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Stabilizing method for reflection interference contrast microscopy

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Abstract

Reflection interference contrast microscopy (RICM) is a technique for measuring the shape and position of microscopic objects in solution; it has many biological and biophysical applications. Use of RICM for long-time acquisitions requires minimizing defocusing effects that are due to thermal and mechanical drift. We present a simple stabilizing method that accomplishes this using an image-analysis-based linear focus function to establish feedback control of the focal position. While implementing this routine, we used RICM for independent measurement of the apparent fluctuation in the vertical position of an immobilized bead: the measured height had a standard deviation of 0.12 nm during a 45 min acquisition while under feedback control, demonstrating the high stability achievable with our approach.

1. Introduction

Reflection interference contrast microscopy (RICM) is an imaging technique that uses optical interferometry to precisely measure distances between microscopic reflecting surfaces. Since it has high resolution, RICM has been applied to a large number of biological and biophysical systems, such as in the study of adhesive interactions involving lipid bilayer vesicles and cells [1–6]. It is a valuable tool for extracting topographic information over a relatively large range of distance (from a few to thousands of nanometers) [6–11]. With the RICM technique, an object in solution is localized very near a glass surface and is illuminated through that surface. Rays reflected from the object and those reflected from the glass interfere, creating a characteristic pattern of fringes that can be captured with a microscope. This pattern contains the information needed to determine the distance of the object from the glass surface, provided the microscope is properly focused on the glass–water interface. Proper focus can be temporarily achieved by analyzing the sharpness of certain image features [12,13]. For example, prior studies utilized the shadow image of a field diaphragm to construct a function of sharpness score versus focal position [7]. Such a function is peak shaped with a maximum value at the proper focal position because the shadow image has sharp edges when in focus.

RICM applications that involve active biological processes can require continuous data acquisition for as long as several hours, during which time the focus must be maintained. In such applications, an obstacle to the use of RICM is the defocusing action of thermal drift and external mechanical vibrations. These effects can be passively minimized by maintaining a constant ambient temperature and by mechanically isolating the microscope [7]; however, passive methods cannot completely prevent objective defocusing. Instead, active methods in

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which the objective or stage is moved by a feedback-loop-controlled piezoelectric actuator [14–16] are needed to maintain accurate focal position for long periods of time.

Automatic correction of the focal position first requires measurement of the current position through a focus function that determines the deviation from the proper focus. Peaked sharpness-based functions, while useful for one-time focusing, are not suitable for frequent focal correction [12] because at least two positions on opposite sides of the peak will have the same sharpness score. Thus, from a single measurement of sharpness, it is difficult to know in which direction to move to correct the focus. Further, in certain low-light conditions, the function can display several prominent local maxima, creating many positions with equivalent sharpness scores, thus increasing the indeterminacy of the focus function [13]. These drawbacks limit the accuracy and speed of active-feedback control utilizing sharpness-based focus functions. A focus function with a monotonic response overcomes these drawbacks because each function value corresponds to only one focal position [14,15]. Thus, focal position can be corrected with a unique, well-determined direction; this feature improves the accuracy and performance of feedback control over peak-shaped focus functions.

We present a simple stabilizing method for RISM using a linear focus function. We determine the absolute focal position using a peak-shaped focus function, then calculate a linear focus function using analysis of the RISM image of a reference object immobilized on glass. We use this linear function to implement optimal feedback control of the focus. To test the stability of the system, we implemented feedback control while independently measuring the vertical position of micrometer-sized spherical beads stuck to glass. Measuring the motion of micrometer-sized beads near glass surfaces has a variety of applications, such as single molecule measurements [17–20] or surface interaction studies [7,9]. Using our method, we found that the RISM-measured height of the stuck bead was stable to within a fraction of a nanometer for a period of 45 min.

2. Experiments

A. Reflection Interference Contrast Microscopy

As illustrated in Fig. 1, RISM images are formed by illuminating the subject with light intensity I_0 . The light waves reflected from the top surface of the underlying planar substrate (intensity I_1) are superposed with those reflected from the bottom surface of the object under observation (intensity I_2). The intensities I_1 and I_2 are related to the intensity I_0 of the incident beam by Fresnel's law. The intensity distribution of the interference pattern for a given wavelength is given by

$$I(x, y) = I_1 + I_2 + 2\sqrt{I_1 I_2} \cos[k\Delta(x, y) + \varphi], \quad (1)$$

where $k = 2\pi n_L/\lambda$ is the wavenumber of the illuminating light and φ is the phase shift of the light reflected from the object. $\Delta(x, y)$ is the difference in the optical path length between I_1 and I_2 , which is determined by the geometry of the object's bottom surface. Due to the incoherence of the illumination, Eq. (1) must be incoherently summed over the range of angles defined by the illumination numerical aperture (INA) [2,8]. In the case of a spherical object, $\Delta(x, y)$ depends on the object's radius R and its separation distance h [7,8], and the interference pattern has a series of concentric rings. For a given R , a change in height δh changes the radius of the rings by a specific amount; this can be calculated numerically by use of the nonplanar interface theory developed by Wiegand *et al.* [8].

B. Experimental Setup

The basic microscope system is shown in Fig. 1. We used an inverted microscope (TE2000-U, Nikon, Yokohama, Japan) with an oil-immersion objective (Plan APO 1.4 NA 100×). The system was mounted onto a vibration-isolating optical table. Monochromatic epiillumination with an aperture and a field diaphragm was provided by a high-pressure Hg lamp filtered by a 540 – 552 nm bandpass filter. By focusing the incident beam on the back focal plane of the objective lens, we ensured that the incident beam uniformly illuminated the image plane. The INA was adjusted with the aperture diaphragm, which was minimized to use the smallest achievable INA of 0.48. The field diaphragm produced a shadow in the image plane [see Fig. 2(b)]. We placed a parfocal lens in front of the CCD camera and adjusted it so that the shadow image and cover glass surface were simultaneously in focus. This shadow image was used to set the focus of the objective manually in the vicinity of the cover glass–water interface. RICM images were then acquired with a CCD camera with a 60 Hz frame rate (CV-A10CL, JAI, Yokohama, Japan) interfaced with a computer by a PCI express frame grabber (PCIe-1429, National Instruments, Austin, Texas). In the sample plane, the distance between neighboring CCD pixels was 92 nm. From each acquired image, lateral center positions of objects (x, y) and radial averaged intensity profiles of the interference image $I(r)$ were calculated and stored in real time by use of custom-written tracking software. Here, radius r is the distance from the lateral center position of an object and is measured in pixels. To implement feedback control of the focal position, the objective lens was mounted upon a piezo-objective positioner (PIFOC, P-725. 2CD, Physike Instrumente, Karlsruhe, Germany) with a 0.75 nm spatial resolution, a 20 ms time response, and a 250 μm travel range.

C. Materials

Two different types of micrometer-scale bead were used: 1 μm diameter polystyrene beads (PS04N, Bangs Laboratories, Fishers, Indiana) were used as reference objects to make focus functions, and 4.5 μm magnetic beads (Dynabeads M-500, Invitrogen, Carlsbad, California) were used to test the stability of the system independently. Figure 2 shows the experimental geometry and interference images of the reference and testing objects. To make reference objects on the cover glass, the polystyrene beads were melted onto the cover glass by use of a 200 °C hot plate for 20 min and were then solidified by cooling to room temperature. The test objects were chosen because of their smooth surface and because these beads adhere well to the cover glass without specific chemical treatment.

D. Focus Functions

An optimal focus function $F^*(z)$ was calculated from the interference image of a reference object on the cover glass [see Fig. 2(b)] to estimate the actual focal position and to determine the correct value during feedback control. Focus function values F_r were defined for different radii r by use of the radially averaged intensity profile $I(r)$:

$$F_r = f_r / f_{r \gg R}, \quad (2)$$

$$f_r = \frac{I(r-1) + I(r) + I(r+1)}{3}, \quad (3)$$

where f_r is the nearest-neighbor average of $I(r)$ at a given r and $f_{r \gg R}$ is the background intensity far from the bead. Normalizing with $f_{r \gg R}$ eliminates sensitivity to variations in the incident illumination intensity. Thus defined, the focus function $F_r(z)$ is a unitless, relative intensity that measures the local change of $I(r)$ with focal position z , and $F^*(z)$ is the optimal focus

function chosen from the various $F_r(z)$. In particular, to determine $F^*(z)$, we used the following algorithm: we fit each $F_r(z)$ with a line over the range of z of interest (typically ± 200 nm) and discarded those with fitted slopes A below $0.5 \mu\text{m}^{-1}$ (i.e. those with little focal position variance). We then determined which fit minimized the fitting residue ε . This procedure guaranteed an optimal focus function with both high-dynamic range and highly linear behavior. In practice, we found that feedback performance is optimized when $\varepsilon < 1.5 \times 10^{-4}$ and $A > 0.5 \mu\text{m}^{-1}$.

Figure 3(a) shows the radial intensity profile $I(r)$ calculated from interference images of a reference object taken at different focal positions. As seen in Fig. 3(a), $I(r)$ increases monotonically with focal position around $r = 7$ (dashed box). Correspondingly, within the range from -200 to $+200$ nm, $F_7(z)$ has a large slope ($A = 0.63 \mu\text{m}^{-1}$) and a small fitting error ($\varepsilon = 1.42 \times 10^{-4}$). Thus, $F_7(z)$ was selected as the optimal focus function, $F^*(z)$, for this reference object [Fig. 3(b)]. In Fig. 3, the profiles and $F_r(z)$ were calculated from 100 nm steps of z . To check the linearity of the focus function between steps, we compared interpolations (cubic spline) between 100 nm steps with experimental data taken in steps of 10 nm (Fig. 4). We found no significant difference between the two, indicating that steps of 100 nm are sufficient to accurately sample the behavior of the focus function.

E. Stabilizing

Initially, the objective was manually focused at the position in which the shadow image of the field diaphragm showed the sharpest edges. Next, $F^*(z)$ was calculated from seven interference images (200×200 pixels) of the reference object obtained in 100 nm steps around the initial objective position. For feedback control, a target value for focus function F^*_{target} must be defined. By moving the objective lens focus with the piezoactuator, we found the focal position at which the shadow image of the field diaphragm shows the sharpest edges as defined by sharpness scores [13,21]. At this position we defined the value of F^* to be the target value F^*_{target} ; target position z_t was calculated by interpolating $F^*(z)$ at F^*_{target} (see Fig. 5).

After $F^*(z)$ was measured and z_t was defined, we implemented the feedback algorithm to maintain the focal position over time. Interference images of the reference particle were continuously acquired at a 60 Hz frame rate, and the current focus value of F^*_c was calculated from each image in real time. The current focal position z_c was estimated by interpolating the measured F^*_c within the previously acquired function $F^*(z)$. Thus, we monitored focal position z_c versus time. To control the focal position actively, we measured the difference between the current focal position z_c and the target focal position z_t . The focal position was corrected by moving the objective with the piezoactuator by an amount Λ , defined as follows:

$$\Lambda = \begin{cases} \alpha(z_c - z_t), & |z_c - z_t| < \delta z_{\text{max}} \\ \text{sign}[z_c - z_t] \delta z_{\text{max}}, & |z_c - z_t| \geq \delta z_{\text{max}} \end{cases}, \quad (4)$$

where α is a gain that controls the focusing response. If the gain is set too high, the system will oscillate because of overcorrection; if the gain is too low, the correction will be insufficient to overcome thermal drift [14]. We found the optimal gain range to be between 0.2 and 0.5. Utilizing δz_{max} prevents the corrected position from going out of the range of the focus function. The correction was applied every 1 s because the drift of the focal position in 1 s is less than 10 nm; the degree of drift was estimated by monitoring focal position z_c versus time without feedback control. Use of this method allows one to maintain the focal position to within ± 10 nm from the target position (see Fig. 6). As shown below, this is more than adequate for effective stabilization of the RISM interferogram. If needed, this algorithm allows the target focal position to be changed in real time simply by redefining z_t .

3. Results and Discussion

The goal of RISM experiments is to relate the measured interferogram to the shape and position of the object. However, the interferogram will change not just if the object moves but also if the focal position changes because of thermal drift and/or mechanical vibration. Thus, the interferogram information about the object can be distorted or obscured by unwanted focal position movement; reducing the drift will improve the accuracy of RISM measurements [16].

In RISM measurements, a change in focal position is not equivalent to an identical change in object position (e.g., a 1 nm change in focal position causes different changes to the interferogram than a 1 nm change in object height). Thus, while recording z_c versus time would give information on fluctuation in the focal position, it does not indicate the stability of RISM measurements. To test the stability of RISM measurements directly, we simultaneously implemented the stabilizing routine while performing independent RISM measurements of the apparent fluctuation in the height of beads immobilized on the glass surface [Fig. 2(b)]. Any measured change in bead height would then be caused by unremoved drift in the apparatus rather than actual bead motion.

A solution of 4.5 μm beads was allowed to sediment onto the glass surface; after some time, the beads became immobilized on the glass (through nonspecific interactions), as judged by the complete disappearance of their Brownian motion. For these spherical objects, the RISM interferogram consists of concentric rings [Fig. 2(b)], and changes in height or focal position change the radius of these rings. According to numerical calculations based on Ref. [8], the radius of the first interference ring is linearly dependent on the actual height, with a 1 nm change in actual height corresponding to a 4 nm change in the radius. Here, actual height is fixed, and we quantify unwanted focal position motion through the apparent height h_{eff} using the same linear factor.

Figure 7(a) shows the change of h_{eff} with time; each data point was calculated in real time from the acquired images over 50 min with a 60 Hz frame rate. For the first 45 min, the focal position was corrected every 1 s with $\alpha = 0.5$ and $\delta z_{\text{max}} = 100$ nm, and the measured h_{eff} was very stable. We turned off the correction routine around $t = 45$ min, and the apparent height of the bead immediately showed large fluctuations. Thus, the routine successfully removed drift from the apparatus. The Fig. 7(a) inset magnifies 6 s of data around $t = 30$ min, which clearly shows that the fluctuation in h_{eff} was well under 1 nm. In fact, during the entire 45 min trace utilizing stabilization, the standard deviation of δh_{eff} was 0.12 nm and the noise was normally distributed [see Fig. 7(b)], while the focal position was maintained within ± 10 nm of the target position.

The reduced fluctuation in measured height relative to that of the focal position is an advantage for applying this method to tracking objects attached or tethered to a glass surface. The measured standard deviation of the apparent motion of the bead is smaller than the spatial scale of many biological materials, such as the thickness of lipid bilayers (~ 2 nm) or the base pair separation in double-stranded DNA (~ 0.34 nm). Thus, we expect our stabilization technique to be useful for the high-resolution study of biomolecular behavior. In particular, a variety of tethered-particle techniques, in which a DNA or protein molecule is used to tether a colloid to a glass surface, have been shown to be powerful probes of the physical behavior of single biomolecules [17–20]. When a force is applied to the particle (using, e.g., optical or magnetic methods), the particle's Brownian motion can be reduced to less than 1 nm. In these cases, we expect RISM particle tracking and the presented stabilization method to improve the resolution (signal-to-noise ratio) of the measurement.

4. Conclusion

We have developed a stabilizing method based on linear focus functions that improves the stability of reflection interference contrast microscopy. By use of this method, the standard deviation of the apparent vertical position of an immobilized bead was 0.12 nm for a period of 45 min. We expect this technique will be useful in applications that involve long-term, high-resolution tracking of objects that move near glass surfaces, such as adhesion studies of lipid bilayer vesicles and cells [2–6,22] or tethered-particle experiments [17–20,23].

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Fig. 1. (Color online) Schematic diagrams of the illumination geometry for RICM: L, Hg Lamp; AD, aperture diaphragm; FD, field diaphragm; F1, incident bandpass filter; F2, imaging bandpass filter; P, parfocal lens; M, beam splitter; PI, piezo-objective positioner; Obj, objective lens. n_o , n_L , and n_G are refractive indices of the object, water, and cover glass, respectively, and CCD represents a CCD camera with a 60 Hz frame rate. The inset shows the optical geometry of reflecting beams near the object. R is the radius of the spherical object and h is the separation distance from the water–cover glass interface.

Fig. 2. (Color online) (a) Schematic diagram of reference (left) and testing (right) objects. z_t is the objective position when properly focused. (b) Image acquired near proper focus. The left and right interference images are formed from the reference and testing objects, respectively. The shadow image of the field diaphragm can be seen in the bottom-left corner.

Fig. 3.

(a) Radial intensity profiles $I(r)$ calculated from interference images of the reference object in Fig. 2. The dashed box illustrates the region that is eventually used for the focus function. $I(r)$ values correspond to 8 bit intensities per CCD pixel. (b) Focus functions calculated from the intensity profiles in (a); note that F_r is calculated as a unitless relative intensity [Eq. (2)].

Fig. 4. (Color online) Comparison of optimal focus functions measured with different methods. $F^*(z)$ was calculated by use of either 100 nm objective steps (filled circles) or 10 nm steps (open circles). A cubic-spline interpolation (solid line) between the 100 nm steps matches the data taken using 10 nm steps.

Fig. 5.
 (Color online) Optimal focus function selected from those in Fig. 3(b) ($r = 7$). z_t was calculated by interpolating $F^*(z)$ at F_{target}^* .

Fig. 6. Focal position versus time during feedback control. The standard deviation of the focal position from raw data acquired at 60 Hz was 9.8 nm.

Fig. 7. (Color online) (a) Change of the apparent separation distance h_{eff} calculated from the testing object (a $4.5 \mu\text{m}$ bead). Raw data acquired at 60 Hz (the frame rate of the CCD camera) are shown; no filtering was applied to these traces. The feedback routine was turned off around $t = 45$ min. The inset shows a magnification of the boxed area. (b) The probability distribution of the change of h_{eff} for 45 min. The distribution is well fit by a Gaussian function (solid line).