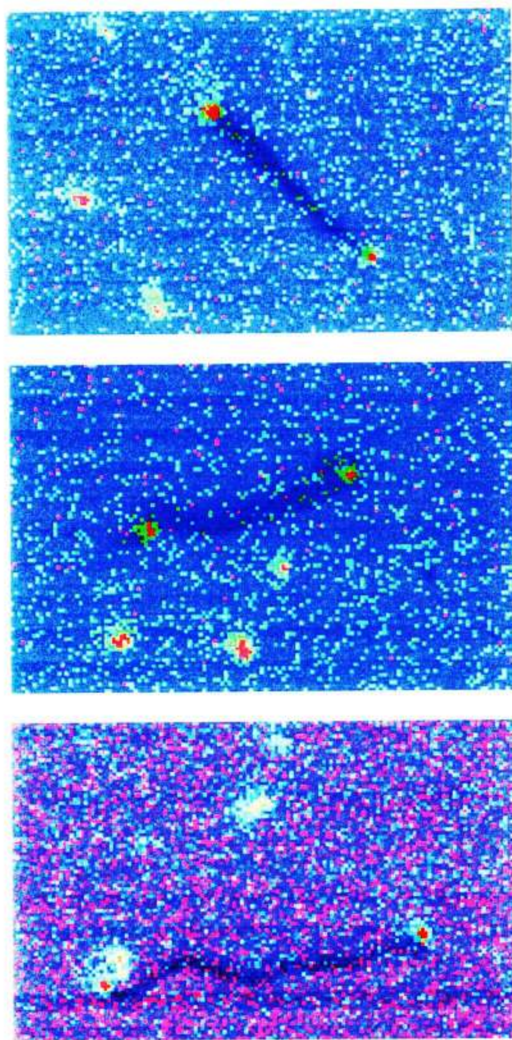

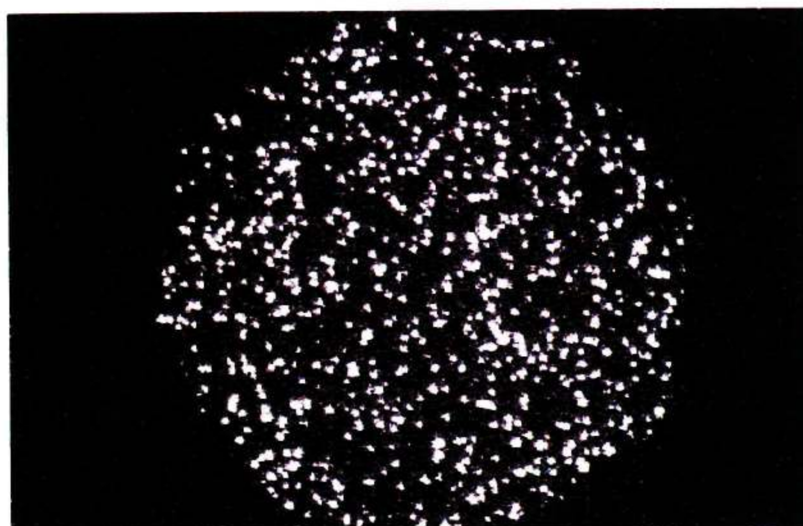

Figure 1, above: Single molecules of TRITC imaged with mercury lamp illumination and an inexpensive cooled CCD camera. From ref 3.

Figure 2, left: Single molecules of DNA whose ends have been labeled with a single fluorescent nucleotide. Lambda phage DNA was labeled with dATP-TMR (tetramethylrhodamine) conjugate and a dTTP, dCTP, dGTP mixture using Klenow fragment DNA polymerase. There is a single dATP incorporation site on each end of lambda phage, so only a single labeled fluorophore is inserted. Samples were purified by dialysis against TE buffer with 10mM NaCl for 8 cycles of one hour each (fresh buffer change every cycle). Samples were deposited on a RCA-cleaned cover slip, blown dry and observed in air. Withholding the enzyme results in no observed incorporation signal.

Thus, to fully realize the benefits of single molecule imaging and in an effort to push the state of the art to "image" information in DNA, we have developed a new type of near field microscope whose resolution is not limited by the diffraction of light. Near field microscopy is by now a mature field; the most common technique is to force light through a sub wavelength aperture and the resulting resolution is determined by the size of the aperture. Using this technique, resolutions as low as 20-30 nanometers have been demonstrated, but typical day-to-day resolutions are closer to 50 nanometers. One runs into the problem that the smaller the aperture, the lower the transmission for light. Also, these tips are invariably coated with metal and one has to worry about light leaking out near the end due to the finite skin depth of the metal. Recently, apertureless approaches to near field microscopy have become popular, in which a sharp stylus interacts optically with the object being imaged and the resolution is determined solely by the sharpness of the tip of the stylus. In 1995 Wickramasinghe's group demonstrated a single wavelength apertureless microscope with apparent resolutions as low as 1 nanometer (4). The contrast mechanism is due to dipole interactions between an AFM tip and the object being imaged. However, one finds that with such single wavelength microscopes there are height-related artifacts and it becomes challenging to separate the height signals from the optical signals. For that reason, we started investigating a fluorescence apertureless microscope and showed proof of principle of a 1-photon fluorescence apertureless microscope in January 1999 (5). Later that year, Xie's group demonstrated a complete apertureless fluorescence microscope that could be used to image plant chromophores with 2-photon excitation (6). However, due to tip-quenching effects the 2-photon microscope can only be used to image samples in which there is energy transfer away from the tip before re-radiation. We later demonstrated a fully working 1-photon fluorescence apertureless microscope with resolution ~ 30 nanometers (7).

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2. Fluorescence Apertureless Near Field Microscope

We exploit the fact that fluorescent molecules transfer energy non-radiatively to proximate metal or semiconductor surfaces. This quenching effect has been studied for many years and may limit the effectiveness of microscopes using electric field enhancement as a contrast mechanism. The ability to use single photon excitation allows greater flexibility in the choice of laser source and reduces the peak power through the objective and on the sample. The use of fluorescence facilitates interpretation of the images and provides compelling evidence that the contrast mechanism is indeed optical in origin. We have previously observed specific spectroscopic effects such as photobleaching, showing that there is no height contamination in the optical image. As with ANSOM, the resolution is determined by the radius of curvature of the probe. AFM tips with 5 nm radius of curvature are now commercially available, and carbon nanotube AFM tips have been demonstrated. It is thus reasonable to expect that the ultimate resolution of FANSOM will be in the 1-5 nm range. There are a number of important possible applications of FANSOM, including imaging of single DNA molecules for optical mapping and fluorescent in situ hybridization, spectroscopy of nanoparticles and quantum well devices, and the possible extension to single molecule Raman imaging.

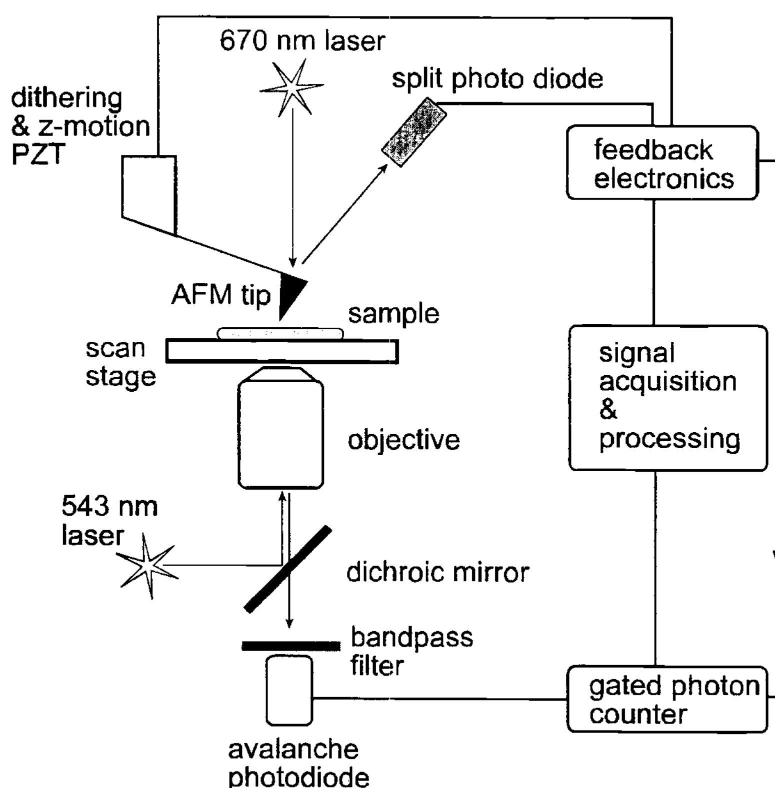

Figure 3. Schematic diagram of FANSOM apparatus. See text for details.

We used our FANSOM to image fluorescent latex particles with sub-diffraction limit resolution. The experimental set-up is shown in figure 3. It consists of a tapping mode atomic force microscope (AFM) on top of an inverted optical microscope, both homemade. The sample is scanned under the tapping AFM tip (resonant frequency ~ 250 kHz), while a green HeNe (543.5 nm, Uniphase 1674P) laser beam is focused to a diffraction limited spot on the tip via a microscope objective. Commercial silicon AFM tips (Nanosensors) were coated with a 60 nm layer of gold by thermal vacuum deposition. Photons emitted by fluorescence are collected by the same objective (Olympus Plan Apo Chromat 60x, 1.4 NA)

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and imaged onto a photon counting avalanche photodiode (EG&G SPCM 100), whose output is in turn processed by a gated photon counter (Stanford Research Systems SR400) to implement a lock-in detection scheme. The gating was triggered by the position of the AFM cantilever such that the counts measured while the tip was closest to the sample were subtracted from the counts measured when the tip was farthest from the sample. This effectively selects a 100 Hz frequency window centered on the 250 kHz resonant frequency of the cantilever and allows discrimination of the far field emission from the photon suppression due to fluorescence quenching at the near field of the tip. The position of the AFM cantilever was measured on a split photodiode using a standard optical lever scheme. The split photodiode signal was used as part of a feedback loop to maintain constant tapping amplitude, and was also used with a phase locked loop to trigger the photon counter gates.

During image acquisition, the sample was scanned in the xy plane and two signals were collected: the photon counter difference output (optical) signal and the AFM z-feedback (topographical) signal. This allows the simultaneous acquisition of topographical (AFM), and near field optical fluorescence (FANSOM) images. Far field optical images using the total number of photons per pixel were also taken during a separate scan. Our samples consisted of 60 nm fluorescent latex beads (Nile Red, Interfacial Dynamics Corp) deposited on an RCA cleaned glass cover slip. The laser beam had a total power of 20 nW focused into a 500 nm diameter spot. We used a set of band pass filters in front of the avalanche photodiode that selected out the region between 580 and 620 nm, excluding both the 543.5 nm excitation light and the 670 nm diode laser used for the AFM feedback.

Figure 4 shows two sets of images collected from a 1 μm^2 field of view. Fluorescent beads are seen using AFM, FANSOM and far field imaging. In one image there is also a non-fluorescing contaminant particle that is visible in the AFM image but not in the corresponding FANSOM or far field images. We estimated the resolution of the FANSOM microscope by taking a line slice through the center of the beads and measuring the full width half maximum of the peak, finding 95 and 120 nm. Given that the bead has a 60 nm diameter, we can estimate the resolution of the FANSOM to be between roughly 30 nm, well below the diffraction limit of 260 nm. Although this method of determining the resolution does not strictly adhere to the classical Rayleigh criterion, it does show unambiguously that the resolution surpasses the diffraction limit. Control images taken with the tip removed show only the expected far field image, with no signal in the FANSOM image. Control images taken with the green laser blocked show no FANSOM image, only the normal AFM image.

We verified that the FANSOM image is not contaminated with height information by observing photobleaching of the bead. Repeated images of the same bead showed a gradual reduction in the level of the FANSOM signal, while the AFM image remained unchanged. We also took images with 100 nm beads excited by a 60 nW laser beam. By positioning the AFM tip directly above a single bead, we were able to observe the near field signal as a function of time. We measured the signal in this case as the height of a peak in the power spectrum of the output of the avalanche photodiode (7). The resonance is present only when the tip and the laser are aligned, and only when the incident laser is present. The signal gradually decreases with time as a result of photobleaching. At the end of the measurement we verified that there had been no significant drift of the AFM tip position. In previous work, we have observed a similar effect with bare silicon tips and shown that it is due to near field effects (5).

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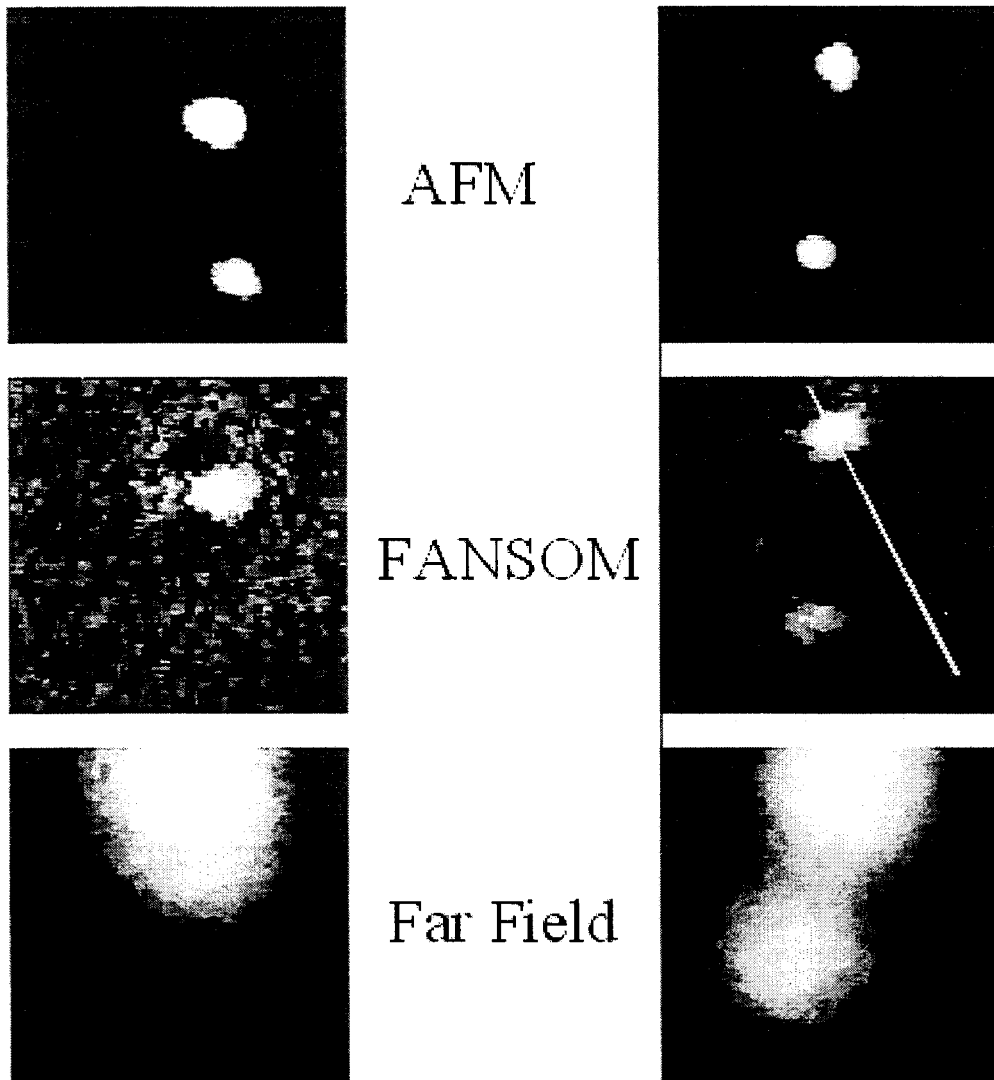

Figure 4. Images of 60 nanometer beads from FANSOM microscope with roughly 30 nanometer resolution. See text for details.

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3. Conclusion

In conclusion, we have demonstrated a 1-photon fluorescence apertureless microscope with a resolution of 30 nanometers. We believe that the resolution can ultimately be reduced below 10 nanometers, since it depends only on the radius of curvature of the AFM tip. We would also like to improve the sensitivity of this microscope in order to image single molecules. We have imaged single fluorophores using the far field mode of our instrument, and have been able to use that data to estimate what the signal to noise would be for near field imaging of single fluorophores. With our current apparatus, we think we would see single molecules with a S/N of about 4. In order to try to improve that figure, we have begun systematically investigating ways to improve the signal we get, by evaluating a larger class of fluorophores and by making improvements to the optical path. We believe it is feasible to improve the signal to noise figure enough in order to get convincing images of single fluorophores, and hopefully one day image fluorescent information encoded in DNA. Since the four bases have distinct Raman spectra, it may be possible to use this kind of microscope with surface enhanced Raman scattering in order to image information in DNA (8).

4.References

1. S.M. Nie and R.N. Zare, "Optical detection of single molecules", *Ann. Rev. Biophys. Biom.* **26**: 567-596 (1997)
2. X.S. Xie and J.K. Trautman, "Optical studies of single molecules at room temperature", *Ann. Rev. Phys. Chem.* **49**: 441-480 (1998).
3. M. Unger E. Kartalov, C.S Chiu, H. Lester and S. Quake, "Single Molecule Fluorescence Observed with Mercury Lamp Illumination", *Biotechniques* **27**: 1008-1013 (1999).
4. F. Zenhausern, Y. Martin and H.K. Wickramasinghe, "Scanning Interferometric Apertureless Microscopy – optical imaging at 10 Angstrom resolution", *Science* **269**:1083-1085 (1995).
5. G.A. Lessard, T.J. Yang, P. Barritault and S.R. Quake, "A Scanning Apertureless Fluorescence Microscope. *Proceedings of the SPIE* **3607**, 158 (1999).
6. E.J. Sanchez, L. Novotny, and X.S. Xie, "Near-field fluorescence microscopy based on two-photon excitation with metal tips", *Phys. Rev. Lett.* **82**: (20) 4014-4017 (1999).
7. T.J. Yang, G.A. Lessard, and S.R. Quake, "An apertureless near-field microscope for fluorescence imaging", *Appl. Phys. Lett.* **76**: (3) 378-380 (2000).
8. S.R. Quake, US Patent 6,002,471 (1999).

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