Phase-based cell imaging techniques for microbeam irradiations

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Abstract

The microbeam facility at Columbia University is expanding current protocols for single-particle, single-cell irradiations, so experimenters can locate and irradiate nuclei and cytoplasm of unstained cells. The ion beamline is located directly under the dish, therefore, any new techniques must use reflection microscopy. Two approaches are being integrated and neither require the removal of the cell growth medium prior to irradiation.

(1) A novel immersion-based Mirau interferometry lens which uses low-coherence light sources to inhibit unwanted fringing is under design. The process requires tens of nanometers or better precision of vertical stage motion, which will be accomplished with our custom high-precision z-stage.

(2) Quantitative Phase microscopy is under testing, also using the z-stage. Future plans include optimization of software routines to decrease time between irradiations.

Both methods will be compared further with the automated location routines which use nuclear and cytoplasm stains.

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1. Background

Columbia University's Radiological Research Accelerator Facility (RARAF) is incorporating two new methods for targeting samples for irradiation with its single-cell/single-particle microbeam: Quantitative phase microscopy-based and

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immersion-Mirau-based targetting. Current RARAF protocols for mammalian cell cultures use nuclear and/or cytoplasmic stains to image and locate cells or cell nuclei. While very low stain concentrations ($\sim$50 nM) as well as control studies to isolate effects from the radiation induced stresses are used, no-stain imaging allows increased flexibility especially in relation to future studies, which will have more subtle endpoints.

The microbeam delivers focused ions into the target from below the sample, so that the light source and microscope elements must all rest above the sample, rendering transmission microscopy unfeasible for our purposes. We are incorporating into the lab both phase-shifting interferometry

(PSI) [1] and Quantitative phase microscopy (QPm) [2,3]. Each technique has advantages and disadvantages and so are both being developed as functional protocols. An encouraging early example of the integration of these techniques into the lab is shown in Fig. 1.

Any technique which requires the growth medium to remain on the cells during irradiation will by design necessitate the use of a below dish detector. RARAF has such detectors and efforts are underway to further improve their use in the Columbia Microbeam.

2. Phase-shifting interferometry

The immersion-based Mirau interferometric objective is schematic representation in Fig. 2. By using a high-pressure mercury lamp through a neutral cube fitted with a 540/25 filter ($\lambda =$ 540 nm, $\Delta\lambda = 25$ nm), we have a coherence length $l_c = 2 \ln 2/\pi \times \lambda^2/\Delta\lambda = 5.1$ μm, which aids in suppressing stray reflections and unwanted fringing which would result from sources having higher coherence lengths [4]. The beam splitter enables standard interferometry and the recombined wave contains an interference pattern which encodes the height of the sample. Since the intensity map is a well known function of the background intensity, the fringe modulation and the phase [5], three
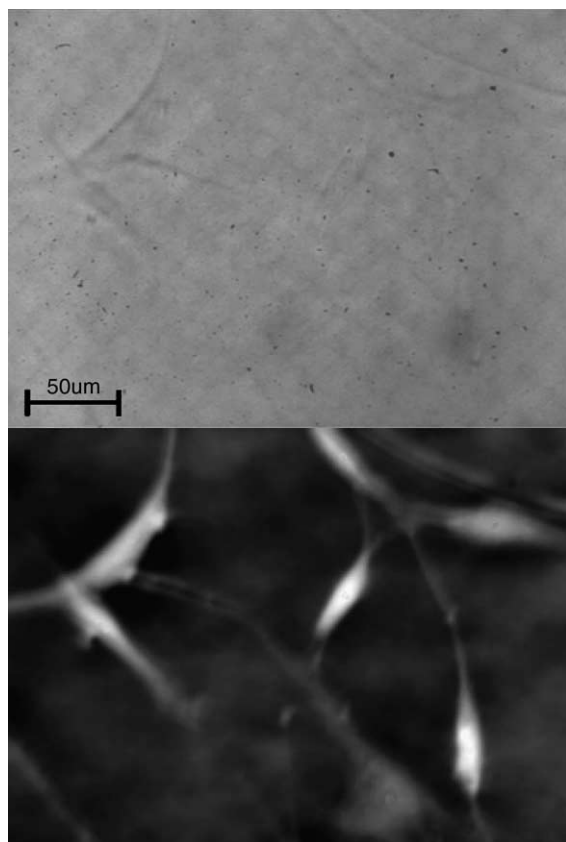

Fig. 1. Example of QPm algorithm applied to normal human dermal fibroblasts. The top half is taken with the 540 nm line of the mercury lamp, in focus, which gets used along with two slightly defocused images to create the final image shown in the bottom half.
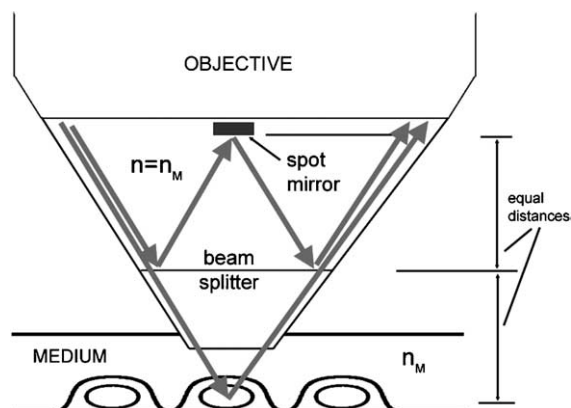

Fig. 2. Schematic showing the plans for an immersion-based Mirau interferometer which is being integrated into the labs at RARAF.

images of the sample taken at separate, pre-determined sub-wavelength distances contain enough information to solve for the modulation image such as in Fig. 4.

Precision of z-motion is critical for this type of application. We have built a custom stage with a dc-actuator and cross-roller bearing-based "macro" stage holding an LP-200 piezo-actuated nano-positioner from Mad City Labs (Madison, WI). This allows samples to be translated with better than 10 nanometer precision in three dimensions with an operating range in X and Y of several millimeters.

This description has assumed thus far that all growth medium were removed from the sample. In practice, this is difficult as a thin layer, remains after the medium removal until it has time to evaporate. While the cells can be kept under a moisturizer, so that they don't fully dry out, it takes several minutes for the liquid medium to evaporate fully and it leaves behind salt in crystalline patterns which interfere with the imaging. Our alternative is to intentionally leave a few millimeters thick layer of the liquid medium which would also simplify the experimental protocol and further remove variables associated with handling the medium. Because the index of refraction of the medium is substantially different from the neighboring air ($n_{\mathrm{medium}}/n_{\mathrm{air}} = 1.33$), each millimeter of medium, which the wave travels through twice, would introduce a path length difference from the reference wave of 660 μm. The path length difference must be well within the coherence length in order to reconstruct the topological image, so an immersion-based approach has been introduced to allow the two light pathways to be tuned, so that the two pathways differ on the order of a micron.

Rather than simply account for the path difference by adjusting the location of the spot mirror, which would introduce a difference in intensity of the interfering wavefronts (and therefore reduce the visibility of the interference patterns) due to transmission loss at the barrier between the medium and air, it was simpler to make the two pathways identical in every aspect. This assures that everything that happens to the reference wavefront also happens to the sample wavefront. The simplest geometry is to have the immersion fluid fill the entire region between the sample and the spot reference mirror.

The selection for the height off the sample of the reference mirror was based upon three criteria: (1) the amount of light the back of the mirror would obscure between the source and both the sample and the reference side of the spot mirror, (2) the shadow cast by the spot mirror and (3) the amount of light collected as a function of the amount of medium, which changes due to Snell's law. The first two factors are reduced by a greater height of the mirror off of the sample, while the third effect is decreased by decreasing the height. With the mirror set to a height of approximately 200 μm above the sample, we found no ill effect on the automated imaging techniques.

Fig. 3 contains a schematic of the final design of the immersion-based Mirau interferometric lens. The alignment of the two angles to keep the reference mirror parallel to the sample plane is accomplished by using a ball-and-socket along with springs and adjustable lever posts. The components are easily removable to allow for frequent cleaning.

Fig. 4 has preliminary results from a non-immersion-based lens. While the results are not as stark as the expected results from the im-
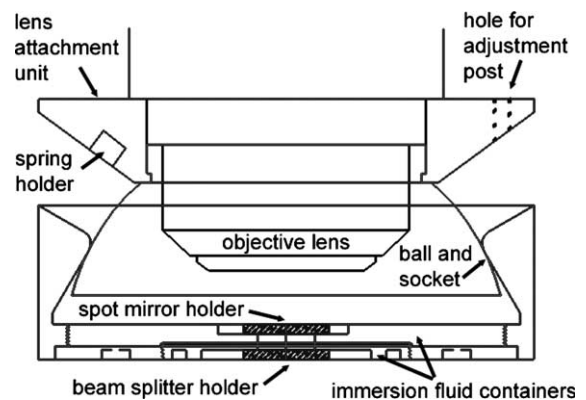

Fig. 3. Drawing of the device used to ensure that the index of refraction is constant for equivalent pathways between the light source and the sample and between the light source and the reference spot mirror as well as for equivalent pathways between the sample and the objective and between the reference spot mirror and the objective.
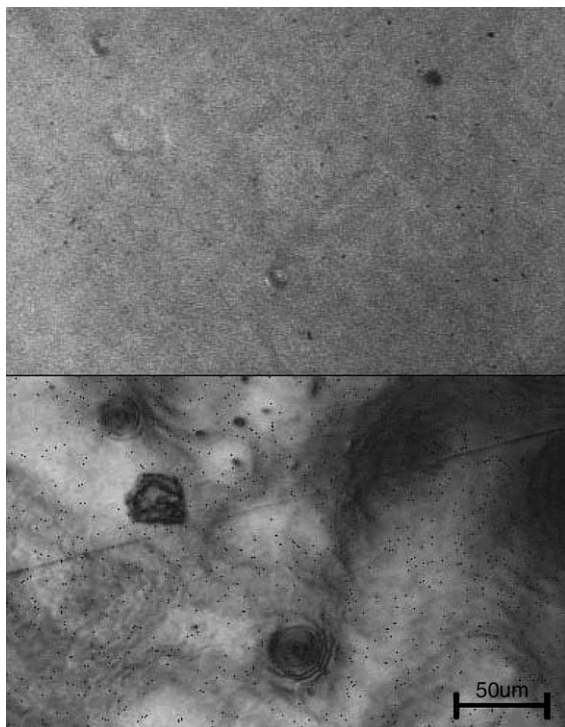
Fig. 4. Preliminary non-immersion Mirau image taken of polystyrene beads (bottom half) as compared to the same beads under room illumination (top half). The results are encouraging and show that an immersion-based system is of definite necessity.

mersion-based lens, it is already apparent that for objects such as mammalian cells, an automated phase-unwrapping routine must be incorporated into the software, so that the automatic imaging systems will be able to recognize the customary cell and cell nuclei shapes as opposed to the ridged appearance of such objects due to the phase information carrying with it an ambiguous $n2\pi$ at every location in the image.

3. Quantitative phase microscopy

Non-interference phase microscopy is a relatively new technique that can generate phase images and phase-amplitude images using a standard microscope, with either transmitted or reflected light [2,3]. By inspecting the intensity of three images, one taken in focus, one focusing

above the focal plane and one focusing below the focal plane, the light transport equation can be solved using Fourier transform methods, so that a phase image can be obtained as well as a phase-amplitude image. To automate the gathering of the three images required, each of which has a different, specified z-position, we also use the custom 3-direction stage used for the PSI imaging. It is worth noting that, this approach does not require an unwrapping of phase as PSI does. Fig. 1 was obtained by using this technique with reflected light and applying false color to the intensity.

Software for generating the phase images or the phase-amplitude images from the three microscope images is distributed by IATIA (Melbourne, Australia). In fact, because the methodology produces linear images of both amplitude and phase, it is possible to use the phase data to emulate other imaging modalities, such as Nomarski interference [3], though this extra step is not needed for our application. Our trials have indicated that, this process is over four times as costly in computer processing time as is PSI and for high throughput which is offered by RARAF [6], this gives some advantage to PSI (without phase unwrapping) over QPm. QPm has been integrated further into the lab and has generated more usable images thus far. Efforts are underway to make the QPm approach more efficient. Further, it should be noted that other than a slight reduction in contrast due to transmission loss and a decrease in effective numerical aperture when more medium is present, this technique is not affected by the presence or lack of growth medium over the cell sample.

4. Future development

The custom immersion-based Mirau device is under construction. Once it is integrated and tested, the next most critical need is to complete the correlation-studies to set benchmarks and protocols for the various thresholds when calculating the final images of unstained cells using either PSI or QPm. To accomplish this, the studies use stained cells through a filter such that, the fluores-

cent light given off by the stain is not passed along to the camera. The response to the 540 nm reflected light is collected and then the same exact sample is imaged using only the traditional stained technique.

5. Conclusions

PSI and QPm are nearly integrated into the available protocols at Columbia University's RARAF and offer the biological experimenter the option of using the microbeam on individual cells and subcellular structures while not incorporating any stains or dyes. PSI is currently a faster technique and therefore may for the time being be more preferable for high cell throughput studies. Both techniques can be used without removing the medium prior to irradiation.

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