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Contract N00014-92-J-1260

R & T NO. 4121090-01

TECHNICAL REPORT NO. 12

COMBINED ATOMIC FORCE AND SCANNING REFLECTION INTERFERENCE CONTRAST MICROSCOPY

by

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Prepared for publication

in

Scanning (1994) (submitted)

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Approved for Public Release

SEPTEMBER 1994

REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: Technical Report No. 12	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER: N00014-01	
4. TITLE (and Subtitle): Combined Atomic Force and Scanning Reflection Interference Contrast Microscopy	5. TYPE OF REPORT & PERIOD COVERED: Technical Report 1/01/94-12/31/94
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): P.E. Hillner, M. Radmacher and P.K. Hansma	8. CONTRACT OR GRANT NUMBER(s): N00014-92-J-1260
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of California, Physics Department, Santa Barbara, CA 93106, Contracts and Grants, 3227 Cheadle Hall	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: R&T No. 4121090-01
11. CONTROLLING OFFICE NAME AND ADDRESS: Office of Naval Research, Electronics and Solid State Physics Program, 800 N. Quincy, Arlington, VA 22217	12. REPORT DATE: JUNE 1994
	13. NUMBER OF PAGES: -3-
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): Office of Naval Research Detachment, 565 South Wilson Avenue, Pasadena, CA 91106	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

"Approved for Public Release: Distribution Unlimited:

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Reports Distribution List for ONR Physics Division Office

18. SUPPLEMENTARY NOTES

Scanning (1994) (submitted)

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

method of combining an Atomic Force Microscope (AFM) with a Reflection Interference Contrast Microscope (RICM);

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A sphere attached to a cantilever is used simultaneously as an atomic force microscope (AFM) tip and as a curved reflective surface for producing scanning reflection interference contrast microscope (RICM) images of fluorescent beads dried onto a glass slide. The AFM and RICM images are acquired in direct registration which enables the identification of individually excited beads in the AFM images. The addition of a sharp, electron beam-deposited tip to the sphere gives nanometer resolution AFM images without loss of optical contrast.

4/1/94

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D.C. Joy
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Dear Dr. D. C. Joy:

please consider our manuscript "Combined atomic force and scanning reflection interference contrast microscopy," by Hillner, Radmacher, and Hansma for publication in *Scanning* as an Original Paper. Three identical copies of the manuscript with laserprinted figures, two sets of glossy figures, and one High Density Macintosh floppy disk with the text and figures are enclosed.

Sincerely,

Paul Hillner

Combined atomic force and scanning reflection interference contrast microscopy

Running title: Combined AFM and RICM

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This work was supported by the National Science Foundation under Grant DMR9221781 (P.E.H.) and by the Office of Naval Research (M.R., P.K.H.).

Keywords: AFM, RICM, electron beam-deposited tip, EBD tip, fluorescence quenching

Summary: A sphere attached to a cantilever is used simultaneously as an atomic force microscope (AFM) tip and as a curved reflective surface for producing scanning reflection interference contrast microscope (RICM) images of fluorescent beads dried onto a glass slide. The AFM and RICM images are acquired in direct registration which enables the identification of individually excited beads in the AFM images. The addition of a sharp, electron beam-deposited tip to the sphere gives nanometer resolution AFM images without loss of optical contrast.

Introduction

Since its invention in 1986 (Binnig *et al.* 1986) the atomic force microscope (AFM) (Rugar and Hansma 1990) has been combined with optical microscopes by several groups (Radmacher *et al.* 1992, Putman *et al.* 1992a, 1992b, 1993, Henderson *et al.* 1994, Schabert *et al.* 1994, Hansma *et al.* 1994). Scanning near-field optical microscopy (Pohl *et al.* 1984, Betzig and Trautman 1992) in particular has been directly combined with AFM (Betzig *et al.* 1992, Toledo-Crow *et al.* 1992, van Hulst *et al.* 1992, 1993, Shalom *et al.* 1992, Radmacher *et al.* 1994). We report here a method of combining an AFM with a reflection interference contrast microscope (RICM), a productive tool in biophysics (Verschueren 1985, Zilker *et al.* 1987, Rädler and Sackmann 1992, 1993). The combination microscope enables a fluorescing object to be located in a scanned RICM image and correlated with surface topography in a simultaneously acquired AFM image.

Method

RICM quantitatively determines an object's shape and distance away from a nearby, usually flat reflective surface from the interference pattern produced by light reflecting from both the object and the surface. However, our scanning RICM uses a sphere as the reflective surface as sketched in Figure 1. A microscope objective focusses a laser on a fluorescent bead dried onto a glass slide. The same objective collects fluorescence. The interference between the fluorescence directly emitted by the bead and the fluorescence reflected by the sphere is observed by measuring the intensity of fluorescence passing through a confocal pinhole. By scanning the sphere across the sample an interference pattern of concentric rings centered at the lateral position of the bead is recorded.

The interference is destructive when the average path length of reflected fluorescence compared to direct fluorescence is $p\lambda/2$ and constructive when the path length difference is $(p+1/2)\lambda/2$, where $p \geq 0$ is an integer and λ is the wavelength of the fluorescence. Thus if the sphere contacts a bead during the scan the corresponding interference pattern will have a dark center. If the sphere is scanned above a bead the intensity of the center of the interference pattern varies sinusoidally with the height of the sphere; bright at $\lambda/4$, and dark at $\lambda/2$, etc. The radial intensity profile of the interference pattern is also a strong function of height.

To produce our images a metal sphere is epoxied to the tip of a standard AFM cantilever (Nanoprobes, Digital Instruments, Santa Barbara, CA; OMCL Series, Olympus, Tokyo, Japan) as shown in Figure 2. We produce the spheres by grinding SmCo (Dexter-Permag, Sunnyvale, CA) or NdFeB (Edmund Scientific, Barrington, NJ) magnets. Electron beam-deposited (EBD) tips (Keller and Chou 1992, Hansma *et al.* 1992, Schiffmann 1993) are grown on some spheres in a scanning electron microscope (SEM) (JSM-5300LV, JEOL, Peabody, MA). A scanned-cantilever AFM (Hansma *et al.* 1994) (a prototype of the Bioscope, Digital Instruments) is mounted on an inverted stereo microscope (Axiomat 135, Zeiss, San Leandro, CA) and controlled with an AFM controller (Nanoscope III, Digital Instruments). The sample, a dilution of 15 nm fluorescently-doped latex beads (Orange Fluospheres, Molecular Probes, Eugene, OR) in pH 6.5, 10 mM Na_2HPO_4 (Fisher Scientific, Pittsburgh, PA), is allowed to dry on a glass slide. An argon-ion laser (532-100A; Omnichrome, Chino, CA) is cleaned with a 512/10 nm bandpass filter (Chroma Technology, Brattleboro, VT) and focussed by a 40X (N.A. 0.60) objective (Achroplan, Zeiss) to excite fluorescence in a 0.75 μm diameter spot. A 560/60 nm bandpass filter (Chroma) follows a 525 nm cut-off dichroic mirror (Chroma) to detect fluorescence and not excitation light nor 670 nm (red) diode-laser light used for tracking the AFM cantilever. Red polycarbonate filters (HT 026; Lee Filters, Burbank, CA) at the diode laser and at the AFM photodetector reduce artifacts in the AFM images caused by scattered excitation light. A 0.3 mm diameter confocal pinhole allows fluorescence to reach a photomultiplier tube (R1387; Hamamatsu, Bridgewater, NJ). The output of the photomultiplier tube is amplified and bandlimited from DC to 1 KHz by a differential amplifier (Model 113; Princeton Applied Research, Princeton, NJ). Images are ported to another computer (Quadra 660AV, Apple, Cupertino, CA) for contrast enhancement (NIH Image, Wayne Rasband, Anonymous FTP: zippy.nimh.nih.gov), though the general features of the interference patterns are visible in the raw data.

Results

Figure 3 shows images of fluorescent beads beads dried onto a glass slide scanned with a 10 μm sphere. The one excited bead (shown by the arrow in the AFM image) appears at the same position in the RICM image. Each

bead shown in the AFM image appears as a dimpled tip image of a small asperity on the bottom of the sphere. The bright center of the interference pattern indicates that the sphere didn't contact the excited bead; the bead is perhaps encased inside a tiny crystal of dried buffer. The distortion of the interference rings at the bottom of the interference pattern is caused by the sphere contacting a high, sharp object on the surface, probably a larger crystal of dried buffer. The sharp object produces a tip image of the sphere in the AFM image. Several other tip images are also visible, which shows that large spheres are unsuitable for high-resolution AFM imaging.

The tip artifacts caused by using large spheres as tips can be alleviated by growing sharp, EBD tips on the spheres. The inset of Figure 2 shows an EBD tip on a sphere. Their small tip radii, typically 10-30 nm, enable high-resolution AFM imaging. The placement of the EBD tip on the sphere is important. By mounting the cantilever in the SEM at the angle that it will be held during AFM imaging and then centering the sphere by eye, the EBD tip can be placed within one micron of the center of the sphere.

Figure 4 shows images of beads scanned with an EBD tip on an 18 μm sphere. The position of the one excited aggregate of beads in the AFM image (arrow) is offset by 900 nm relative to the center of the interference pattern in the RICM image. As the EBD tip scans over the ~100 nm tall aggregate, the sphere is correspondingly raised which causes a shift in the phase of the the interference pattern, enough in this case to induce a dark spot in the bright center. The position of the dark spot in the RICM image clearly shows the 900 nm offset of the EBD tip relative to the center of the sphere.

Figure 5 shows images of another excited aggregate of beads (shown by the arrow in AFM image) scanned with the same sphere. This aggregate also produces a large enough shift in the phase of the interference pattern to create a dark spot at the same 900 nm offset. Two other surface features produce additional dark spots in the interference pattern as well. Note that the bead just to the right of the aggregate in the AFM image is too small to produce a dark spot in the RICM image. To verify that the dark spots are caused by the coupling of surface topography into the RICM image, the cantilever was raised just above the surface and scanned without feedback. The dark spots in the interference pattern dissappeared. Had the height of the EBD tip spaced the sphere $\lambda/4$ closer or farther from the sample, a dark center of the

interference pattern would have resulted. In this case the slight phase shifts due to surface topography would induce bright spots in the dark center.

One additional phenomenon, fluorescence quenching, may also contribute to the dark spots observed when the EBD tip contacts the excited aggregates shown in Figures 4 and 5. When an excited fluophore is in close proximity ($<\lambda/4$) to a conductive material (such as a metal or semiconductor), the optical energy stored in the fluophore partially non-radiatively couples to phonons and surface plasmons in the conductive material thereby quenching the emitted fluorescence. Quantitatively, the apparent quantum yield of a fluophore drops exponentially with decreasing distance to a nearby conductor; dropping to ~10% of its free space value at 20 nm and to ~1% at 2 nm for a typical fluophore (Shu *et al.* 1988). EBD tips, being highly carbonaceous, are somewhat conductive (Akama *et al.* 1990) and hence capable of quenching fluorescence. However, based on the large size (~100 nm) of the excited aggregates, fluorescence quenching probably plays only a minor role, since less than 20% of the volume of the aggregate is close enough to be significantly quenched by the EBD tip.

Conclusion

We have demonstrated a novel scanning microscope in which optical and topographic images of nanometer scale objects are acquired in direct registration. We have also operated the microscope in fluids which greatly reduces the loading force on the sample during imaging. However, when mobile fluorescent material is present in solution, it often collects on the metal sphere and prevents RICM imaging. Hydrophobized glass spheres could be a solution to this problem. If so, the microscope may prove to be a useful tool for the localization of fluorescent labels in AFM images of biological samples.

Acknowledgments

This work was supported by the National Science Foundation under Grant DMR9221781 (P.E.H.) and by the Office of Naval Research (M.R., P.K.H.). We thank D. A. Walters for SEM imaging and growing the EBD tips and M. Bower for helpful discussions.

Figure Captions

Figure 1. Schematic of combined atomic force and scanning reflection interference contrast microscope.

Figure 2. SEM image of a metal sphere epoxied to an AFM cantilever. Inset: enlargement of an EBD tip grown on the sphere.

Figure 3. Fluorescent beads imaged with a 10 μm sphere. (a) AFM image. (b) RICM image. Scan size: 10 μm x 10 μm. Imaging time: 26 s. The interference pattern centers on the bead indicated by the arrow. Note the large AFM tip images of the sphere.

Figure 4. Fluorescent beads imaged with an EBD tip on an 18 μm sphere. (a) AFM image. (b) RICM image. Scan size: 10 μm x 10 μm. Imaging time: 26 s. The interference pattern corresponds to the aggregate of beads indicated by the arrow. Note the high AFM resolution.

Figure 5. Fluorescent beads imaged with an EBD tip on an 18 μm sphere. (a) AFM image. (b) RICM image. Scan size: 10 μm x 10 μm. Imaging time: 26 s. The interference pattern corresponds to the aggregate of beads indicated by the arrow. Note the topography-induced dark spots in the RICM image.

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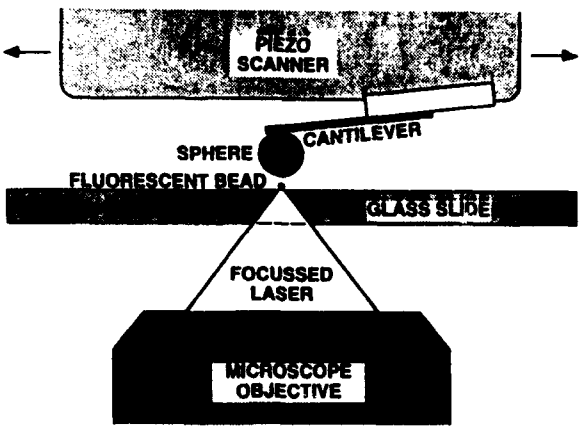
PIEZO
SCANNER
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FLUORESCENT BEAD
GLASS SLIDE
FOCUSSED
LASER
MICROSCOPE
OBJECTIVE

Figure 1

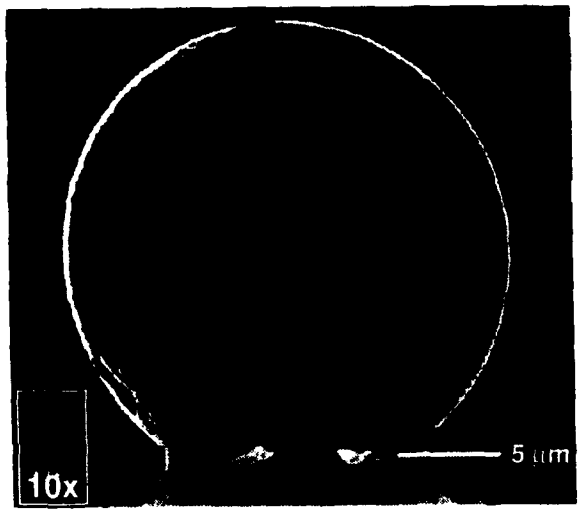


Figure 2

(a)

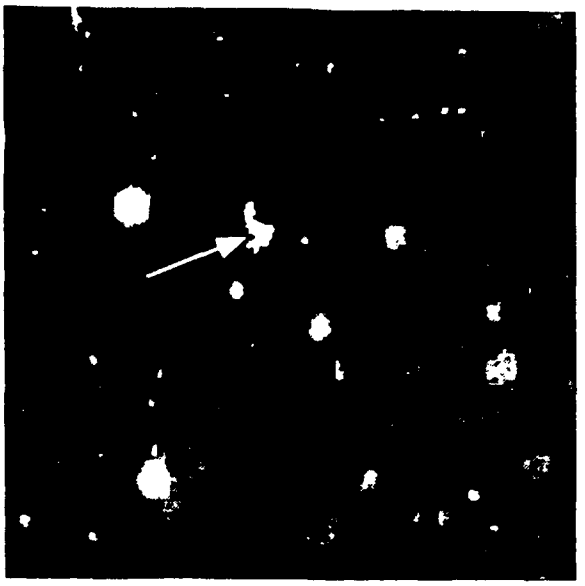
(b)

Figure 3

(a)

(b)

Figure 4



(a)

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Figure 5