An automatic focus/hold system for optical microscopes

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A system for maintaining long-term focus of samples under high-magnification quantitative observation in an epi-illumination optical microscope is described. A negative feedback signal is generated from focus-dependent changes in the backreflection of an off-axis HeNe laser. This reflection is intercepted by a small prism downbeam from the standard trinocular head, and detected by a small two-photodiode array. Spontaneous drifts in sample focus (presumably due to thermal and mechanical relaxations) are detected as a nonzero difference signal, which is used to drive a dc motor mechanically coupled to the fine-focus knob of the microscope. This system has several advantages: (1) it is completely compatible and nonobstrusive with concurrent data acquisition of sample intensities; (2) it requires no alteration of the sample, sample stage, or objective; (3) it monitors the focal position of sample areas very near to those under observation; (4) it is inexpensive. In an experimental test, the system can hold a thin glass coverslip sample (a common substrate for biological cell cultures) to within 0.5 μm of its preset focus position, well within the depth of focus of the microscope. Without the system, such samples typically drift several micrometers over periods of 10 min. In response to a disturbance of the focus knob, the system can restore the focus to within 0.5 μm of the preset position.

I. INTRODUCTION

Long-term observation of thin samples by high magnification, short depth-of-focus microscope optics is often hampered by a drifting focus, apparently due to slow thermal fluctuations and stress relaxations in the sample. We describe an optically based automated negative feedback technique for holding a sample in focus on a microscope stage for an arbitrarily long time. The system requires no modification of the sample stage and is compatible with concurrent acquisition of light intensity data (either total light flux or imaging) from the region near the center of the field of view. We have successfully employed this focus/hold system in quantitative fluorescence microscopy experiments in biophysics on samples which would drift out of focus within a few minutes without the system engaged.

This system requires only inexpensive components as accessories to a standard epifluorescence microscope equipped with a trinocular head and photometer unit: a 10-mW helium-neon laser, a two-detector photodiode, a dc motor with built-in gear reduction, and some simple optics and electronics.

The negative feedback focusing signal is derived from an optical signal. A HeNe laser beam is transmitted off axis through the microscope's epi-illumination system to a near focus on the glass/water or glass/air interface (generally a coverslip) at the sample plane. As the sample drifts out of focus, the image of the HeNe beam reflection both moves laterally and changes its size. The lateral motion is monitored by a two-detector photodiode whose output is connected to a difference amplifier which drives a small dc motor connected to the fine-adjust focus knob of the microscope.

Some other techniques that have been used to keep samples in focus on a microscope stage for long-term experiments involve monitoring some distance and using changes in the distance to provide an error signal to adjust the focus. Fay *et al.*[1] use an eddy current sensor attached to the microscope nose piece to monitor distance to the stage. Lanni[2] uses the distance-dependent capacitance between the sample holder and a collar attached to the objective to provide the error signal for piezoelectric elements which support the sample holder. Shack *et al.*[3] describe a method for keeping a translating microscope slide in focus by placing a holographic grating in the pupil of the objective lens. The special grating produces two defocused first-order images which are detected by separate sensors which provide focus correction signals.

The focus/hold system described here has several desirable features. First, the system is inexpensive, compatible with existing epi-illumination, imaging, and photodetection microscope optics, and requires no modification of the sample, sample holder, objective, or stage. Second, the region of the sample held within this focus range is very close to the area of observation for data acquisition, since the HeNe laser beam passes through the same microscope objective used to observe the sample. Third, we find experimentally that this technique can hold the coverslip to within ± 0.5 μm of its original preset focus position; this range is within the depth of focus of the microscope.

II. OPTICS

The optics of the focus-hold system consists of two segments: illumination of a small part of the sample plane (usually at or near a glass/water interface in cell biological studies) by a HeNe laser beam focused near the region of observation, and continuous monitoring of the reflection of

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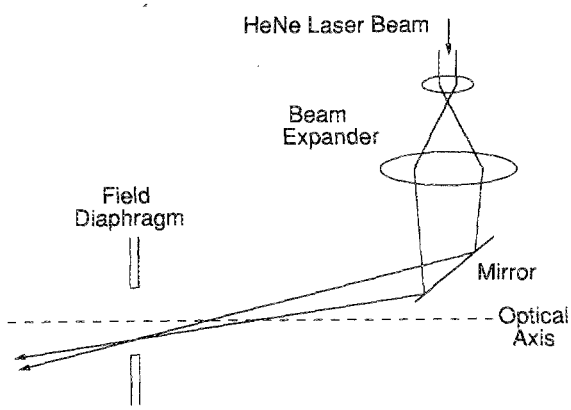

FIG. 1. HeNe beam used for the focus-hold system is expanded and focused to an off-center point at the field diaphragm as it enters the microscope. The beam path is not parallel to the optical axis as it enters the microscope. Note that the optical axis of the microscope is not obstructed by the HeNe optics.

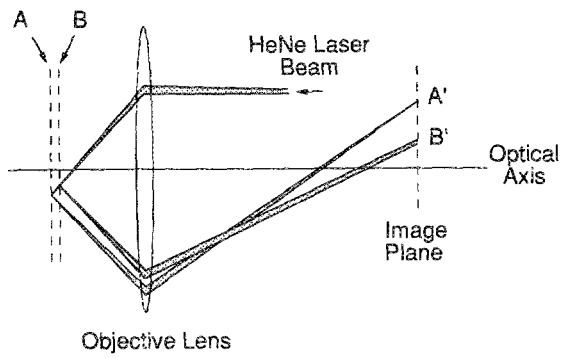

FIG. 3. Depiction of how movement of reflecting surface near object plane results in lateral movement and some defocusing of reflected HeNe beam at a detector located off axis at an image plane. With reflecting plane at *A*, the HeNe beam hits detector focused at *A'*. If reflecting plane moves to *B*, the HeNe beam hits detector defocused ats *B'*.

the HeNe beam by the photodiodes. Neither segment interferes with the normal epifluorescence data collection functions of the microscope.

A 10-mW HeNe laser provides the monitoring light for the focus/hold system. Figures 1 and 2 show the path of the HeNe laser beam. In Fig. 1, the HeNe beam is directed toward the microscope at a slight angle with respect to the optical axis so that the HeNe optical elements do not obstruct the active epi-illumination light path. Before entering the microscope (in our case, a Leitz Diavert inverted fluorescence microscope), the HeNe beam passes through two lenses which expand the beam and then focus it to an off-center point at the field diaphragm plane of the microscope. Figure 2 shows the path of the beam after it passes through the field diaphragm and encounters the dichroic mirror of the microscope epi-illuminator system. Because the HeNe beam wavelength is longer than the reflection

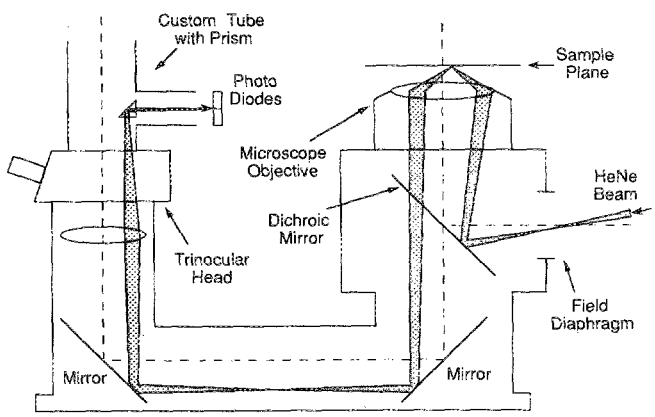

FIG. 2. Path of the HeNe beam (shaded region) through the microscope and through the custom adaptor tube to the photodiode array. Only those transmitted and reflected beams which go to the photodiode array are shown. Note that the optical axis (dashed line) is unobstructed by the focus/hold system. For clarity, we show the HeNe beam off axis (with respect to the optical axis) in the plane of the diagram. Diagram not to scale.

edge wavelength of the dichroic mirror, the beam partially reflects off both the front and back surfaces of the dichroic mirror, and passes through the microscope objective toward a focus as two off-center adjacent spots at the sample plane. These two spots then partially reflect off the glass/water interface at the sample plane.

The reflected HeNe light is gathered by the objective, along with the transmitted, scattered or emitted light of the sample itself, and is transmitted through the dichroic mirror/barrier filter module and the trinocular head. Immediately on top (downbeam) of the trinocular head is a custom-built adapter tube. This tube contains a small off-axis right-angle prism, fixed in position, which intercepts the off-axis HeNe light and directs it out a port to a small photodiode array about 10 cm away. Since the prism is small (5×5 mm), and located off axis, the center of the microscope's field of view is unobstructed by the prism. A blackened pipe is attached to the adapter tube at the port to reduce the amount of stray room light entering the port. The entire path from the port to the photodiode array need not be enclosed if experiments are performed in a darkened room; leaving the space in front of the photodiode array open allows for easy accessibility during adjustment.

In our system, the photodiode array is very simple: it consists of two photodiodes separated by a 0.13-mm gap enclosed in a single package (United Detector Technology Model PIN-Spot/2D). The array is positioned so that the two adjacent HeNe reflections straddle the boundary between the two diodes, falling in approximately equal portions on each diode and producing a difference signal near zero. With the diode array properly oriented, a drift in the focus of the sample plane results in a lateral translation of the HeNe reflection spots from their centered position toward one of the diode elements, thereby producing a large difference signal (see Fig. 3). A drift in the opposite direction will produce a difference signal of the opposite sign.

The lateral translation of the HeNe spots at the diode array arises from the off-axis course of the HeNe beam with respect to the optical axis. As the sample focus drifts,

the HeNe spots at the diode array also defocus, but significant lateral motion occurs before much defocusing of the spots. (See Fig. 3.)

A HeNe laser of significantly less than 10 mW will not be adequate due to the low reflectivity at the dichroic mirror and the glass/water interface. However, samples with a glass/air instead of glass/water interface produce a stronger HeNe reflection, thereby permitting use of a lower-power HeNe laser.

Filters may be needed to block errantly scattered HeNe light from interfering with the normal detection of sample light in the epifluorescence microscope. For example, in our biophysics experiments, we use a photomultiplier tube atop a standard commercial photometer unit (Nikon Model PFX) with an adjustable image plane diaphragm to selectively monitor emission from fluorescein-labeled biomolecules located in the central region of the field of view. The intensity of fluorescence is often too low to be seen by eye through the trinocular eyepieces. In contrast, the off-axis directed HeNe light which scatters into the central region is easily visible. We find that a 6-mm thickness of Schott BG-18 colored glass sufficiently attenuates the HeNe 633-nm line while passing half of the fluorescein fluorescence intensity (at ~500–530 nm) to the photomultiplier. In our particular setup, the BG-18 colored glass, cut into two 3-mm-thick, 9-mm-diam circles, is placed directly on top of the rectangular image plane diaphragm in the photometer unit which is located just downbeam from the right-angle prism that deflects the directly reflected HeNe light to the diode array. In other commerical photometer units, there are special filter ports at an appropriate location just before the main photodetector.

A neutral density filter is slid in front of the HeNe laser to attenuate the HeNe beam during visual observation of the field of view. In addition, a long-pass filter may be placed in the deflected HeNe light path if necessary to block sample fluorescence from getting to the photodiode array.

III. ELECTRONICS

The difference signal from the two-detector photodiode array is filtered and amplified to drive a dc motor attached to the fine-adjust focus knob. Figure 4 shows the schematic of the circuit. The two signals from the two-detector photodiode go through high-gain/low-pass amplifiers, A_1 and A_2, and then into a difference amplifier A_3. After variable gain amplification in A_4, the difference signal enters a driver circuit which provides sufficient current to the dc motor M. The 1-MΩ feedback on A_4 provides high gain to the driver transistor bases in order to quickly bias them into conduction at which point the 220-kΩ feedback keeps the driver transistor emitters from saturating too easily. The voltmeter V (± 10 V) allows the user to monitor and thereby minimize the difference signal as the HeNe spot is adjusted to fall equally on the two diode detectors prior to engaging the focus/hold system. Switch S disengages the motor from the circuit. The six diodes at the output limit the voltage to the motor to about ± 2 V. The driver transistors are current limited to protect the diodes.

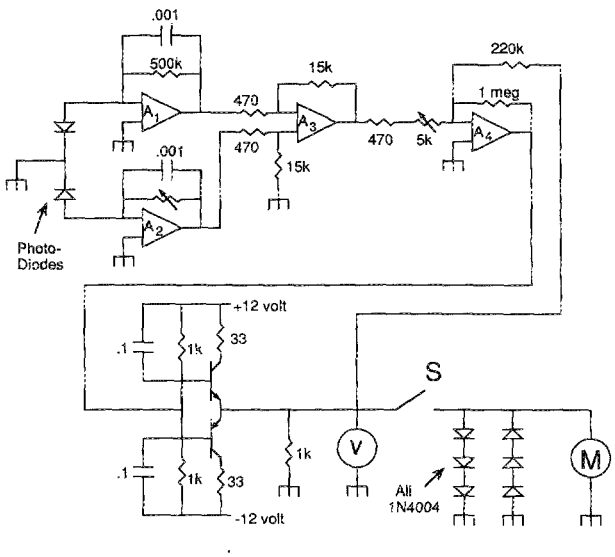

FIG. 4. Schematic of the electronic circuit which uses input from the two-detector photodiode to drive a dc motor which is connected to the microscope's fine-adjust focus knob. A_1 and A_2 are LM307 op-amps, A_3 and A_4 are a LM1458 dual op-amp, and the *NPN* and *PNP* driver transistors are NTE49 and NTE50, respectively. The feedback resistor on A_2 is adjusted close to 500 kΩ so that the output voltage of the driver stage is zero when no light hits the photodiodes. All resistances are in ohms and capacitances in microfarads. *S*—switch, *V*—voltmeter; *M*—dc motor.

The feedback resistor on A_2 is a trimpot covering a range around 500 kΩ and is adjusted so that the output voltage of the circuit is zero when no light hits the photodiodes.

The photodiode array and all electronics up to the output of the driver transistors are housed in a 2 in.×3 in.×4 in. aluminum box, with the face of the photodiode array flush mounted at a hole in the box. The box itself is mounted on an *x-y* translator which is rigidly attached to the custom adaptor tube containing the HeNe beam deflecting prism on top of the trinocular head. The translator allows the box (and therefore the photodiode array) to be positioned precisely relative to the HeNe reflection spots.

The dc motor is a servomotor (JR Servo NES-507) with its feedback electronics disconnected. This motor has built-in gear reduction. It starts up at about 0.7 V, drawing about 100 mA, and rotates at a few seconds per revolution.

IV. ADJUSTMENT AND OPERATION

For the initial setup, rough positions for the beam expander (which controls the HeNe spot location on the sample plane) and the deflecting prism must be established so that the spot is off center but its reflection can be captured by the prism. Whether the HeNe beam is properly intercepted can be checked by scattering from an index card temporarily inserted in the open space between the prism adapter tube and the photodiode box. This initial setup should be done with an objective and sample similar to those intended for the actual experiment. For continuing work with the same objective, this setup need not be repeated even if samples are changed.

The next step is to focus and finely center the HeNe spot at the center of the two-detector photodiode. The electronics should be turned on, but the switch S should be left open to disengage the motor so the focusing knob can be turned manually. The sample is then focused. Focusing the HeNe spot is accomplished by making fine adjustments to the longitudinal position of the second lens of the beam expander while watching the spot on a white card just in front of the photodiode. The HeNe spot is centered at the photodiodes by monitoring the voltmeter V while adjusting the x-y translator that mounts the photodiode box so that the voltage is zero. When the spot is focused and centered the motor can be engaged by closing S.

The focus of the spot should be checked every time a new sample is put on the stage. Fine centering has to be done every time the sample is refocused or translated on the stage. With practice, both of these tasks are easily done in a few seconds.

In our setup the photodiode is not quite at an image plane. Therefore, as the beam expander is adjusted to focus the HeNe spot at the photodiode, the spot becomes slightly defocused at the sample plane. If desired, parfocality between the sample plane and the photodiode face can be restored by an appropriate lens between the prism and photodiode. The lateral position of this lens can be adjusted to center the spot at the photodiode, rather than moving the photodiode itself.

It is not necessary for the in-focus plane to be at the glass/water interface at which the HeNe reflection occurs; the HeNe reflection can still be focused at the photodiode. Therefore, microscope experiments on cell cultures can still employ the focus/hold system while the microscope is focused on a part of a cell not immediately adjacent to the coverslip. In practice, the focus/hold system holds the glass/water interface to within 0.5 μm of its original position even if this interface is 10 μm from the object plane.

The sensitivity of the focus-hold system can be varied by adjusting the gain of A_4 (see Fig. 4). For the highest sensitivity, the gain should be set just below the level that causes the motor to oscillate. The motor must be rigidly coupled to the fine-adjust focus knob, otherwise the motor will go into oscillation even with low-gain settings.

We determined the effectiveness of the system by setting the focus/hold system and noting the orientation of the focus knob, then disengaging the motor and manually defocusing the knob, then reengaging the motor and noting the orientation to which the focus knob returned. The calibration of the focus knob (assuming negligible play in the gears) showed that the sample was reproducibly returned to within 0.5 μm of its original position. This result agrees with the observation that while the focus-hold system is operating, the magnitude of the "correction" rotations of the knob correspond to vertical motions of the stage of about 0.5 μm.

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