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Simultaneous immersion Mirau interferometry

Oleksandra V. Lyulko,[a] Gerhard Randers-Pehrson, and David J. Brenner
Radiological Research Accelerator Facility, Columbia University, 136 S. Broadway, Irvington, New York 10533, USA

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A novel technique for label-free imaging of live biological cells in aqueous medium that is insensitive to ambient vibrations is presented. This technique is a spin-off from previously developed immersion Mirau interferometry. Both approaches utilize a modified Mirau interferometric attachment for a microscope objective that can be used both in air and in immersion mode, when the device is submerged in cell medium and has its internal space filled with liquid. While immersion Mirau interferometry involves first capturing a series of images, the resulting images are potentially distorted by ambient vibrations. Overcoming these serial-acquisition challenges, simultaneous immersion Mirau interferometry incorporates polarizing elements into the optics to allow simultaneous acquisition of two interferograms. The system design and production are described and images produced with the developed techniques are presented. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4803181]

I. INTRODUCTION

Non-invasive imaging methods are becoming increasingly popular in cell biology because of the growing complexity of the endpoints.[1–6] This becomes particularly critical when the imaging technique is used for targeting cells for a chemical or radiation assault and subsequent long term tracking. Perhaps the most common method of cell visualization, fluorescent staining, particularly using UV-illumination, potentially induces cytotoxic and phototoxic damage in addition to the cellular response to the assault under study.

As an example, the Columbia University Radiological Research Accelerator Facility (RARAF) provides the radiation research community with single-particle, single-cell microbeam irradiation facilities for studying the biological effects of exposure to ionizing radiation. A component of each of these microbeam irradiation platforms is an imaging system that enables the targeting of individual cells or subcellular structures. Users typically provide their own cell lines and endpoint of interest, which in turn requires an imaging technique at RARAF to work on all cell types, including those that do not contain fluorescent proteins. To address the concerns from the RARAF users and collaborators that fluorescent stains and UV-light may alter cellular response to irradiation, significant emphasis in recent years has been placed on developing label-free imaging techniques[7,8] that would work in an epi-illumination imaging mode.

In the charged-particle microbeam irradiation platforms at RARAF, energetic particles are delivered to the target through the beam exit window below the sample. The beam line terminus occupies the entire space below the microscope stage in this configuration, while the microscope objective is located above the dish[8,9] (Fig. 1). Due to these geometric constraints, the use of transmitted illumination at the microbeam endstation is not feasible and the imaging system requires epi-illumination. After several epi-illumination non-stain imaging techniques have been tested, Mirau interferometry was chosen for its compact arrangement suitable for incorporation into the microbeam endstation and simplicity of alignment and operation.

In developing a Mirau interferometry technique for cell imaging we have progressed through several stages: from using a commercial Mirau attachment in air to using it with medium and to building a custom immersion Mirau interferometer. This work resulted in Immersion Mirau Interferometry (IMI)—a non-invasive interferometric technique for imaging of biological targets in medium.[8] Furthermore, to enable interferometric imaging in the presence of ambient vibrations, we developed Simultaneous Immersion Mirau Interferometry (SIMI)—a vibration-insensitive modification of IMI. This paper describes the evolution from Mirau interferometry to IMI and then to SIMI, discusses its principles, presents the design of the apparatus and image-based results obtained with the SIMI interferometer.

II. BACKGROUND AND MOTIVATION

A. Mirau interferometry

A Mirau interferometer is a compact system, comprising a beam-splitting surface and a reference mirror which are parallel to each other and normal to the axis of the microscope objective.[8,10] The test (sample) and reference paths can be equalized by adjusting the position of beam-splitting and reference surfaces with respect to the objective. The data acquisition technique is based on the principles of Phase-Shifting Interferometry (PSI): a series of interferograms is acquired at different times as the path differences between the test and reference beams are incremented by fractions of wavelength; the image of the sample is reconstructed point-to-point using corresponding pixel intensities from the individual interference patterns.

If δ_i is the increment of the phase of the test beam for the ith measurement, then the resulting intensity at a point (x, y)

[a]Author to whom correspondence should be addressed. Electronic mail: ovl1@caa.columbia.edu

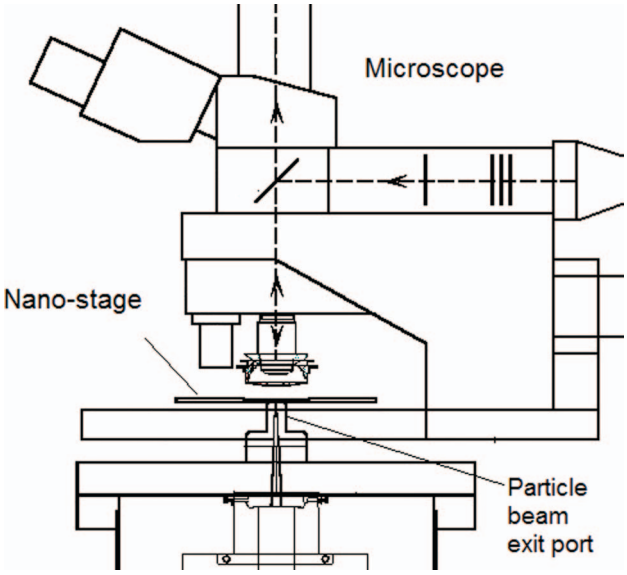

FIG. 1. Configuration of the RARAF microbeam endstation.

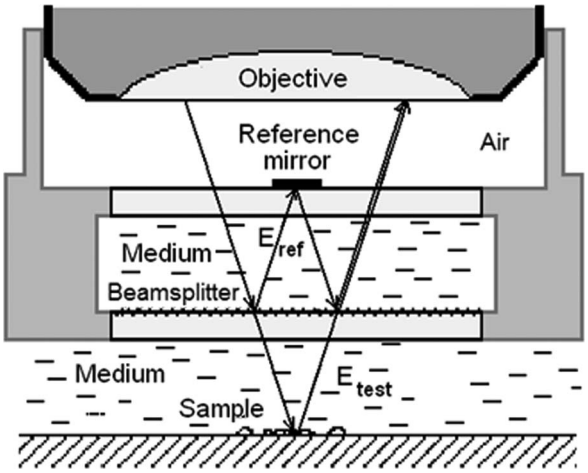

FIG. 2. Schematics of immersion Mirau interferometric attachment.

can be described by the formula:

$$I_i(x, y) = I_b(x, y) + I_m(x, y)\cos[\varphi(x, y) + \delta_i], \quad (1)$$

where $I_b(x, y)$ is the average (background) intensity, $I_m(x, y)$ is the fringe or intensity modulation, and $\varphi(x, y)$ is the phase difference between the test and reference beams at a given point. The most common PSI data collection technique, the "four-step algorithm"[11] with $\delta_i = 0, \pi/2, \pi, 3\pi/2$, $i = 1, 2, 3, 4$, which simplifies the intensity equation (1), was implemented at RARAF. Equation (1) can be solved at each point for the intensity modulation:

$$I_m = \frac{1}{2}\sqrt{(I_1 - I_3)^2 + (I_4 - I_2)^2} \quad (2)$$

or for the phase:

$$\varphi = \tan^{-1}[(I_4 - I_2)/(I_1 - I_3)]. \quad (3)$$

Solving the system of Eq. (1) for the phase requires phase unwrapping due to the 2π phase ambiguities.[12] The phase carries information about optical thickness of the sample, while targeting cells for irradiation does not require information about the cells' vertical profile. Thus, to determine the coordinates of the cells in the x-y plane one can solve the system for the intensity modulation $I_m(x, y)$ and identify the cell contours without phase unwrapping, which simplifies processing and increases throughput.

B. Transition to the immersion mode (IMI)

Under the standard protocol for an irradiation experiment at RARAF, when the cell dish is positioned on the stage of the endstation, most of the cell growth medium is aspirated from the dish and the cells remain covered with only a thin layer of medium. In this scenario, the liquid slowly evaporates, causing interference fringes to drift, which distorts the image acquisition. To make the interferometric imaging feasible, a novel approach has been developed, IMI, where the cells remain in the growth medium, the apparatus is submerged in the liquid and the space between the beam-splitter and the reference mirror contains liquid with the same index of refraction (Fig. 2).[8]

C. Vibration-insensitive imaging approach (SIMI)

Another modification resulted from by the sensitivity of interferometric measurements to the vertical position of the sample. At RARAF, the microbeam endstation is an integral part of the particle beamline and thus cannot be vibration isolated. In the presence of minute (subwavelength) vibrations, the stage positions cannot be maintained with the desired precision for interferometry imaging and the quality of the reconstructed image is significantly reduced. The problem of vibrations can be resolved by acquiring all interference patterns at the same time to "freeze" vibrations.[13–15] SIMI employs polarization elements integrated with its optics to encode multiple phase shifts in a single beam.

III. SIMULTANEOUS IMMERSION MIRAU INTERFEROMETRY

A. Design

The schematics of the SIMI system are presented in Fig. 3, inset. A $\lambda/8$ waveplate, oriented along the x-axis, is introduced to the test arm of the Mirau interferometric attachment below the beam-splitter that separates the test and reference beams. When the incident light is linearly polarized at 45° with respect to the x-axis, the x-component of polarization of the test beam is delayed by $\pi/4$ (1/8 of the wavelength) each time the beam passes through the waveplate. Therefore, as a result of the double traversal of the waveplate, the x-component of the test beam experiences a 1/4-wavelength phase shift with respect to the y-component. Using a polarization-splitting element (a beam-splitter or a beam-displacer), the x-components of both test and reference beams can be spatially separated from the y-components and recorded. Since the x-component of one of the beams is delayed by 1/4-wavelength, the interference pattern, formed by

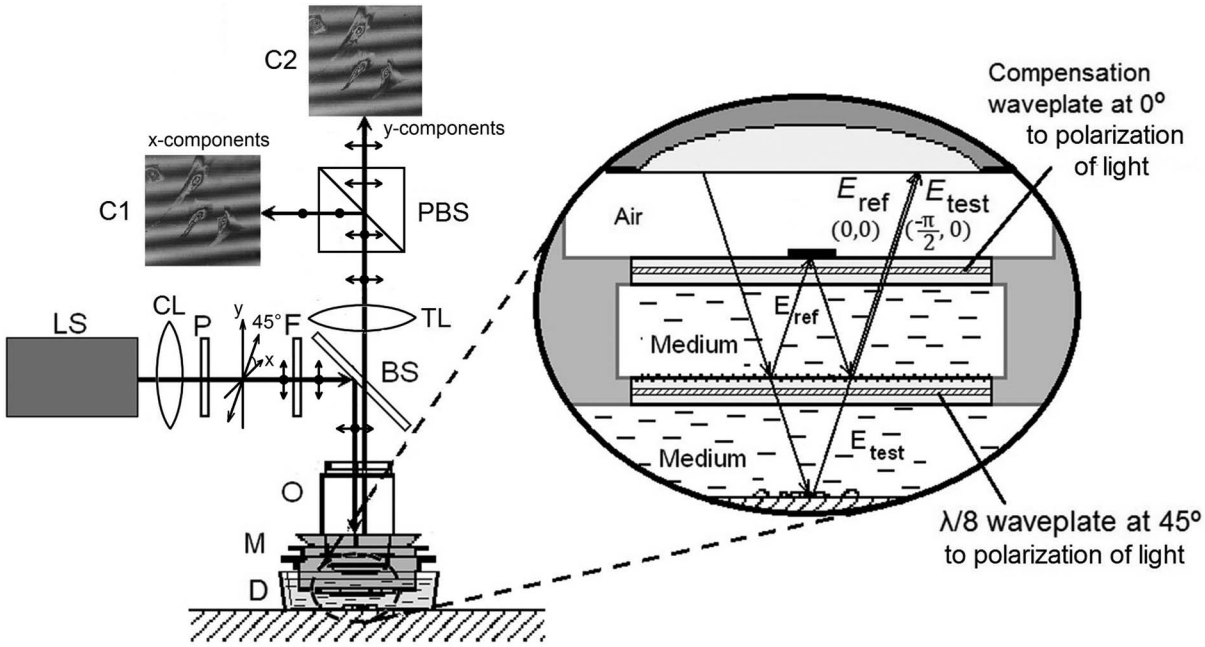

FIG. 3. Components of SIMI: The light from a light source (LS) passes through a collimator lens (CL), a linear polarizer (P), and a band-pass filter (F) is reflected by a 50/50 beam-splitter (BS) and directed to the objective (O) with mounted SIMI interferometer (M). The interferometric attachment fits into the microbeam Petri dish (D). A λ/8 waveplate, placed in the test arm of the interferometer, introduces a quarter-wave phase shift to one component of the test beam (inset). Successive application of a polarization beam-splitter (PBS), that follows a tube lens (TL), enables separation of two interference patterns with different phase that are recorded on camera sensors (C1, C2). The arrows denote the directions of polarization.

the x-components, will have fringes shifted by 1/4 of their width.

The system of Eq. (1) for interferogram intensities has three unknowns at each point of the image—the fringe modulation, the background, and the phase. In the geometry of the Mirau interferometer, with the beam-splitting plane normal to the incident beam, the SIMI technique described above allows for encoding of only two interferograms. Provided that the phase shift between interferograms is equal to $\pi/2$, the set of Eq. (1) takes the form:

$$I_1(x, y) = I_b(x, y) + I_m(x, y)\cos[\varphi(x, y)], \tag{4a}$$

$$I_2(x, y) = I_b(x, y) - I_m(x, y)\sin[\varphi(x, y)]. \tag{4b}$$

Additional information can be added by taking a background image out of focus, at a position where interference fringes are not visible:

$$I_3(x, y) = I_b(x, y) \tag{5}$$

and subtracting Eq. (5) from each of the Eqs. (4). After elimination of the background intensity, only the fringe modulation $I_m(x, y)$ and the phase $\varphi(x, y)$ are left. After the range of pixel intensities of interferograms are normalized in the interval from -1 to 1, the fringe modulation can be found as

$$I_m(x, y) = \{[I_1(x, y) - I_3(x, y)]^2 + [I_3(x, y) - I_2(x, y)]^2\}^{1/2}. \tag{6}$$

The phase can be calculated as

$$\varphi(x, y) = \tan^{-1}[(I_1 - I_3)/(I_3 - I_2)]. \tag{7}$$

B. Interferometric attachment

The Mirau interferometers available commercially (e.g., Leica, Nikon, Zeiss) are designed and mostly used for surface metrology in air.[13,16] In the described application, a Mirau interferometer was intended to be used for interferometric imaging in a fully immersion mode. The use of a "dry" attachment is limited because of leakage and interaction of the medium content with the surface of the attachment body and

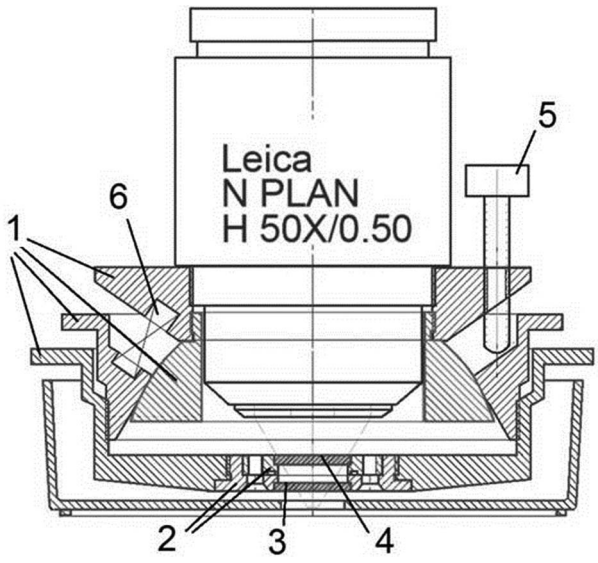

FIG. 4. Design drawing of immersion Mirau interferometric attachment: 1—attachment body, 2—optics holders, 3—beam-splitter, 4—mirror, 5—adjustment screw, and 6—spring.

the optics coatings. Thus, the immersion modality required a specially designed interferometric attachment.

A custom immersion Mirau interferometric attachment for a microscope objective was designed and built in-house.[8] The attachment is compatible with a threaded Leica N PLAN H 50X/0.50 objective (Fig. 4) with a working distance of 9.5 mm in air. The ball-and-socket mechanism with springs and adjustable lever posts regulates the inclination of the reference mirror and the beam-splitter with respect to the sample plane. The shape of the attachment body is designed to fit into the microbeam dish. The optics holders contain two windows: one with the beam-splitter and another with the spot mirror. To fill the space between the two windows with liquid for immersion imaging, the optics holders were assembled while submerged in a bath with distilled water. Interchangeable beam-splitters with different reflectance values facilitate adjustment of the intensities of the test and reference beams, which allows imaging of a variety of samples with different indices of refraction. Beam-splitters with reflectances of 10% and 25% in air, corresponding to 4% and 15% in water, respectively, were used for cell imaging. The optics holders can be easily disassembled for cleaning and re-filling with liquid.

C. Fabrication of optical components

SIMI utilizes the body of the IMI attachment with the modified optics which incorporates λ/8 waveplates. Polycarbonate phase retarders were chosen for SIMI because of their superior retardance properties compared to that of mica and quartz: good accuracy and wide angular field of view, i.e., little dependence of retardance on the angle of incidence.[17] To protect the polycarbonate waveplates from exposure to water, which may change their retardance properties, each waveplate was cemented between two thin glass windows with optical adhesive (Fig. 3, inset). The glass windows are thin enough to accommodate the thickness of polymer and the optical epoxy while the total thickness of the assembly matches that of the windows of the IMI attachment.

Identical assemblies containing the same waveplate film were used in both the test and reference arms. The lower waveplate creates the phase shift between the x- and y-components of the test beam and the upper waveplate is introduced to restore identical optical paths in the test and reference arms.[13] To preserve the polarization components of the light incident on the beam-splitter and the lower (main) waveplate, the slow axis of the upper waveplate is oriented at 0° with respect to the light polarization.

The top side of the lower assembly has a beam-splitter coating. The coatings were chosen so that their reflectance and transmission properties, as well as the phase shift upon transmission and reflection, are independent of the polarization of light. A reference spot mirror is coated on the top side of the upper assembly.

D. Splitting the polarization components to create interferograms

The x- and y-polarization components of both the test and reference beams can be spatially separated with a polarization-splitting element (a polarization beam-splitter or a beam-displacer) placed after the modified Mirau interferometer (Fig. 3). The outputs of the polarization-splitting element form two separate interferograms with a $\pi/2$ phase shift due to the phase shift between the x-component of the test beam and the x-component of the reference beam.

A polarization beam-splitter separates the orthogonal components of polarized light by reflecting the component perpendicular to the plane of incidence; the interferograms are then formed on two separate sensors. The drawback of such an arrangement is that any misalignment between the two sensors affects the quality of the reconstructed image.

A beam-displacer, on the other hand, allows capturing of both interferograms with a single camera. A beam-displacer is made of uniaxial birefringent material (calcite) with two possible velocities and two possible directions of propagation for each incident beam. The beam with the ordinary polarization component is transmitted without deflection. The beam with the extraordinary polarization is steered in the birefringent medium and emerges parallel to the ordinary beam with a certain separation between the two beams. Thus, the output beams of a beam-displacer are parallel to each other and can be sent to the sensor of a single camera. This approach simplifies alignment; however, the image resolution is limited because the separation distance of the beam-displacer defines the width of the field of view.

E. Optics alignment

To equalize the test and reference paths of the SIMI interferometric attachment, the height of the reflecting surfaces with respect to the objective is adjusted by rotating the focusing ring of the attachment. This procedure changes the orientation of the axes of the incorporated waveplates. The alignment of the direction of polarization of the incident light along the axes of the waveplates is then done by rotating the polarizer. The angular position of the polarization-splitting element and the camera sensor(s) about the vertical axis can be adjusted using a custom adaptor for one or two cameras to ensure optimal splitting of the polarization components of the output light. The adaptors built in-house consist of the main body with inside mounts for a beam-splitter or a beam-displacer and rotatable C-mounts for the cameras (Fig. 5).

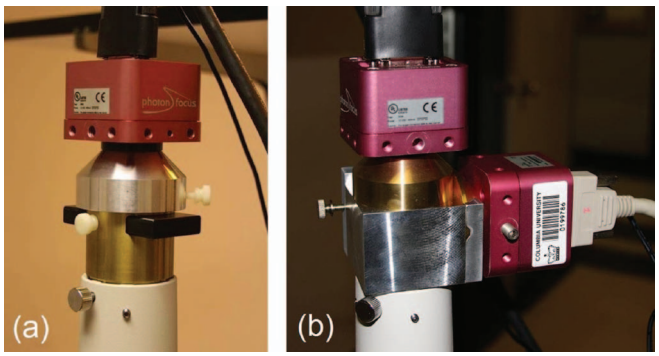

FIG. 5. Modifications of the camera adaptor of the SIMI microscope module: (a) adaptor for one camera with a polarization beam-displacer fitted inside; (b) adaptor for two cameras containing a polarization beam-splitter.

IV. IMAGING RESULTS

Imaging with the developed SIMI system was performed on a Nikon Eclipse 50i microscope with a rotatable polarizer and a beam-displacer with a separation distance of 4 mm (BD40, Thorlabs, Newton, NJ) or a beam-splitter (Polarizing Cube Beamsplitter, Edmund Optics, Barrington, NJ) incorporated into one of the custom camera adaptors described above. The microscope was mounted on a table with no vibration isolation. The interferograms were recorded using CMOS cameras (MV-D1024-CL, 10.6 μm pixels size, Photonfocus AG, Lachen, Switzerland). The samples were illuminated with an X-Cite 120 Series light source (EXFO, Life Sciences & Industrial division, Mississauga, Ontario, Canada). Monochromatic light was selected using a band-pass filter and directed to the objective by a 50/50 neutral density dichroic mirror. The SIMI attachment was mounted onto a threaded Leica 50× microscope objective (Leica Microsystems, Wetzlar, Germany).

Before the production of the assemblies that incorporate the waveplates and precision windows, the concept of simultaneous Mirau interferometric imaging in immersion mode was tested using a prototype SIMI assembly. The prototype assembly consisted of the immersion Mirau interferometric attachment built for IMI and polycarbonate $\lambda/8$ waveplates (Bolder Vision Optik, Boulder, CO) cut to fit inside the optics holders and applied directly to the windows. The attachment was filled with distilled water and submerged under the PBS (phosphate buffered saline) solution containing the cell sample.

After the polarizing assemblies had been manufactured, subsequent experiments were performed using the immersion Mirau attachment with the custom-manufactured polarization optics. Each window contains a layer of polycarbonate waveplate that was laminated between two pieces of glass. Similarly, the optics holders were filled and secured while submerged in water. Before filling, the attachment was kept in an ultrasonic bath for a few minutes to remove all the air that may have been captured in the device. During the imaging, the attachment was submerged in the liquid covering the cells.

The cells for experiments were grown either on glass slides or on thin mica substrates attached to the bottoms of the wells of microbeam dishes (modified Petri dishes, typically used for microbeam experiments, with a hole in the bottom covered by a thin layer of polypropylene or other material on which the cells are plated).[9] Cleaved mica sheets were found to be among the most suitable substrates for plating cells for interferometric imaging at our irradiation workstation because of their smooth surface, suitable for focusing of the interferometer, and low thickness, allowing the charged particles to traverse both the substrate and the cell. Mica sheets of thickness of 0.0002 in (5.08 μm) were used. The unstained live cells were cultured in Minimal Essential Medium (MEM)-based cell growth medium. The medium contains Phenol Red dye as a pH indicator. The cells could be left in the same medium and imaged with red light (605 nm) or transferred to pre-warmed PBS or clear (without phenol red) medium just prior to imaging for better transparency and imaged with green (540 nm) or red light.

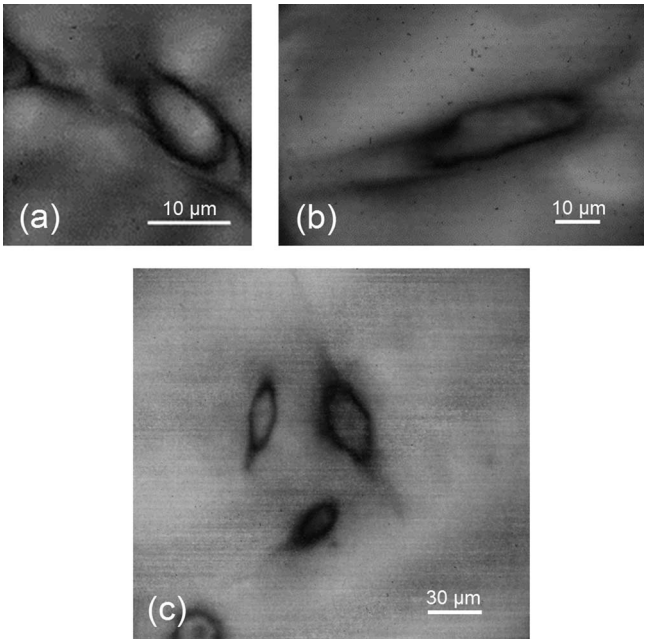

FIG. 6. SIMI images of live cells. (a) 3T3 cells were plated on mica substrates, illumination wavelength 540 nm; (b) WI 38 cells were plated on glass slides; illumination wavelength 605 nm. In both cases the cells were cultured in cell growth medium and imaged in PBS; to create each image, two interference patterns were formed on a single camera sensor. (c) HT 1080 cells plated on glass slides; illumination wavelength 540 nm. The cells were imaged in clear medium using two camera sensors.

The experimental results are presented in Fig. 6. Panel (a) shows an image of live 3T3 cells (mouse embryonic fibroblasts) acquired with the prototype SIMI configuration with a beam-displacer and a single camera. The cells were plated on mica substrates attached to the bottoms of microbeam dishes, in cell growth medium and transferred to PBS pre-warmed to 37 °C for imaging. The cells were illuminated with green light of 540 nm wavelength with the filter bandwidth of 25 nm (Omega Optical Inc., Brattleboro, VT). The image presented in Fig. 6(a) was reconstructed using a single pair of interferograms and a background image.

Images produced by the completed SIMI attachment are presented in Figs. 6(b) and 6(c). Fig. 6(b) shows WI 38 cells (normal human lung fibroblasts). The cells were cultured on glass slides in cell growth medium that was substituted by PBS pre-warmed to 37 °C prior to imaging. The sample was illuminated with red light selected by a 605/40 band-pass filter. The polarization components were separated with a beam-displacer and pairs of interferograms were recorded on a single sensor. The image shown is the average of five consecutively acquired images reconstructed from interferograms. The image presented in Fig. 6(c) was created using a beam-splitting cube and two identical cameras to create interferograms. HT 1080 fibrosarcoma cells were cultured on glass slides in cell growth medium which was substituted by clear medium (MEM, Life Technologies, Grand Island, NY) before imaging. Green light (540 nm with 25 nm bandwidth) was used for illumination. The presented image is the average of four images reconstructed from consecutively acquired pairs of interferograms. The intensity modulations for the images in Figs. 6(a)–6(c) were retrieved using Eqs. (4)–(6).

V. DISCUSSION

The developed technique provides the possibility for label-free imaging of live cell cultures in liquid medium in reflected light. The imaging can be done in the presence of vibrations. In the images reconstructed from interferograms, the outlines of the cells are visible and detectable by the automated image processing system.

One limitation of the current design is that optical elements of the threaded Leica 50× objective are corrected for spherical aberrations caused by 2 mm of glass, as it was designed for use with the interferometric attachment in air. Filling the attachment with water and immersing introduces additional spherical aberrations which causes significant reduction in the image quality. An improvement would be to use a microscope objective with the optics corrected for imaging through water. To accommodate the addition of interferometric attachment within the typically short working distance the reference mirror coating may be plated directly onto the front element of the objective.

Next, in the implementation of the SIMI system utilizing a single camera to avoid the alignment of two sensors, a calcite 40-mm-long beam-displacer was used with the separation distance of 4 mm. That defines the distance between the two interference patterns, restricting the maximum size of field of view to less than 400 pixels and resulting in low image resolution. Alternatively, one can use a lateral displacement beam-splitter, that would have to be placed in the infinity space of the microscope to ensure that both beams converge at the focal plane of the tube lens, or a cube beam-splitter with two separate cameras.

Finally, the introduction of the compensation waveplate to the reference path changes the amplitudes of the polarization components of the test beam emerging from the interferometer, which can be shown using Jones matrix analysis.[18–20] Due to this ellipticity, the phase shift between the two interferograms is affected. During the experiments, we adjusted the apparent phase shift as close as possible to $\pi/2$ by slightly rotating the direction of polarization of the incident beam and the angular position of the beam-displacer. The dark areas visible on the background of the images could be due to the imprecision of the phase shift.

VI. CONCLUSION

This work describes a novel technique, SIMI, for imaging live cells in liquid medium using reflected light. Fluorescent staining of the cells or UV illumination are not required, which eliminates possible cytotoxic and phototoxic damage and increases the sensitivity of the results from irradiation experiments.

The SIMI approach has evolved from IMI when the need arose to perform imaging in the presence of ambient vibrations. The simultaneous acquisition of two interferograms is enabled by using polarization optics to encode different phase shifts into a single interference beam. A special interferometric device mountable onto a microscope objective was designed, constructed and successfully used in experiments. To our knowledge, SIMI is the first constructed system for imaging live cells in medium without coverslips utilizing polarization Mirau interferometry. Although the motivation for this development was to design a non-invasive imaging technique for an irradiation workstation, both IMI and SIMI can be implemented in a variety of other imaging applications.

ACKNOWLEDGMENTS

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