THE RADIOLOGICAL RESEARCH ACCELERATOR FACILITY

An NIH-Supported Resource Center

WWW.RARAF.ORG

Director: David J. Brenner, Ph.D., D.Sc.
Associate Director: Gerhard Randers-Pehrson, Ph.D.
Manager: Stephen A. Marino, M.S.

Research Using RARAF

The "bystander" effect - the response of cells that are not directly irradiated but are in close contact with, nearby, or only in the presence of irradiated cells - has been the focus for many of the biological studies at RARAF over the past two decades. Both the Microbeam and the Track Segment Facilities continue to be utilized in various investigations of this response to radiation exposure. The number of biological experiments investigating the mechanism(s) by which the effect is transmitted increased slightly this year compared with last year, with newer experiments continuing to use the Microbeam Facility to examine damage to sub-nuclear structures (e.g., mitochondria, telomeres, and specific chromosomes) and other radiation effects. Research into bystander effects *in vivo* continued this past year with irradiations of the ears of mice.

Experiments

Listed in Table I are the experiments performed using the RARAF Singletron between January 1 and December 31, 2013 and the number of shifts each was run in this period. Fractional shifts are assigned when experimental time is shared among several users (e.g., track segment experiments) or when experiments run for significantly more or less than an 8-hour shift. Use of the accelerator for experiments was 27% of the regularly scheduled time (40 hours per week), up slightly from 23% last year.

Table I. *Experiments Run at RARAF January 1 - December 31, 2014*

Exp No.	Experimenter	Institution	Exp. Type	Title of Experiment	Shifts Run
110	Tom K. Hei	CRR	Biol.	Identification of molecular signals of alpha particle-induced bystander mutagenesis	37.5
112	Yigal Horowitz	Ben Gurion Univ.	Phys.	TLD track separation and distinction testing for dosimetry	5.0
113	Alexandra Miller	AFRRI	Biol.	Role of alpha particle radiation in depleted uranium-induced cellular effects	2.5
128	Mark Axelrod	Landauer, Inc.	Phys,	Testing of new radiation detector formulations	4.5
153	Howard Lieberman	CRR	Biol.	The role of Rad9 in mediating global gene expression in directly irradiated and bystander cells	2.5
163	Lubomir Smilenov	CRR	Biol.	Bystander effects in the hairless mouse ear	3.0
164	Lubomir Smilenov	CRR	Biol.	Mouse irradiation using IND spectrum neutrons	2.0
165	Helen Turner	CRR	Biol.	Mouse/blood irradiation using IND spectrum neutrons	1.0
169	Vincent LiCata	LSU	Biol.	The denatured states of a thermophilic versus a mesophilic DNA polymerase after charged particle irradiation	1.5
170	Ciaran Morrison	National Univ. of Ireland	Biol.	Live cell imaging of centrosome kinetics following microbeam irradiation	1.5
173	Ekaterina Dadachova	Albert Einstein College of Medicine	Biol.	Comparison of fungal cell susceptibility to external alpha particle beam radiation versus alpha particles delivered by ^{213}Bi-labeled antibody	2.0
174	Gordana Vunjak-Novakovic	Columbia University	Biol.	Micro proton induced x-ray emission of bone/cartilege grown on artifical scaffolds	3.0

Sixteen different experiments were run during the year. Four experiments were undertaken by members of the CRR, supported by grants from the National Institutes of Health (NIH), the National Cancer Institute (NCI), the National Institute of Allergies and Infectious Diseases (NIAID) and the National Institute of Biomedical Imaging and Bioengineering (NIBIB). Twelve experiments were performed by external users, supported by grants and awards from the Department of Energy (DoE), the Department of Defense (DoD), the NIH, the National Aeronautics and Space Administration (NASA), the National Science Foundation (NSF), the National Cancer Institute (NCI), the Economic Community Cooperation Programme, and internal funding from the Georgetown University Department of Radiation Medicine. Brief descriptions of these experiments follow.

A group led by Tom Hei of the CRR continued experiments investigating the effects of cytoplasmic irradiation and the radiation-induced bystander effect (Exp. 110). Using the Microbeam Facility, Jinhua Wu investigated mechanisms by which cytoplasmic stimuli modulate mitochondrial dynamics and functions in human small airway epithelial cells (SAECs). Their recent studies have shown that mitochondrial fragmentation induced by targeted cytoplasmic irradiation of human SAECs is mediated by up-regulation of dynamin-regulated protein 1 (DRP1), a mitochondrial fission protein. To further explore the role of mitochondria in modulating the biological activities of high-LET radiation, autophagy in SAECs was examined. Autophagy was observed as early as 30 minutes after cytoplasmic irradiation with 10 alpha particles and peaked at 4 hours based on LC3B punctae formation (Fig. 1). Sequestration of free radicals by DMSO abolished the induction of LC3B punctae formation, suggesting that activation of autophagy is free radical dependent. Autophagy led to an increase of γ-H2AX foci that was dramatically reduced by choloroquine (CQ) or 3-methyladenine (3-MA), which are known inhibitors for autophagy. The DRP1 inhibitor mdivi-1 also significantly reduced autophagy, indicating that it plays a key role in activation of autophagy. DRP1 knockout HCT116 cells showed little or no autophagy after cytoplasmic irradiation, further confirming its role in autophagy induction. DRP1-dependent up-regulation of autophagy-initiating protein beclin-1 was also observed. Finally, a sustained activation of ERK was detected, suggesting potential involvement of the non-canonical MEK/ERK pathway in regulating autophagy in cytoplasmic irradiated cells.

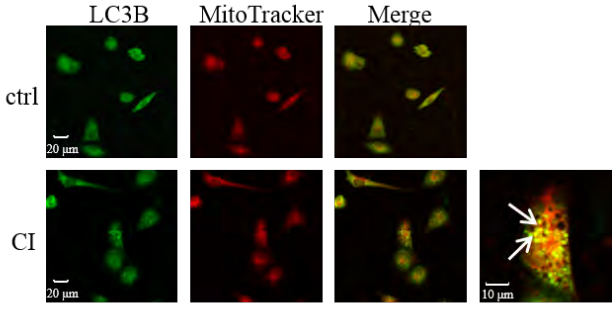

Figure 1. *Mitophagy and non-selective autophagy mediated by cytoplasmic irradiation. Co-localization of autophagosome (LC3B labeled in green) and mitochondria (MitoTrackerRed) after cytoplasmic irradiation (4 hr). ctrl, control. CI, cytoplasmically irradiated.*

Yigal Horowitz returned this year to continue his study of TLD dosimetry. The current experiment looks at the effects of track density on read out accuracy of TLD dosimeters. This work was performed on the Track Segment facility, delivering ^{4}He ion (alpha) doses of several kGy to produce very high track density. The TLDs were returned to Yigal in Israel for study and development of plans for further experiments.

Alexandra Miller of the Armed Forces Radiobiological Research Institute (AFFRI) continued studies using the Track Segment Facility to evaluate depleted uranium (DU) radiation-induced carcinogenesis and other late effects using *in vitro* models and to test safe and efficacious medical countermeasures (Exp. 113). One objective of this study has been to determine if phenylbutyrate (PB), a histone deacetylase inhibitor and epigenetic effector, can mitigate neoplastic cell transformation induced by different qualities of radiation, and if so, to identify which adverse epigenetic mechanisms are involved in carcinogenesis and potentially reversed by PB. This also would be of interest for Space missions and alpha particle exposures from accidental releases. Track segment irradiations with ^{4}He ions were performed on human small airway epithelial cells (SAECs) and growth rate, transformation, and genomic instability were quantified. Irradiation of SAECs overcame contact inhibition and caused an increase in transformation frequency and induction of gene amplification, i.e., genomic instability. Treatment with PB following irradiation resulted in a significant suppression of transformation frequency and gene amplification. Studies are ongoing to evaluate the impact of PB treatment on changes in DNA methylation caused by irradiation with ^{4}He ions.

In a second part of the Miller study, rodent bone marrow stromal cells were irradiated and co-cultured with unirradiated hematopoietic progenitor cells (FDC-P1). The FDC-P1 cells were monitored for the ability to grow in agar to assess neoplastic transformation. The data have demonstrated that co-culturing irradiated bone marrow stromal cells with FDC-P1 cells causes an increase in neoplastic transformation of FDC-P1 cells that involves the process of cell-cell communication. Additional mechanistic studies have shown that antioxidant processes are also involved in the non-targeted effect in FDC-P1 cells. Further studies with this model are ongoing evaluating involvement of non-targeted effects in multiple exposures at low doses (5 cGy).

Mark Axelrod from Landauer, Inc. continued to collaborate with our team in the development of dosimetry tools. The experiment this year was determining the dose response of new, proprietary film and chip dosimeters developed by Landauer. The work used our track segment facility to deliver proton and ^{4}He ion irradiations with different LET values ranging from 10 keV/um up to 125 keV/um to verify the range of sensitivity to particle irradiation of these dosimeters. Landauer is performing irradiations with gamma and x rays for comparison to our particle irradiations. The ultimate goal is for Landauer to be able to provide discriminated particle/EM dose characterization from a single film badge.

Howard Lieberman, Shanaz Ghandhi, Sunil Panigrahi, and Kevin Hopkins continued investigations of the effects of Rad9 on radiation-induced changes in gene expression in human cells directly irradiated or as bystanders (Exp. 153). Using shRNA against RAD9, the expression of RAD9 was knocked down in the human prostate cancer cell line DU145. The RAD9 knocked-down DU145 cells were seeded onto double-ring "strip" dishes (described above) and irradiated with ^{4}He ions using the Track Segment Facility. Both irradiated and bystander cells were of the same type and the signaling was through cell-to-cell contact and also through factors released into the medium.

Additional irradiations were made using spacer dishes in which cells were plated on Mylar glued to a stainless steel ring and the ring was inserted into a standard track segment dish so that the cells on the ring were not in direct contact with the cells irradiated on the dish. In these experiments, the DU145 cells were irradiated, WPMY1 or prSMC cells were the bystander cells, and signaling was only possible through molecules secreted by the irradiated cells. After irradiation, bystander response was measured by the micronucleus assay. An increase in micronucleus count was observed in the RAD9 knockdown cells as compared to the parental cells, supporting a role for RAD9 in the radiation-induced bystander effect. Future studies will investigate the mechanisms involved in this process.

Lubomir Smilenov and Manuela Buonanno of the CRR are examining the bystander effect *in vivo*, irradiating mouse ears using the Microbeam Facility (Exp. 163). A special fixture has been designed and constructed in the CRR machine shop to position anesthetized mice so that a region on one ear can be irradiated with the microbeam, with the other ear serving as a control. The mouse ear has an average thickness of 250 μm. A 3 MeV proton beam with a range of ~134 μm was defocused to a diameter of ~35 μm and scanned in a line a few mm long in order to irradiate a large number of cells. At chosen times after irradiation, mice were sacrificed and a punch of the ear was collected. Tissues were then fixed, paraffin-embedded and cut in 5-μm sections perpendicular to the direction of the charged particle beam. As expected, cells in the epidermal layer opposite to the γH2AX-positive region, well beyond where the beam penetrated, did not exhibit foci. The average width spanned by γH2AX-positive cells exceeded 150 μm, however, significantly larger than the proton beam width. These results suggest that microbeam proton irradiation induced DNA damage in bystander cells *in vivo*. Ongoing experiments aim at investigating the kinetics of DNA repair focus formation and apoptosis in microbeam-irradiated ears. Further, the biological effects of smaller-diameter proton microbeams will be investigated.

Lubomir Smilenov and Helen Turner both made use of our new improvised nuclear devise (IND)-spectrum neutron irradiation system to study the effects of IND-spectrum neutrons on mice and human blood samples. This work was supported by the Columbia Center for Medical Countermeasures against Radiation (CMCR) for the development of biodosimetry approaches for a radiological event. Human blood samples were given up to 2 Gy of IND-spectrum neutron dose or 4 Gy of x rays using the Westinghouse orthovoltage x-ray system. Mice were irradiated with up to 2 Gy of neutrons, and comparison mice were given up to 4 Gy of x rays. Some mice were also given 1 Gy of neutrons and then an additional dose of x-ray to simulate a mixed field. The mice were sacrificed and blood was collected and scored for micronucleus and γH2AX foci to determine dose response. The results for this initial study will guide the future irradiations for the complete study of neutron exposures by the CMCR.

Vincent LiCata of Louisiana State University has continued his investigation into whether proteins from radiation resistant organisms are radiation resistant in isolation. This project examines homologous DNA binding proteins from organisms that live under very different conditions, one being the extremely radiation-resistant bacterium Deinococcus radiodurans. Protein stability and function are assayed at different radiation doses to determine whether the DNA polymerase (and eventually other isolated proteins) from D. radiodurans are better able to withstand radiation exposure than are homologous proteins from non-radiation resistant organisms. In this study, the protein is spread evenly under a cover slip and irradiated using the Track Segment facility to doses of 5 and 10 kGy and then analyzed for fragmentation and DNA end rejoining capacity.

Ciaran Morrison of the National University of Ireland, in collaboration with Brian Ponnaiya, continued a study probing the mechanisms involved in unscheduled centrosome duplication after exposure to ionizing radiation at defined nuclear sites using the Microbeam Facility. Experiments using DT40 chicken lymphocytes were conducted to test the ability of the cells to adhere to a substrate, and to assess the viability of these cells over the time span proposed for the experiments (48 h). Cells were seeded onto poly L-lysine-coated 60 mm dishes and imaged for irradiation on the microbeam endstation.

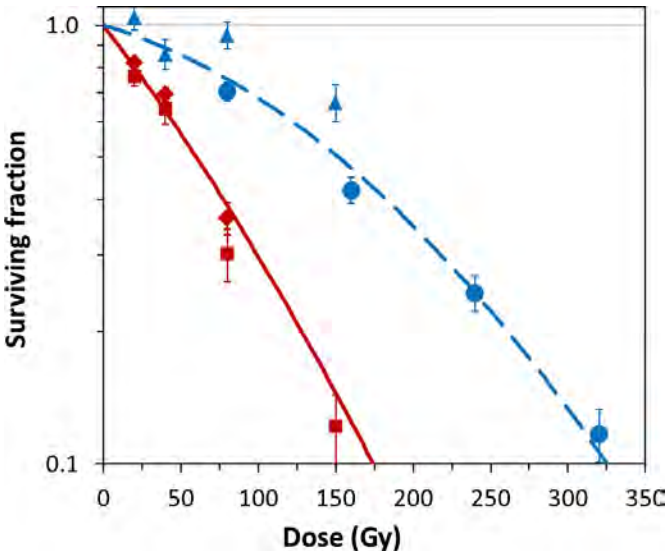

Figure 2. *C. neoformans clonogenic survival curves for external-beam α-particles (red symbols and line) and γ-rays (blue symbols and line). Different symbol shapes of each color represent independent repeat experiments. Curves represent best-fit linear-quadratic dose responses, and error bars are 95% confidence intervals.*

Ekaterina Dadachova at the Albert Einstein College of Medicine has been developing radioimmunotherapy (RIT) for treatment of Cryptococcus neoformans infections using ^{213}Bi-labeled antibodies specific to the cryptococcal capsule. She is performing a comparison of fungal cell susceptibility to external α-particle beam radiation versus α particles delivered by the bismuth-labeled antibodies (Exp. 173). Fungi grown to stationary phase in defined minimal medium were suspended in solution. As for other experiments, the solution was formed into a thin layer with a known uniform thickness under a cover slip. The fungi were irradiated with doses of 1 to 80 Gy of 125 keV/μm ^{4}He ions. Results so far (Fig. 2) indicate that: a) C. neoformans is more sensitive to external beam α particles than to external γ rays; b) α particles delivered by the capsule-binding antibodies may be more cytocidal to the C. neoformans cells than external beam α particles.

In addition to these experiments that use ionizing radiation, the ultraviolet (UV) microspot is being used by Kimara Targoff in the Division of Pediatric Medicine of Columbia University as an irradiation modality to observe the consequences of the ablation of single cells in the developing embryonic zebrafish heart (Exp. 162). Unlike the charged particle microbeams, the UV microspot only produces damage in the focal spot (approximately 1 μm diameter, 1.5 μm long), thus producing minimal effect elsewhere along the beam path. Cardiomyocytes in the ventricles of the hearts of zebrafish embryos are transfected with red fluorescent protein (RFP). The exposure protocol involves imaging cells on the top ventricular surface of the heart, where the incident laser first transits the ventricle, in live embryos 52 hours post fertilization and identifying individual cell nuclei as

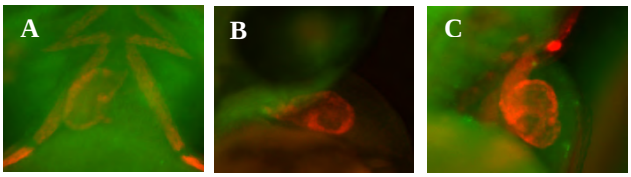

Figure 3. *Lateral views of control (panel A) and 4 and 24 hours post-UV irradiated (panels B and C respectively) Zebrafish embryos following immunostaining for Caspase 3 (green) and MF20 (red). MF20 was used to visualize the ventricles.*

targets. Each cellular target exposure is a sequence of three co-planar UV microspot scans over a 17.5 x 17.5 μm^2 area about each cell nucleus. The incident laser wavelength is tuned to 700 nm (350 nm during two-photon absorption) and the total exposure energy per cell is 27 mJ, corresponding to 18 mW exposure power during the 1.5 s exposure time. Consequences of UV microspot damage are monitored by live imaging (Fig. 3), in situ hybridization, and immunostaining.

Development of Facilities

Development continued on a number of extensions of our irradiation facilities and capabilities for imaging and irradiating biological specimens:

- Focused particle microbeams
- Focused x-ray microbeam
- Neutron microbeam
- IND spectrum neutron source
- Advanced imaging systems
- Targeting and manipulation of cells
- New cell analysis tools
- Small animal systems

Focused particle microbeams

The electrostatically focused microbeam was consistently operated with a 1-2 μm diameter beam and 0.5 μm when called for by a particular experiment. We have continued the process of performing a test run of the microbeam system on the evening preceding an irradiation day. These test runs have become a vital point of development for new techniques and training of operators for the microbeam system.

The Super-Microbeam development continued with the design and purchase of the super conducting solenoid magnet from Cryomagnetics, Inc. We will be taking delivery of the magnet in 2015 and installing it during the summer. The construction of the scanning Stimulated Emission Depletion (sSTED) super resolution microscope extension was begun with the purchasing of the required optical components. The interface of the sSTED development with the multi-photon microscope was begun with a low power alignment laser with the full 2 W continuous wave (CW) laser to be purchased in mid-2015.

The permanent magnet microbeam (PMM) was used as a secondary charged particle microbeam endstation for

the development of our Flow and Shoot (FAST) microfuildics irradiation system and the capillary electrophoresis (CE) system. The PMM has all of the irradiation capabilities of the electrostatic microbeam except the sub-micron beam spot size.

Focused x-ray microbeam

The x-ray microbeam uses characteristic Ti Kα x rays (4.5 keV) generated by proton-induced x-ray emission (PIXE). PIXE produces a nearly monochromatic x-ray source (extremely low bremsstrahlung) of the characteristic target x-ray energy. This allows these x-rays to be focused using a Fresnel zone plate to a spot size of 5 μm from a proton beam size of ~50 um in diameter.

The x-ray microbeam is stationed at a dedicated horizontal beamline at RARAF with the x-ray beam focused up in the vertical direction with the same microscope and stage geometry used in the charged particle microbeam systems, allowing for easy intercomparison between the microbeam types.

Neutron microbeam

The neutron microbeam at RARAF is the world's first neutron microbeam that can irradiate single cells. Incident protons near the reaction threshold (1.881 MeV) of the ^{7}Li(p,n)^{7}Be reaction generate neutrons that are severely forward coned in the laboratory frame of reference. By placing the target cells close to the lithium target it is possible to limit this cone to a single cell target. The Neutron microbeam uses a proton beam at 1.886 MeV focused to 10 μm on the lithium target. This results in a neutron spot size at the cell targets of 36 μm diameter with neutron energies ranging from 10-50 keV and a dose rate of 27 mGy/min.

The neutron microbeam is located in the accelerator bay at RARAF on a dedicated horizontal beam line. The proton beam is focused using a single quadrupole quadruplet with the spot size measured using an ionization counter and a knife-edge occlusion measurement. The center of the proton beam, visualized using a thin scintillator, is the center of the neutron beam. The proton beam measurements are made with a thin Havar metal window, which is exchanged with the lithium target for diagnostics.

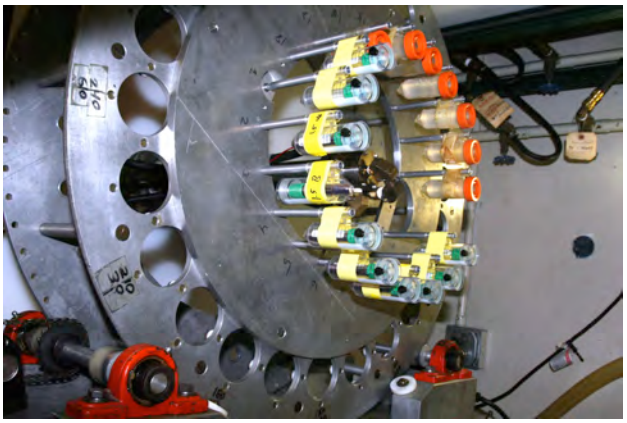

***Figure 4**. Irradiation of blood samples at the Hiroshima spectrum neutron source*

The neutron spot size is measured using CR-39 track-etch plastic coated with a thin layer of lithium carbonate heavily enriched with ^{6}Li. The neutrons interact with the ^{6}Li through the ^{6}Li(p,α)^{3}H reaction, producing energetic α and ^{3}H recoils that are easily observable as pits in the etched CR-39 using a microscope.

IND-spectrum neutron source

The IND-spectrum irradiator, based on the neutron energy spectrum of the Hiroshima bomb, was completed in the past year and irradiations have begun for both whole blood (Fig. 4) and small animals.

This fast neutron irradiation source was designed to generate the neutron spectrum seen from the "Little Boy" atomic bomb at Hiroshima at 1.5 km from ground zero. This field is generated through the reactions ^{9}Be(d,n)^{10}B and ^{9}Be(p,n)^{9}B using a mixed beam of monoatomic, diatomic and triatomic protons and deuterons. The RARAF Singletron uses a gas mixture of hydrogen to deuterium of 1:2, which feeds into the radio frequency plasma ion source. This irradiator is on the 0º beam line, as any bending of the beam to get to a target would separate the six different beams, preventing generation of the spectrum.

The neutron spectrum was verified using two proton recoil detection systems. A 2" diameter 2" thick liquid scintillator for energies >1 Mev and a 1.5" diameter spherical gas proportional counter with 3 atmospheres of hydrogen gas for <1 MeV. Using MCNPX-PoliMi Monte Carlo simulations to calculate the exact response functions of the detectors, it is possible to reconstruct the spectrum from the readout of the detectors in the neutron field.

The dose rate has been calibrated to deliver 0.25 Gy of neutrons in 10 minutes (with a gamma-ray contribution of an additional 15%). This dose rate allows the delivery of 1 Gy in less than 1 hour and provides the opportunity to rotate animals or other samples to achieve dose symmetry.

Advanced imaging systems

We continue to develop new techniques to obtain two- and three-dimensional images of cells, reduce UV exposure, and improve resolution.

<u>Real-time imaging</u>

Short-term effects that happen within seconds up to the first few minutes after irradiation set the stage for later effects. Real-time imaging and observation of the short-term responses will give insight to experimenters into their end points. Techniques have been developed using our EMCCD camera and our fast switching SOLA LED light source to acquire images with several frames per second to observe the short-term effects of irradiation on a timescale of minutes to hours following irradiation.

Multi-photon microscope with the UV microspot

The multi-photon microscope was developed several years ago and integrated with the charged particle microbeam irradiator. This microscope, through the long wavelength incident laser, allows in-depth imaging of 3D tissues and small animals, such as *c. elegans* and zebrafish embryos. This is achieved using the sectioning capability of the multi-photon effect, in which the photon density increases to generate constructive interference producing a 3D voxel of photons with half the original incident wavelength and twice the energy, which can locally excite fluorophores and/or produce other fluorescent effects (e.g. auto fluorescence and second-harmonic generation). This 3D voxel is then scanned through a single layer and stepped through the sample using the nanoprecision z-stage, generating a stack of 3D slices of the sample, which are reconstructed into 3D images.

By increasing the intensity of the laser, there can be a 3 photon interference at the area of constructive interference, resulting in a voxel of UV light with a third of the incident wavelength (and three times the energy). This is how we produce our UV microspot. The UV microspot can be used to induce damage within a 3D target. This UV microspot is currently being used to irradiate developing zebrafish embryo ventricles with Dr. Targoff (see above).

STED

We are developing a Stimulated Emission Depletion (STED) super resolution microscope system with optical resolution of 70 nm in combination with out super microbeam to achieve compatibility between imaging resolution and beam spot size. The STED system at RARAF builds off the multi-photon microscope using it as the primary excitation laser. A second CW laser is added in parallel with the multi-photon laser. Using polarization optics, the second laser projects a donut shaped point spread function around the excitation spot of the multi-photon system. With proper selection of the second laser wavelength and sufficient intensity, the second laser will deplete the fluorescent states around the excitation spot allowing fluorescence to be produced only from the center of the donut, which will be reduced to nanometer sizes.

We have already shown that it is possible to perform STED imaging on live cells using a water immersion lens and the STED super resolution microscope at Dr. Liao's Lab in the Mechanical Engineering Department. This system, while not optimized for water immersion optics, gave a resolution of <100 nm.

We have completed the design of the STED system for RARAF and have begun purchasing the required optics. A small, low powered alignment laser with the correct wavelength was purchased to allow the construction and alignment of the STED system without the safety hazard presented by the higher power laser, which will be purchased in 2015.

Targeting and manipulation of cells

We have the capability to fabricate microfluidic devices in hard plastics, such as acrylic, and soft plastics, such as polydimethylsiloxane (PDMS). The micro-milling machine installed at RARAF has software to produce parts designed using the Solid Works computer-aided design (CAD) program. This system has been used to manufacture the single-cell dispenser and the microfluidics chips for the cell sorter and microFACS systems (described below). Several new microfluidic systems are being developed to target, manipulate and analyze cells.

FAST

The Flow And ShooT (FAST) microfluidic irradiation system has continued in development. This system flows non-adherent cells, such as lymphocytes, though a microfluidic channel over the microbeam window where the Point and Shoot magnetic deflector tracks, targets and irradiates the cells as they pass the beam location.

The current design is for a PDMS microfluidic chip with a 200 μm wide by 20 μm deep channel to be vacuum coupled to the microbeam exit window. The channel is thin enough for particles to pass completely through the chip and into the gas ionization counter mounted on the microscope objective. The channel is imaged with 10's of frames per second for cell imaging, locating and tracking for inputs to the Point and Shoot irradiator. We have increased the analysis speed of this process and can track cells with flow rates up to 2 cm/second. The tracking prediction accuracy of the system is within 1.5 μm of the actual new position of the cells 95% of the time.

Cell dispenser

Development of the automated cell dispenser has continued with further study of the electrical properties of the fluid channels, geometry of the detection elements and the design of the output channels to assist in the flow characteristics of the dispensing pulse. The basic design is cells flowing in a polymethyl methacrylate (PMMA) microfluidic channel approach a T intersection of channels. Immediately before the intersection are parallel electrodes that sense the cell by the change in the impedance of the fluid in the channel. When a cell is detected, a speed determination is made from the detection signal and a pulse is sent through the intersecting channel to dispense the cell out of the chip.

Work has been performed looking at the conductivity of the cell medium with the geometry of the impedance electrodes for detection of cells. This work continues with the optimal spacing of the electrodes for the media and flow rates to be determined ultimately by the throughput needs in terms of number of cells/minute.

The original design of the dispenser with a long delay output channel demonstrated that the dispenser with the sensor technology worked as theorized. The dispensing channel has been shortened and several designs are being tested for dispensing droplet size control and accuracy of

dispensing. These design changes are required for optimization and integration into our other microfluidic devices.

MicroFACS

Development of the microfluidic Fluorescence-Activated Cells Sorting (microFACS) system has continued to combine flow cytometry and sorting with our other microfluidic irradiation and dispensing technologies.

The microFACS system uses Dean vortex, drift flow focusing to entrain the samples into a sheath flow focused column for flow cytometry detection in the main channel. The sample is illuminated with a laser through fiber optic coupling, with the fluorescent output also detected through fiber optic coupling. The combination of fiber optics and microfluidics will allow for the microFACS to be coupled to the other microfluidic systems in close proximity to the microbeam endstations.

The primary development on the microFACS was the modeling using COMSOL Multiphysics (Fig. 5) and construction of different introduction arcs for the drift flow focusing. Longer input arcs allow for slow flow on the output channel without loss of focusing. The current design has a 180° arc with an output flow rate of 5 cm/sec. Ultimately, matching this speed to that of the FAST (currently 2 cm/sec) will allow for an integrated microfluidic sorting and irradiation system.

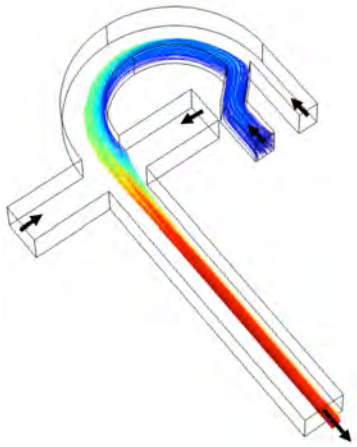

Figure 5*. COMSOL model showing the Dean flow focusing of the sample input. The color of the trajectories corresponds to the velocity: slower (blue) to faster (red). The arrows denote flow directions. The column diameter at the exit is 60 μm.*

AMOEBA

Our Automated Microbeam Observation Environment for Biological Analysis (AMOEBA) is an automated system for precise regulation and control of environmental conditions for biological samples before, during, and after microbeam irradiations. The AMOEBA system will establish, maintain and change culture conditions (e.g. temperature, pH, pCO2, drug concentrations) as required by our microbeam users. This system will have automated feedback, fluid-flow control systems for all of the needed parameters through a distributed electronics control package.

The AMOEBA is being designed in two comparable systems: the standard AMOEBA for dish based microbeam experiments and the μAMOEBA for microfluidics based irradiation experiments. Initial testing has verified that we can control temperature (37 ± 1 °C) and pH over long periods with minimal feedback circuitry. We have begun using these basic controls for monitoring cell kinetics involving DNA repair, cell-cycle progression and chromosomal domain dynamics.

New cell analysis tools

CE-LIF

We have begun the construction of our Capillary Electrophoresis – Laser Induced Fluorescence (CE-LIF) system to provide our users with the capability of measuring reactive oxygen species within individual cells immediately after irradiation. The nanoliter input volumes make this system ideal for single-cell, small-scale biochemical analysis.

The basic idea of CE is to take advantage of two superimposed flow modalities experienced by the analytes in a long, thin fused silica capillary: (1) Electrophoretic flow, responsible for separating the analytes of lysed cells by charge and Stokes radius; (2) Electroosmotic flow, which drives the buffer and analytes (regardless of polarity) toward the detector. The electroosmotic flow is much stronger than the electrophoretic flow, ensuring that all analytes will reach the detector. Coupled to CE, laser-induced fluorescence (LIF) provides highly sensitive detection, particularly for brightly fluorescent molecules.

Preliminary studies with our collaborator, Dr. Jonathan Sweedler from the University of Illinois (Urbana-Champaign) has demonstrated that single cell CE-LIF has sufficient sensitivity to measure radiation relevant endpoints (e.g. glutathione levels and oxidation of redox-sensitive dyes, such as DHR-123). These preliminary studies are guiding the development of the CE-LIF system for sensitivity at the RARAF Microbeam Facility.

Single-cell Raman spectroscopy

We plan a unique coupling of Raman spectroscopy with a microbeam irradiator to provide non-invasive, label-free identification and assessment of the distribution of bio- molecules and chemicals within a single cell in real time. Raman will allow for the determination of conformational changes to biomolecules resulting from radiation damage in live single cells in culture or 3D tissues before, during and after irradiation. This will be the first real-time coupling of Raman spectroscopy in conjunction with a microbeam irradiator.

The Raman spectroscopy system to be integrated with our Microbeam Facility will be a Renishaw InVia Raman spectroscopy system. An identical system is available in the Electrical Engineering Department on the Columbia

University main campus, where we have acquire preliminary data and demonstrated the ability of the system to acquire data using water immersion optics. With this system, we have successfully measured the Raman spectrum of single cells in 3D full-thickness human skin models (MatTek Corp.).

Small animal systems – Transgenic mouse model

For many years now, investigations of radiation-induced bystander effects have been conducted in cell cultures and 3-D systems *in vitro*. The next logical step was to develop and implement microbeam irradiation protocols to study effects in living organisms. We have now developed a mouse ear model for *in vivo* bystander studies. With an average thickness of 250-300 μm, this model can be used to investigate radiation-induced bystander effects with a 3-MeV proton microbeam having a range of 134 μm.

Using gentle suction, the ear of an anesthetized mouse is flattened onto the underside of a flat plate of a custom-made holder. The flattened mouse ear is then placed over the microbeam port and cells along a line of the ear are irradiated with the proton microbeam. At chosen times after irradiation, mice are sacrificed and a punch of the ear is collected. Tissues are then fixed, paraffin-embedded and cut in 5-μm sections perpendicularly to the direction of the line of irradiation. The sections are then analyzed for biological endpoints (i.e., formation of repair protein foci, apoptosis) as a function of the distance from the irradiated line.

Using γH2AX focus formation assessed by immunohistochemical analysis as an endpoint, we found that proton irradiation induced γH2AX foci *in vivo* relative to controls. As expected, keratinocytes positive for γH2AX foci were observed in only one of the two epidermal layers of the mouse ear. Cells in the epidermal layer opposite to the irradiated γH2AX positive region did not exhibit foci. Assuming that the nuclei of mouse keratinocytes are 9-11 μm in diameter, a larger number of cells than expected showed foci. In a particular experiment, although the irradiated line was ~35 μm wide, the average width spanned by γH2AX-positive cells exceeded 150 μm. These results suggest that microbeam proton irradiation induced DNA damage in bystander cells *in vivo*.

Singletron Utilization and Operation

Table II summarizes accelerator usage for the past year. The nominal Singletron availability is one 8-hour shift per weekday (~248 days per year), however the accelerator is frequently run well into the evening, often on weekends, and occasionally 24 hours a day for experiments or development. Total use for experiments and development this year was 50% of the regularly available day shifts.

Accelerator use for radiobiology and associated dosimetry was about 22% more than last year and about 2/3 the average for the last 5 years. About 71% of the use for all experiments was for charged particle microbeam irradiations, 21% for track segment irradiations, and 8% for neutron irradiations. Approximately 25% of the experiment time was for studies proposed by external users, and 75% was for internal users.

On-line facility development and testing was about 29% of the available time, primarily for development and testing of the neutron microbeam and the IND neutron spectrum system, and for testing the charged particle microbeam prior to scheduled irradiations. This was about 15% less than the average for the last five years and about the same as last year due to an emphasis on development not requiring accelerator use.

The accelerator was opened once this year to replace the source bottle after 16 months of use.

Table II. *Accelerator Use, January 1 - December 31, 2014*
Normally Scheduled Shifts

Radiobiology and associated dosimetry	27%
Radiological physics and chemistry	4%
On-line facility development and testing	29%
Microbeam Training Course	1%
Safety system	2%
Accelerator-related repairs/maintenance	5%
Other repairs and maintenance	1%
Off-line facility development	55%

Training

REU

Since 2004 we have participated in the Research Experiences for Undergraduates (REU) project in collaboration with the Columbia University Physics Department. This is a very selective program that attracts highly talented participants. For 9-10 weeks during the summer each student attends lectures by members of different research groups at Nevis Laboratories, works on a research project, and presents oral and written reports on his or her progress at the end of the program. Among other activities, the students attend a seminar about and take a tour of RARAF.

This year, Steven Harrellson from Missouri State University participated in the program and worked with Alan Bigelow on the study of radiation effects on hemoglobin in human blood analyzed using Raman spectroscopy.

Microbeam Training Course

The fourth annual RARAF microbeam training course “Single-Cell Microbeams: Theory and Practice” was

given May 19-21, 2014. There were nine students, which is about the largest number we can reasonably handle.

Dr. Marcelo Vazquez of Loma Linda University Medical Center continued his service as Course Director. He has had significant experience from his prior employment at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL) where he helped establish the first NASA Space Radiation Summer School and ran the course for three years.

The microbeam training course was publicized by e-mail notifications using the contact lists for the previous courses and the 2012 Microbeam Workshop and by announcements on the RARAF and the EURADOS web sites.

Applicants and Students

- We received 16 applications. The prospective students were from the U.S, Europe and Asia and, as in the previous courses, covered a wide range of educational levels.
- The nine applicants selected for the course are listed in Table III and shown in Fig. 6.
- As before, candidate selection was made by the RARAF Local Executive Committee, with scores applied based on a set of predetermined criteria.

Course

DAY 1:

- Followed essentially the same format as the first two courses, and featured a guest lecture by Dr. Eduoard Azzam from Rutgers University.
- As before, a live demonstration was given of the immediate production of a focus in cells with GFP-tagged XRCC1 protein using the charged particle microbeam.
- The day ended with a session on the planning and experimental design of microbeam irradiations.

DAY 2:

- Followed the format of last year with demonstrations, hands-on activities (Fig. 6), and intense debriefings.
- In addition, the students were tasked with designing an experiment based on their own scientific interest using knowledge obtained during the course to create a RARAF beam time request proposal.

DAY 3:

- Followed the format of the previous years, with lectures and group discussions and an in-depth tour of the x-ray, and neutron microbeams and the UV microspot.
- The lectures were followed by an intense discussion on user/facility interfacing.
- The students made presentations of their individual or team beam-time proposals for review and critique by the instructors.
- The course ended with an informal closing ceremony and the delivery of a certificate of completion to each student.

Each student took home a notebook containing copies of all the slides from the lectures as well as the instructions for all the physics and biology procedures that were demonstrated and that they had performed.

A virtual course created from the lectures and demonstrations from the past two years is described under "Dissemination" below. A paper on the design of and our experience with the training course was also published this year.

Table III. *Students for the third RARAF Microbeam Training Course.*

Name	*Position*	*Affiliation*
Daniel Adjei	Ph.D. Student	Dept. of Physics, King's College, London
Alisa Kobayashi	Technical Staff Member	Research Development and Support Center, National Institute of Radiological Sciences, Japan
Nicolas Colangelo	MD/Ph. D. student	Rutgers University – New Jersey Medical School
Ruqun Wu	Primary Research Experimenter	Institue of Modern Physics, Chinese Academy of Sciences
Jörgen Elgqvist	Researcher	Medical Radiation Physics/Lund Bioimaging Center, Lund University
Maurizia Di Paolo	Fellowship Researcher	University of L'Aquila
Hakim Belmouaddine	Ph.D. Student	Radiation Sciences and Biomedical Imaging, Univ. of Sherbrooke, Quebec, Canada
Pia Fredericia	Ph.D. student	Center for Nuclear Technologies, DTU Risø, Roskilde, Denmark
Velauthapillai Nivethan	Research Assistant	Institute of Medical Science, Univeristy of Toronto

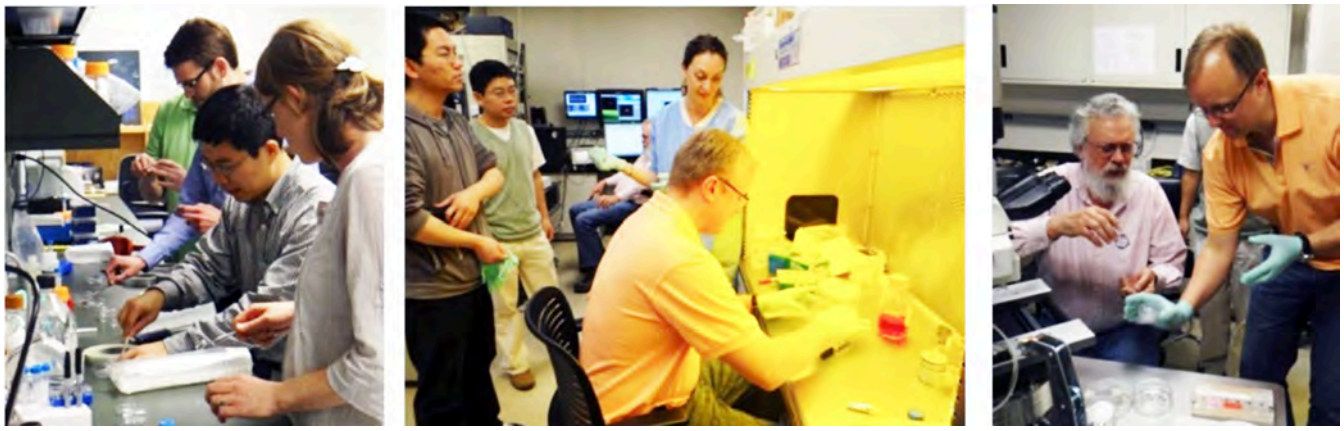

Figure 6. *Microbeam Training Course students engaging in hands-on training.*

Group Training

In addition to training individuals at RARAF, staff members also participate in training courses presented at other facilities as a means of introducing microbeam concepts and experiments to a broader audience. As for the past two years, Gerhard Randers-Pehrson lectured on "High/low LET microbeams" at the NASA Space Radiation Summer School, Brookhaven National Laboratory, Upton, NY, on June 11, 2014.

Dissemination

Web site

A new RARAF website design was created in 2013 to provide clear and effective presentation and improve access to content. Functional menus (including a home page rotating-picture menu) were designed to make navigation through the content easy and interesting, with a hierarchical structure from general information, suitable for a general or non-science audience, to more detailed technical content.

The site contains information on microbeams in general, as well as detailed technical information on our various microbeams; *in-vitro* and *in-vivo* endpoints that we use; details of available on-line and off-line imaging capabilities; microfluidic systems we are developing; other charged particle and neutron irradiation facilities available at RARAF; our on-line training course materials; publication lists; information on RARAF contacts and directions to the facility. The site is periodically updated to include new irradiation facilities, cell handling and analysis capabilities, publications and other information.

Virtual training course

We have developed an on-line virtual microbeam training course, based on the three-day microbeam training courses. This on-line course was designed to give interested physicists and biologists who could not attend in person a thorough introduction to microbeam technology.

The goal of the online course, as for the face-to-face course, is to facilitate a better understanding of how microbeams work, what experiments can be performed using a microbeam, why these experiments are of biological interest, and how to design / perform these experiments.

The on-line curriculum material consists of audio podcasts and the same handouts that the face-to-face students received. The audio of each podcast is synched with the accompanying PowerPoint slides (viewable on a video iPod, tablet, PC or Mac, or smart phone), as well as a PDF version of the slides. High-resolution video (720p, with audio) was also used to document demonstrations of all aspects of a microbeam experiment, from making microbeam dishes to irradiating cells and performing online analyses. After extensive editing, this resulted in about 4½ hours of video footage. Additional material is added to the on-line course for new course presentations or lecturers.

The on-line training course can be accessed through the RARAF YouTube channel (http://www.youtube.com/user/RARAFcourses), or through the RARAF website (www.RARAF.com). The videos can be viewed on any Internet-enabled device supporting YouTube format.

Tours

In addition to training students, tours of the Facility (Fig. 7) provide a general introduction to the research performed at RARAF and the irradiation facilities that are available. This year we gave tours to more than 30 scientists, students, and members of the public.

As an example, eleven high school seniors who had been offered priority admission to Columbia as physics majors, some of whom were Columbia I. I. Rabi Scholarship winners, toured RARAF in April along with Dr. John Parsons from the Physics Department at Nevis Labs.

Figure 7. *Dr. Harken giving a tour for a group from Shanghai Jiao Tong University.*

Personnel

The Director of RARAF is Dr. David Brenner, the Director of the Center for Radiological Research (CRR). The accelerator facility is operated by Dr. Gerhard Randers-Pehrson, the Associate Director of RARAF.

Dr. Charles Geard, a Senior Biologist Emeritus, continues to visit RARAF frequently lending his considerable expertise.

Dr. Brian Ponnaiya, an Associate Research Scientist, is the biology advisor for RARAF. He collaborates with many of the external users and coordinates with the CRR, where he spends about half his time.

Dr. Alan Bigelow, an Associate Research Scientist, developed the multiphoton microscopy system, which includes the UV microspot irradiation facility, and is working on the development of the Raman spectroscopy and AMOEBA systems.

Dr. Guy Garty, an Associate Professor, developed the Flow and Shoot (FAST) system and is developing the CE-LIF system. He spends about half his time working on Project 1 of the CRR National Institute of Allergy and Infectious Diseases (NIAID) project, for which he is the project manager.

Dr. Andrew Harken, an Associate Research Scientist, is responsible for the x-ray microbeam. He is also working on the imaging of cells without stain using a highly sensitive EMCCD camera, the STED system for extremely high-resolution spectroscopy, and the microFACS system.

Dr. Yanping Xu, an Associate Research Scientist, is developing the neutron microbeam. He is also developing the accelerator-generated IND-spectrum neutron source.

Dr. Manuela Buonanno, a Postdoctoral Fellow in radiation biology, collaborates with many of our external users and performs the assays for the mouse ear microbeam irradiations.

Dr. David Welch, a Postdoctoral Fellow, is responsible for the development of new microfluidic tools and interfaces for microfluidic irradiation tools. His expertise in microfluidics has been of considerable assistance in the development of our microfluidics applications.

RECENT PUBLICATIONS OF WORK PERFORMED AT RARAF

1. Brenner DJ, Vazquez M, Buonanno M, Amundson SA, Bigelow AW, Garty G, Harken AD, Hei TK, Marino SA, Ponnaiya B, Randers-Pehrson G, Xu Y (2014) Integrated interdisciplinary training in the radiological sciences. *Br. J. Radiol* **87**: 20130779.
2. Ghandhi SA, Ponnaiya B, Panigrahi SK, Hopkins KM, Cui Q, Hei TK, Amundson SA, and Lieberman HB (2014) RAD9 deficiency enhances radiation induced bystander DNA damage and transcriptomal response. *Radiat Oncol* **9:** 206.
3. Halm BM, Franke AA, Lai JF, Turner HC, Brenner DJ, Zohrabian VM, Dimauro R (2014) γ-H2AX foci are increased in lymphocytes in vivo in young children 1 h after very low-dose X-irradiation: a pilot study. *Pediatr Radiol.* **44(10):** 1310-1317.
4. Lyulko OV, Garty G, Randers-Pehrson G, Turner HC, Szolc B, and Brenner DJ (2014) Fast image analysis for the micronucleus assay in a fully automated high-throughput biodosimetry system. *Radiat Res* **181:** 146-161..
5. Welch D and Christen JB (2014) Real-time feedback control of pH within microfluidics using integrated sensing and actuation. *Lab Chip,* **14**: 1191-1197. ■