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The Columbia University microbeam II endstation for cell imaging and irradiation

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Abstract

The Columbia University Microbeam II has been built to provide a focused ion beam for irradiating designated mammalian cells with single particles. With the interest in irradiating non-stained cells and cells in three-dimensional tissue samples, the endstation was designed to accommodate a variety of imaging techniques, in addition to fluorescent microscopy. Non-stained cells are imaged either by quantitative phase microscopy (QPm) [IATIA, Box Hill North, Victoria, 3129, Australia [1]] or by Mirau interferometry. In conjunction with the focused microbeam, a proposed multiphoton microscope will offer the opportunity to probe live cells at selected depths in tissue samples; dynamic events in such tissue samples are more representative of biological system response than those in cell cultures.

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1. Introduction

Studies at Columbia University's Radiological Research Accelerator Facility (RARAF) concentrate on fundamental investigations into the radiobiological effects on mammalian cells through controlled single-cell single-particle microbeam irradiation [2]. Some of the facility's prime directions are to develop new techniques for imaging cells, to create new tools for the detection of single-molecule events in living cells, and to generate new strategies for dramatically increasing the resolution of imaging dynamic cellular processes. The first-generation irradiation platform at RARAF, microbeam I, was an apertured ion beam; its infrastructure concepts were incorporated by microbeam II, the facility's present cell irradiator, that now delivers an ion beam produced by a 4.2 MV Van de Graaff accelerator and focused by an electrostatic lens system [3]. The heart of the

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microbeam II endstation is a Nikon Eclipse E600-FN research fluorescence microscope. Along with standard fluorescence microscopy techniques, this endstation is equipped with ion beam diagnostic tools to profile and locate the beam. New cell-imaging techniques for non-stained cells and for bulk tissue samples are being incorporated into this platform.

2. Focused ion beam diagnostics

A precision stage at the focused microbeam endstation is used for profiling the beam during the focusing routine. Our voice-coil stage [4] is presently used for this purpose, however a precision *xyz*-stage is being incorporated into the endstation to enable the new cell imaging routines and techniques. The new stage system comprises two components: a custom coarse *xy*-stage, designed and constructed in-house and a 3-axis, piezoelectric-actuated, fine motion stage, the LP-200 low-profile nano-positioner from Mad City Labs (Madison, WI).

Ion beam profiles are measured by individually passing *xy*-crossed, knife edges through the beam at the desired focal plane, while monitoring the ion energies with a solid-state detector; we use a few microns thick, cleaved silicon knife edge for profiling a helium beam and a 13 μm thick nickel knife edge for profiling a proton beam. Threshold settings on a single-channel analyzer enable the distinction between the unobstructed ion counts from those that penetrate the thin silicon. An auto-focusing routine that uses the downhill simplex method [5] successively measures ion beam sizes for sets of three combinations of electrostatic lens voltages to home in on the best lens focal setting. On a related note, a secondary electron ion microscope (SEIM) under construction will expedite the beam spot measurements in the future [6]. When the ion beam focus is determined, it's *xy*-location reference is found for the imaging system by scanning a fluorescent bead from a position over the ion beam focus in an outward rectangular spiral pattern, using the same energy-loss technique that was used during the auto-focus routine. When the fluorescent bead position is determined to be at the center of the ion beam, the solid state detector is removed and an image of the bead is acquired to set the origin values for the imaging system.

3. Cell imaging

Much of the cell-imaging routine was adopted from microbeam I operations, which is thoroughly described in a previous source [2]. With fluorescent microscopy, this routine applies to cells stained with nuclear or cellular dyes. Although the concentrations of the stains used in this protocol are low (~50 nM), there are pros and cons to studies using stained cells. The obvious advantage is that the cells are easily imaged through standard fluorescence microscopy. The disadvantage is that a foreign substance has been added to the cell, and that could potentially disrupt the natural cellular response to radiation. Our research has shown that the dye concentrations used do not effect certain results of the radiobiology studies [7], however, to eliminate the possibility of unwanted influence from dyes, there is a strong interest to conduct microbeam irradiation experiments on non-stained cells.

Non-interference phase microscopy is a relatively new technique that can generate phase images and phase-amplitude images using a standard microscope [1,8]. In practice, to obtain a quantitative phase image one collects an in-focus image and very slightly positively and negatively defocused images, and uses these data to estimate the differential in the illuminating field intensity distribution with respect to the defocus of the image. Information of the specimen is contained within the transport of the illuminating field. Fourier transform-based software (QPm) for generating phase images or phase-amplitude images from three microscope images is distributed by IATIA (Melbourne, Australia). Trial testing of the QPm technique was carried out on unstained, normal human fibroblast cells that had been plated for two days. With a microscope set for transmitted Köhler illumination and after establishing a reference focus, images of the cells were acquired at the focal plane and at 8 μm above and below the sample plane. After QPm processing, the cell

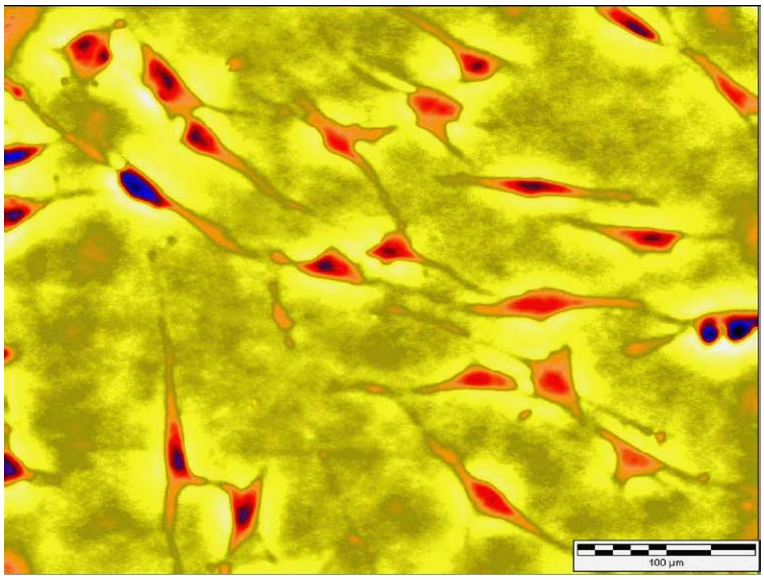

Fig. 1. Image of unstained, normal human fibroblast cells after QPm processing and false-color rendering. The cell nuclear borders are revealed in the false-color rendition.

outlines were distinctly noticeable in gray-scale, but the cell nucleus was difficult to discern. Evidence of cell nuclear borders increased when the resulting images were rendered in false color, see Fig. 1.

Interferometry is another technique for two-dimensional imaging of living cells that have not been stained, and is possible with a Mirau interferometric objective, shown schematically in Fig. 2.

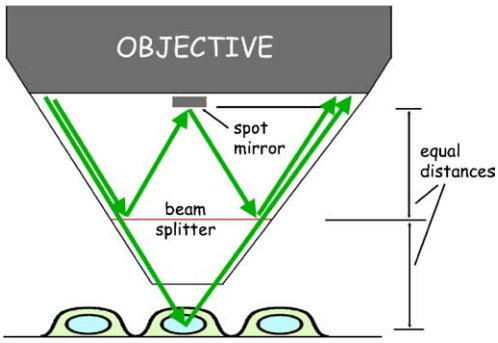

Fig. 2. Shematic of Mirau interferometric objective lens. At the beam splitter the source light is split into a reference path (reflected) and a sampling path (transmitted on to the cell objects).

In Mirau interferometry, a reference path and a sampling path recombine to form an interference image. By changing the z-position of the sample, interference images are acquired at a sequence of path (phase) differences: 0, $\lambda/4$, $\lambda/2$ and $3\lambda/4$. The combined images can then produce a topographic image by solving for the phase shifts at each point [9]. Initial successful trials to image topographic samples like glass beads were made using a Leica Mirau objective (N PLAN H 50X/0.50). The light source was a high-pressure mercury lamp through a neutral cube fitted with a 540/25 filter (λ = 540 nm, $\Delta\lambda$ = 25 nm) providing a coherence length $l_c = 2\ln 2/\pi \times \lambda^2/\Delta\lambda = 5.1$ µm. A low coherence light source such as this is preferred because it suppresses possible undesired fringing from stray reflections [10].

4. Multiphoton microscopy

In order to detect and observe the short-term molecular kinetics of responses to radiation in single living cells within 2D and 3D scenarios, we plan to combine a multiphoton microscope with a single-cell single-particle microbeam irradiator.

A multiphoton microscope is a 3-D, non-destructive imaging tool that, when compared to conventional confocal microscopy, has greater penetration depth and has reduced phototoxicity and photobleaching in the sample bulk [11]. The guiding principle of the multiphoton microscope is: when two photons are spatially and temporally coincident within the excitation cross section of a fluorophore molecule, they can act as one photon with twice the energy to excite an electronic transition. An aspect of our custom design is that the microscope is mounted on a pivot arm where it can be operated in an on-line or off-line mode. In the off-line mode, the multiphoton microscope is placed above an additional photomultiplier tube (PMT) which increases light-gathering efficiency and offers particular benefits for transmitted signal from second harmonic generation (SHG), a second-order non-linear optical process, occurring at regions or interfaces that lack inversion symmetry and is complementary to multiphoton excitation [12]. A schematic view of the major components of our multiphoton microscope design is shown in Fig. 3.

On the microbeam II table, the laser pulses exit the light guide, are deflected by xy-scanner mirrors in the scanning head into the microscope, and reflect off a low-pass dichroic mirror down to the back aperture of the objective lens, as shown in Fig. 4. Multiphoton excitation and SHG occur in a thin layer of the sample at the focal plane of the microscope objective. Fluorescent light emitted isotropically can be collected by assorted PMT's placed after each optical path from the sample.

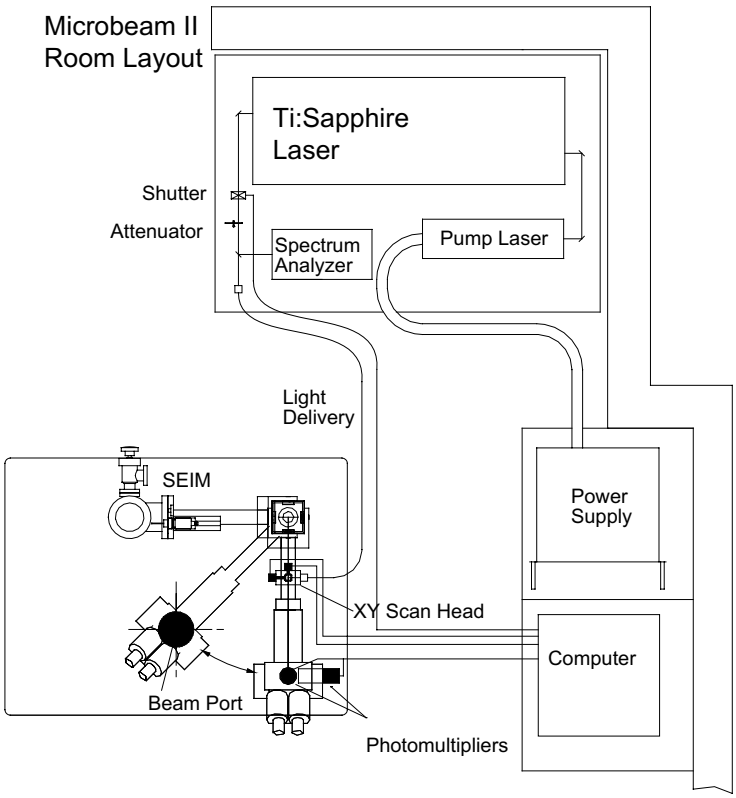

Fig. 3. Schematic layout of the RARAF endstation. A silhouette of the multiphoton microscope and SHG imaging system is shown in the online position above the beam port through the microbeam II table. The scanning light source is a tunable, scanning mode-locked Ti:sapphire laser system (λ = 700–1000 nm). The SEIM is also mounted on a pivot arm.

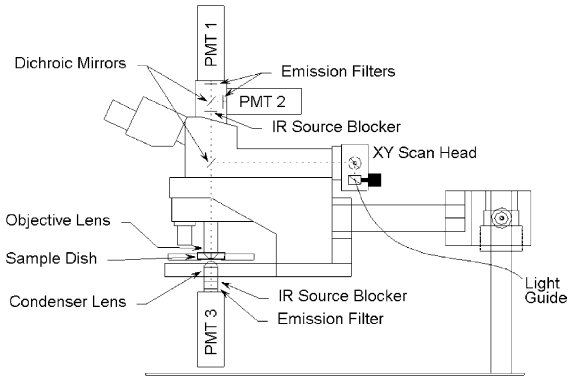

Fig. 4. Schematic to show the light path in a modified Nikon E600-FN research microscope for multiphoton microscopy and SHG microscopy. This arrangement depicts the off-line mode. PMT 3 is not used in the on-line mode.

With the proper light filters installed, images are acquired by correlating fluorescent emissions from a sample with the position of the scanning excitation laser.

5. Conclusions

A description of the microbeam II endstation at RARAF has been given with suggestions for two methods for imaging non-stained cells. In addition, implementing the proposed multiphoton microscope will advance our capabilities to observe short-term kinetics of radiation response in live cells. Imaging dynamic events in 3D tissue samples will enhance our understanding of biological system response to radiation.

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