

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.

**[0056]** Features can be aligned by translation through the laser beam to generate a calibration curve. There can be an alignment of two or more independent structures relative to each other, or relative to a shift of a known center, or the structures can be raster scanned with respect to each other. Various methods are known. In one example, the center of that structure can be chosen and called 0. One can then move one structure 10.1 nm relative to the other structure (e.g., a lens) by moving either structure 10.1 nm. The calibration curve can also be attained by moving the laser beam through the structure.

## EXAMPLES

### 2.1 Experimental Apparatus

**[0057]** The apparatus used for these experiments, illustrated in FIG. 1A, was a custom built atomic force microscope mounted on top of a research grade optical microscope enhanced for mechanical stability. A full detailed discussion of the optics and instrumentation required for 3D back-scattered optical detection has previously been described by Carter, A. R., King, G. M. & Perkins, T. T. *Back-scattered detection provides atomic-scale localization precision, stability, and registration in 3D*. Opt Express 15, 13434-13445 (2007), incorporated herein by reference, as well as its application to ultrastable atomic force microscope at ambient conditions, described in Carter, A. R., King, G. M. & Perkins, T. T. *Back-scattered detection provides atomic-scale localization precision, stability, and registration in 3D*. Opt Express 15, 13434-13445 (2007) and 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), also both incorporated herein by reference.

**[0058]** Briefly, we used a custom built atomic force microscope mounted on top of an inverted microscope (FIG. 2). A high numerical aperture microscope objective was used to separately focus a pair of lasers onto a commercial atomic force microscope tip (OTR-4,  $k=0.08$  N/M (Veeco)) and a silicon fiducial mark ( $r=250$  nm,  $h=40$  nm) fabricated onto a glass cover slip. The back-scattered signals were efficiently separated from the inward-propagating lasers by an optical isolator formed by a polarizing beam splitter (PBS) and a quarter waveplate ( $\lambda/4$ ). Next, a dichroic mirror separated the signals onto two different quadrant photodiodes (QPD).

**[0059]** Movement of the fiducial mark in x and y relative to the detector beam caused a corresponding change in the distribution of light on the quadrant photodiode. Thus, the difference between the left and right halves measured the x signal, and the difference between the top and bottom halves yielded the y signal. Vertical motion (z) was deduced by the sum signal, which is the total light falling upon the four quadrants of the quadrant photodiode. The resulting quadrant photodiode voltages were amplified using custom built electronics and digitized. The sample's and tip's dimensional positions were controlled via a feedback loop using a pair of closed loop, direct drive, 3D piezoelectric transducer (PZT) stages (P363.3CD and P733.3DD, Physik Instrumente). For force sensing, we reflected a 785 nm laser off the backside of the atomic force microscope cantilever and detected its deflection with a quadrant photodiode using a standard opti-

cal lever arm. Meyer, G. & Amer, N. M. *Novel Optical Approach to Atomic Force Microscopy*. Appl. Phys. Lett. 53, 1045-1047 (1988).

**[0060]** Field-programmable gate array (FPGA) cards (PCI-7833R and PCI-7831R, National Instruments) were used to provide the computational power to parameterize and minimize crosstalk via linear algebra-based algorithms, as described in Lang, M. J., Asbury, C. L., Shaevitz, J. W. & Block, S. M. *An automated two-dimensional optical force clamp for single molecule studies*. Biophys. J. 83, 491-501 (2002). and Churnside, A. B., King, G. M., Carter, A. R. & Perkins, T. T. *Improved performance of an ultrastable measurement platform using a field programmable gate array for real-time deterministic control*. Proc. of SPIE 7042, 704205 (2008), both incorporated herein by reference. Field-programmable gate arrays (FPGAs) also provided for more rapid (500 Hz) and therefore precise controlling of all six axes of motion, leading to tip control of  $<0.04$  nm in 3D in air.

**[0061]** We used Olympus OMCL-TR400PSA tips for the initial phase of the experiment. These are symmetric pyramidal shaped tips.

### 2.2 Sample Preparation

**[0062]** We used patches of bacteriorhodopsin (BR) to demonstrate this technique. Our protocol for adsorption of patches of BR from *Halobacterium salinarum* (Sigma) was adapted from Muller, D. J. & Engel, A. *Atomic force microscopy and spectroscopy of native membrane proteins*. Nat. Protoc. 2, 2191-2197 (2007). and Goncalves, P., Agnus, G., Sens, P., Houssin, C., Bartenlian, B & Scheuring, S. *Two-chamber AFM: probing membrane proteins separating two aqueous compartments*. Nat. Methods 3, 1007-1012 (2006), both incorporated herein by reference, for use on glass cover slips (Corning). Silicon posts were added as fiducial marks, as described 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.

### 2.3 Initial Alignment of Sample and Tip

**[0063]** We sequentially aligned the stage to one laser and the tip to the second laser.

**[0064]** First, the sample was positioned in 3D with respect to the laser focus that tracked the fiducial mark embedded in the substrate. We aligned the 845 nm laser focus to a silicon disk on the cover slip by using an automated routine that controlled the sample piezoelectric transducer (PZT) stage. The general shape of the lateral signals (e.g. x and y axes) was the derivative of the Gaussian, which allowed automated alignment (See Carter, A. R., King, G. M. & Perkins, T. T. *Back-scattered detection provides atomic-scale localization precision, stability, and registration in 3D*. Opt Express 15, 13434-13445 (2007), incorporated herein by reference.) The exact functional form is not critical. The vertical signal, which can be offset amplified for enhanced sensitivity, was referenced to a maximum or minimum in the offset amplified sum signal. Typically, the final position of the fiducial mark was chosen to reside halfway between the maximum and minimum as the region of highest sensitivity.

**[0065]** We next raster scanned the sample around this alignment point through a  $\sim 200 \times 200 \times 100$  nm<sup>3</sup> volume that constituted the calibrated dynamic range. We then used a feed-