

back loop to hold the sample stationary with respect to its laser or to perform scanning based on the parameterized back-scatter detection (BSD) signals. Three dimensional alignment improves registered tip exchange but is not necessary. Following the above protocol in two dimensions can lead to registered exchange with slightly reduced registration.

[0066] FIG. 3 illustrates the alignment of the tip. The tip was aligned relative to the tip detection laser. Different substrates and being in air and water yielded similar results. (a) Quadrant photodiode (QPD) signals as the tip was scanned along the x-axis through the detection laser. These records were measured through microscope cover glass with the tip in air (blank) and submerged in water (grey). On-axis signals (solid lines) and lateral crosstalk signals (dashed lines) are displayed. (b) Analogous records acquired through a thin metal film (2 nm Ti+6 nm Au) on glass. Records were digitized with the same electronic gain settings as in FIG. 3, but with a silicon-nitride tip. These proof-of-principle signals show that tip-based back-scatter detection (BSD) is compatible with different environments (aqueous) and different substrates (thin metallic surfaces).

[0067] Next, we coarsely centered the atomic force microscope tip on the 810 nm laser focus using a CCD camera (not shown). We then touched the tip to the surface and retracted it 60 nm with the tip's piezoelectric transducer (PZT) stage. We then aligned the tip with respect to its laser. In one method, we can minimize crosstalk between signals (V_x , V_y , and V_z) (FIG. 3) as previously discussed in King, G. M., Carter, A. R., Churnside, A. B., Eberle, L. S. & Perkins, T. T. *Ultrastable atomic force microscopy: atomic-scale lateral stability and registration in ambient condition*. Nano Lett. 9, 1451-1456 (2009), incorporated herein by reference. This alignment was achieved by dithering the atomic force microscope tip along each axis sequentially and changing the center position of the dither in 3D. This alignment can be automated based on the tip signal. Different alignment algorithms may yield slightly different offsets, but the critical issue is that the alignment is reproducible so that any offset is not time varying and can be quantified and removed.

[0068] For stabilized imaging after this alignment, the tip signals in 3D (V_x , V_y , V_z) were finally calibrated around this location. In one realization, the tip is raster scanned in 3D through its detector beam using a closed-loop piezoelectric transducer (PZT) stage. The resulting back-scattered signals (V_x , V_y , V_z) corresponding to stage movements (xPZT, yPZT, zPZT) (FIGS. 4a, b) could be scaled using a parameterization. For instance, one can use a 4th order polynomial. Feedback to the tip's piezoelectric transducer (PZT) stage kept the tip stationary relative to its laser focus.

[0069] As an alternative alignment, the tip and sample can be aligned to their respective lasers using the sum signal from the quadrant photodiode alone. As shown in FIG. 4, the tip is iteratively scanned in one dimension (x or y) while the sum of the power falling on the quadrant photodiode is observed (FIG. 3). The lateral position of the tip is then adjusted to extremize this signal. The same process works in the vertical axis as well. It is possible to use different algorithms on different tips. Again, any offset can be predetermined and removed.

2.4 Imaging

[0070] We imaged in contact mode at a constant force. The imaging mode is not central to our technique. Multiple imaging modes are possible: constant force, dynamic modes, force

volume etc. As is traditional in contact mode atomic force microscopy, the feedback to the stage constituted the topographic imaging signal. We averaged 2 ms of data at each pixel into a single point (unless otherwise stated) and used a 5 nm pixel spacing. To quantify the registration between successive images, we used a two dimensional (2D) cross correlation analysis and tracked the measured maximum in the cross correlation between conditions.

2.5 Re-Aligning the Same Tip

[0071] We retracted the tip and dewetted it and then started the whole process again using OTR-4. It is important to note that the sample does not need to be stabilized during this interchange. Generally, the drift of the sample during such a process did not lead to an ambiguity in which fiducial mark was being used. If necessary, one could either (a) identify a unique registration mark by counting lateral and vertical marks from a corner in the array of fiducial marks, (b) use an external interferometer, or (c) individualize fiducial marks in an asymmetric pattern either at the level of spacing between marks and/or the marks themselves.

[0072] To demonstrate the exchange, we imaged a patch with a highly unique and asymmetric feature membrane patch caused by scraping the tip through the membrane patch. In the proof-of-principle alignment, two dimensional cross correlation between FIG. 5a and the image of the same sample after rewetting (FIG. 5b) showed a registration of [25 nm, 0 nm], in x and y respectively. More careful control of imaging conditions, improved signal to noise of the images, and scanning over smaller regions can improve this alignment.

[0073] The atomic force microscope images shown in FIG. 5 demonstrate the instrument's ability to return to a feature over the course of a day. (a) Image acquired at 12:07 PM. (b) Image acquired with the same tip, after removing the tip from the fluid and re-immersing it, 3:04 PM. (c)-(e) Images after replacing tip with new tip, at 4:22, 4:32, 4:39, and 4:48 PM. (f) Acquired after raising the tip out of the fluid and re-immersing it in fluid.

[0074] We used biological material for this proof-of-principle demonstration. Higher precision would certainly be achieved using small fiducial marks (e.g., 5 nm gold beads). In the images shown in FIG. 5, there is clear degradation of the soft sample that limited the registration.

2.6 Exchanging the Same Type of Tips

[0075] We next removed the tip holder completely, put in a new tip and the repeated the whole process again. This yielded the images shown in FIGS. 5c-e. The registration between images FIG. 5A and FIG. 5C separated over three hours and different tips [-35 nm, -19 nm] was based on the image analysis on the central portion of the image. Again, it is immediately clear the same individual structure is being imaged. Also, the degradation of the sample is the limit to the cross correlation analysis. Harder samples and/or more gentle imaging modes will lead to higher precision during this process.

[0076] Also it is clear that exchange of tips led to a sharper, higher quality image in FIG. 5c compared to FIG. 5b. This demonstrates the usefulness of replacing a degraded tip for a new high quality tip. Note the improvement in sharpness of the image from FIGS. 5a to 5b. Most likely a protein contaminant present during imaging shown in FIG. 5a was removed during dewetting. Similar behavior is seen between