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Immersion Mirau interferometry for label-free live cell imaging in an epi-illumination geometry

Oleksandra V. Lyulko*, Gerhard Randers-Pehrson, and David J. Brenner

Center for Radiological Research, Columbia University, New York, NY 10533

Abstract

In cell biology studies it is often important to avoid the damaging effects caused by fluorescent stains or UV-light. Immersion Mirau Interferometry (IMI) is an epi-illumination label-free imaging technique developed at the Columbia University Radiological Research Accelerator Facility. It is based on the principles of phase-shifting interferometry (PSI) and represents a novel approach for interferometric imaging of living cells in medium. To accommodate the use of medium, a custom immersion Mirau interferometric attachment was designed and built in-house. The space between the reference mirror and the beam splitter is filled with liquid to ensure identical optical paths in the test and reference arms. The interferometer is mountable onto a microscope objective. The greatest limitation of standard PSI is the sensitivity to environmental vibrations, because it requires consecutive acquisition of several interferograms. We are developing Simultaneous Immersion Mirau Interferometry (SIMI), which facilitates simultaneous acquisition of all interferograms and eliminates the effects of vibration. Polarization optics, incorporated into the design, introduces a phase delay to one of the components of the test beam. This enables simultaneous creation and spatial separation of two interferograms, which are combined with the background image to reconstruct the intensity map of the specimen. Our results of imaging live and fixed cells with IMI and SIMI show that this system produces images of a quality that is sufficient to perform targeted cellular irradiation experiments.

Keywords

Interferometry; phase shifting; vibration; polarization; simultaneous interferometric imaging; dynamic imaging; label-free imaging; no-stain imaging; live cell imaging.

1. INTRODUCTION

In contemporary cell biology studies it is often important to avoid confounding factors, such as the potential stress caused by fluorescent stains or the phototoxicity of UV-light. Consequently, there has been an increasing demand for no-stain no-UV imaging in the last couple of decades^{1, 2, 3}.

The Columbia University single-ion microbeam at the Radiological Research Accelerator Facility (RARAF) originated as a tool to deliver single alpha particles to the nuclei of single cells^{4, 5}. Recent developments in microbeam technology at RARAF have resulted in far-reaching improvements in the precision of particle delivery⁶. With a typical beam diameter less than one micron it is now possible to irradiate subcellular structures such as the nucleus, the cytoplasm⁷ or an individual mitochondrion⁶. Our facility's instrumentation initiatives are

focused on improving the precision, accuracy, and usability of technology for irradiating individual cells, either in a monolayer or within 3-D tissue. In particular, rapid automated imaging is central to our technologies. Novel imaging techniques are available along with conventional fluorescent imaging. For instance, multiphoton technology⁸ provides opportunity to study radiation affects on tissues and small animals⁹. Implementation of label-free imaging¹⁰ will give our users a choice to exclude the influence of fluorescent dyes.

Most of the available no-stain imaging techniques require transmitted light. The microbeam endstation at RARAF is designed so that the microbeam vacuum window, through which charged particles are delivered to the specimen, is located immediately beneath the sample (Fig. 1). Therefore illumination with transmitted light cannot be implemented. Imaging techniques must use reflected light, i.e. epi-illumination.

2. METHODOLOGY AND DESIGN

2.1 Mirau Interferometry

Immersion Mirau Interferometry (IMI) is an epi-illumination non-stain imaging technique developed at RARAF and is a modification of Mirau Interferometry. It is based on the principles of phase-shifting interferometry^{11, 12}. A schematic of Mirau interferometer is shown in Fig. 2a. The Mirau interferometric attachment can be mounted to an objective lens of a microscope. The beam splitter divides the source light into the reference path (reflected and directed onto the reference mirror) and the test path (transmitted onto the sample). The position of the attachment between the objective and the specimen is adjusted so that the test and reference pathlengths are equal. The reference mirror is a circle in the middle of the comparison face with a diameter equal to the diameter of the light spot focused on the sample, i.e. large enough to cover the entire field of view, but not too large to obscure the incident and emerging beams significantly. The two paths recombine in the plane of the beam splitter, return through the objective and form an interference pattern in the image plane. The intensities at each point of these patterns are functions of background intensity, fringe modulation and height (phase), and three or more such images provide enough information to solve for the topographic image of the sample.

We chose a Mirau interferometer over other types of interferometers because of its advantages and the applicability to our applications:

1. The device's compactness allows us to integrate it into the microbeam endstation more easily than other two-beam interferometers (for example, Michelson or Linnik¹¹).
2. It is suitable for work at magnifications in the range from 10x to 50x.
3. The test and reference beams share a common path up to the beam splitter¹³, therefore, they carry the same aberrations caused by the microscope objective and there is no net optical path difference when interference is produced.
4. The accuracy of this type of interferometer is on the order of several nanometers.

In our implementation the sample is illuminated using a high-pressure mercury lamp. A neutral (50/50 dichroic) microscope cube is fitted with a 540/25 excitation filter ($\lambda=540$ nm, $\Delta\lambda=25$ nm). Thus the coherence length is $l_c=2\ln 2/\pi \lambda^2/\Delta\lambda=5.1$ μ m. The low coherence results in suppressing stray reflections and unwanted fringing¹⁴. The vertical position of the sample is changed by quarter-wavelength increments to acquire four interference patterns with relative phases 0, $\lambda/4$, $\lambda/2$, and $3\lambda/4$. The precision vertical motion of the sample is

performed by LP-200 nano-positioner from Mad City Labs (Madison, WI) with a z-axis resolution of 1.4 nm.

The intensity at a given point (x,y) of the interference image is described by a system of equations

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

where $I_b(x,y)$ is the background intensity, $I_m(x,y)$ is the fringe modulation, $\varphi(x,y)$ is the phase at a point (x,y) and δ_i is relative phase shift. Generally, at least three interferograms are required to solve the equations for the fringe modulation or for the phase^{10, 11}. In our case i varies from 1 to 4.

The image analysis was implemented in Visual Basic using Matrox Imaging Library (Matrox Imaging Systems Ltd, Dorval, QC, Canada).

2.2 Immersion Mirau Interferometry

The IMI technique that we have developed is a novel modification of Mirau interferometry. The standard Mirau interferometry technique, described above, is complicated by the thin layer of growth medium which remains over the cells under current RARAF protocols. Given the spatial sensitivity of interferometry, evaporation of this thin layer causes the path length of the test wavefront to vary constantly. Under these conditions the interference pattern would change during the image acquisition. Our solution is to intentionally leave layer of medium a few millimeters thick in the dish and to submerge the attachment (Fig. 2b). Leaving the medium in the dish also simplifies the experimental protocol and improves cell viability, thus allowing for more accurate results from irradiation experiments. Because of a significant difference in the indices of refraction of the medium and of the neighboring air ($n_{\text{medium}}/n_{\text{air}}=1.33$), each millimeter of medium would introduce a path length difference from the reference wave of 660 μm . However, to ensure interference conditions, the path length difference needs to be within the coherence length of 5.1 μm . Moreover, since the test beam changes direction when entering the liquid, the geometry of the interfering paths changes and it is not possible to adjust the pathlengths so that the interferometry is restored. To ensure that the test and reference paths are identical, we decided to fill the entire space between the sample and the spot mirror, which includes the inner space of the interferometric attachment, with cell medium (Fig. 2c).

2.3 Immersion Mirau interferometric attachment

To demonstrate the principles of IMI we first modified a commercial interferometric attachment (Leica Microsystems, Wetzlar, Germany), such that it served as a water immersion lens¹⁰. However, this device was designed for the standard “dry” interferometry and its use in an immersion mode is limited.

To perform IMI on a routine basis, a specially designed interferometric attachment was required. The custom-designed immersion-based Mirau attachment is presented in Fig. 3. The apparatus accommodates the endstation arrangement: it mounts to a threaded microscope objective (we use a Leica objective) and the shape is designed to fit the microbeam dish. The optics holder contains a beam splitter and a spot mirror. The alignment of the two angles to keep the optics parallel to the sample surface is accomplished by a ball-and-socket adjustment mechanism along with springs and adjustable lever posts. The components (most importantly, the optics holder with the beam splitter and the spot mirror) are easily removable to allow for cleaning.

To enable interferometry, high precision optics is needed: we used fused silica windows of high surface quality with flatness $1/20$ of the wavelength. The windows were custom-manufactured by The Optical Corporation (Oxnard, CA). Coatings were completed by Navitar Coating Labs (Newport Beach, CA). Each beam splitter has a layer of protective material to prevent chemical interaction of the coating with the cell medium. Four interchangeable optics holders were built with 5%, 25%, 50% and 85% reflectance beam splitters. Each unit can be disassembled and re-filled with liquid.

2.4 Simultaneous Immersion Mirau Interferometry is a vibration-insensitive technique

Immersion Mirau Interferometry uses temporal phase shifting. The interference patterns required to reproduce the image are acquired at different times and if vibrations exist, the phase shifts between consecutive frames are perturbed from the desired values and the image of the specimen cannot be properly reconstructed. Thus IMI is very sensitive to vibrations. Our experiments revealed that, although the microbeam endstation is mounted on a 27" thick concrete floor, ambient low-amplitude vibrations are present at the site. The measured level of vibrations is insignificant for all other requirements of the microbeam. A vibration survey was performed by a company specializing in vibration isolation systems (Halcyonics, Inc., Menlo Park, CA), whose representative noted that it is very rare for a building to have such a low level of vibrations. Nevertheless, the background vibrations sufficiently reduce the image quality of IMI. A solution to this problem is to acquire all interference fringes at the same time in an approach we are calling Simultaneous Immersion Mirau Interferometry (SIMI).

The fundamental idea of simultaneous interferometric imaging is to use an interferometer in conjunction with polarization optics to encode multiple phase shifts into the components of a single beam. A technique for simultaneous acquisition of four phase-shifted interferograms is described by Brock et al.¹⁵. The system described uses a Twyman- Green interferometer and linearly polarized light from a laser. The reference path contains a $\lambda/8$ waveplate with the axes oriented so that there is a $\pi/2$ phase shift between the x and y components of the reference beam. After beam recombination, two polarization beam splitters are used to separate the light into four separate interferograms. We adapted this principle with some modifications to accommodate the geometry of the Mirau interferometer.

The principles of the simultaneous Mirau interferometric system are illustrated in Fig. 4 and Fig. 5. A linear polarizer (P in Fig. 4a) is inserted in the output beam of a mercury light source and is oriented so that the light becomes linearly polarized at a 45° angle with respect to the x axis. A neutral beam splitter (BS) directs the light into the objective (O) with the interferometric attachment (M). A $\lambda/8$ waveplate is incorporated into the optics of the interferometer below the beam splitter (Fig. 5a). The waveplate creates a $\pi/2$ phase difference between the x and y components of the test beam. Therefore, the output of the interferometer contains two interferograms with a relative phase shift of $\pi/2$. The outputs of the polarization beam splitter (PBS) form two interferograms on two separate CMOS sensors (C1 and C2) (Fig. 4a).

As described above, phase-shifting interferometry generally requires at least three interferograms to solve the system of equations (1) for the fringe modulation or for the phase. The geometry of the Mirau interferometer allows us to produce only two interferograms with the technique described above. We reduce the number of unknowns by taking a background image just outside of the range of the fringe visibility. Subtracting the background image from each of two equations of the form (Eq. 1), we eliminate the background intensity variable I_b and are left with two variables: the fringe modulation I_m and the phase ϕ . Thus, acquiring two interferograms is enough to reconstruct the image, provided that the background is also acquired.

When two cameras are involved, even a slight misalignment or miscalibration creates a discrepancy between the corresponding interferograms that reduces the quality of the final image. A more reliable method is to capture both interferograms with a single camera. To direct both beams onto a single sensor, we use a calcite beam displacer (Fig. 6) instead of a beam splitter. This is a uniaxial birefringent crystal that separates the input beam into two orthogonally polarized beams¹⁶ (similarly to polarization beam splitters), but the output beams are parallel and they can be sent to a sensor of a single camera. A schematic of the configuration where a beam displacer is used is presented on Fig. 4b.

In our experiments we have used a 16 μm thick mica waveplate (B&M Mica Co., Flushing, NY) that was attached directly onto the sample. The drawback of mica is that the retardance varies from one plate to another. Another option is to use polymer waveplates, which have better retardance tolerance. The thickness of the polymer material is of the order of 100 μm . When a layer of polymer is introduced into one of the arms of the interferometer, the test and reference paths are no longer identical because of the difference in the refractive index of polymer and air. To restore interference, it is necessary to introduce a compensation layer of material with the same thickness and index of refraction to the other arm of the interferometer¹⁷. The arrangement in this case is shown in Fig. 5b.

3. RESULTS

Fig. 7 shows an example of an image produced by IMI using a custom-built immersion Mirau interferometric attachment. The unstained live fibroblast cells were submerged in PBS (Phosphate Buffered Saline). The experiment was done on a vibration-isolated table. The outlines of the cytoplasm and nuclei are distinguishable.

We have built and tested a prototype of the Simultaneous Mirau imaging system that operates in the non-immersion mode. Fig. 8 and Fig. 9 demonstrate the results of our preliminary work using a two-camera simultaneous Mirau imaging system (left panels), with a comparison to corresponding fluorescent images of the same samples (right panels). In this preliminary study, we used WISH cells (human amniotic epithelial cell line) and NIH 3T3 cells (mouse embryo fibroblast cell line). The unstained cells were fixed and imaged with the simultaneous Mirau interferometric system on glass slides in air. The same samples were subsequently stained with DAPI and Red Counterstain C and imaged with a fluorescent microscope (Zeiss Imager Z1). There is a good correspondence between the images produced by fluorescent imaging and by Simultaneous Mirau Interferometry. On the Mirau interference images, cell structures can be seen such as nuclei, cytoplasm and nucleoli.

4. CONCLUSION

We have developed Immersion Mirau Interferometry and demonstrated its feasibility in a non-vibration environment. This technique addresses our users' concerns that fluorescent staining or UV-light may alter cellular response to irradiation. It does not require fluorescent labeling or UV-illumination of the specimen and also allows imaging of biological targets which are in medium. The spin-off method, Simultaneous Immersion Mirau Interferometry, provides opportunities for live cell imaging in any environment where vibration isolation is not possible. The future work involves constructing the polarization Mirau attachment designed to work in the immersion mode.

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Fig 1.
Arrangement of the microbeam endstation at RARAF. The exit window of the beamline is located immediately beneath the sample.

Fig 2.

Principles of Mirau and Immersion Mirau Interferometry; a) Mirau interferometer; b) when the interferometer is submerged into liquid, the test and reference paths are not identical; c) Immersion Mirau Interferometer.

Fig 3.

a) Custom-built immersion Mirau interferometric attachment; b) the attachment is positioned on the microscope.

Fig 4.

Layout of the Simultaneous Mirau imaging system: a) Two-camera arrangement. The light beam is split into two perpendicular beams by a polarization beam splitter (PBS) and sent into two separate cameras (C1 and C2); b) One-camera arrangement. The light beam is separated into two parallel beams by a polarization beam displacer (BD) and can be sent onto the sensor of a single camera. Note: the paired interferograms are the results of an actual experiment with fixed fibroblast cells - notice the quarter-wavelength phase shift.

Fig 5. Schematics of the simultaneous Mirau interferometer: a) A $\lambda/8$ waveplate with its fast axis at 45° to the light polarization placed in the test arm introduces a $\pi/2$ delay between the x and y components of the test beam; b) a second waveplate oriented at 0° equalizes the test and reference path lengths.

Fig 6. Polarization beam displacer. θ_e - the angle of separation between the ordinary and extraordinary rays; ϕ - the angle between the normal to the refracting surface and the optical axis of the crystal.

Fig 7.
Unstained live fibroblast cells imaged with IMI.

Fig 8.
WISH cells – note the cytoplasm in these cells is very thin. Left panel: No-stain / no-UV
Simultaneous Mirau interferometric image shows cell nuclei and nucleoli. Right panel:
Corresponding fluorescent image.

Fig 9.
3T3 cells. Left panel: No stain / no UV Simultaneous Mirau Interferometric image shows cytoplasm and a micronucleus. Right panel: Corresponding fluorescent image.