ON THE IMPLEMENTATIONS OF EXPERIMENTAL METHODS USING FLUORESCENCE MICROSCOPY IN MODERN RADIOBIOLOGY

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Medical Physics in the
School of Mechanical Engineering

Georgia Institute of Technology
December 2010

ON THE IMPLEMENTATIONS OF EXPERIMENTAL METHODS USING FLUORESCENCE MICROSCOPY IN MODERN RADIOBIOLOGY

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Date Approved: November 11, 2010

ACKNOWLEDGEMENTS

I wish to thank Dr. Huichen Wang and the members of his lab Dr. Lin Lin Tian, Dr. Minli Wang, Dr. Yi Wang, and Xiaobing Tang for their kindness, patience, and assistance with every step of this project.

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SUMMARY

This thesis is intended as an introductory lab manual on the experimental methods using fluorescence microscopy in modern radiobiology research. It is written for those who are unfamiliar with biology research. It first covers the proper use of laboratory equipment and growth of cell cultures in the lab. Subsequent chapters provide overviews of relevant modern experimental techniques for the quantification of radiation induced DNA damage in cells, and detailed protocols for performing these procedures. Techniques covered include immunostaining with fluorescent antibodies, the comet assay, and plasmid DNA transfections. Results of some straightforward experiments using these techniques are presented.

CHAPTER 1

INTRODUCTION

The field of radiation oncology research requires the work of many specialists, who are each experts in their own field of study. There are the physicists, who are known for constantly tinkering with the treatment machines, striving always to increase the accuracy of treatments, and devising new delivery methods to increase the radiation dose to the tumor while sparing the healthy tissue as much as possible. They think in Grays and millimeters, and see radiation beam geometry. Then there are the biologists, who work tirelessly in their labs trying to better understand the formation of cancer, and the mechanisms that make cells susceptible or resistant to radiation therapy. Their world consists of cell lines growing in dishes, DNA molecules, and DNA damage repair proteins.

A consequence of this degree of specialization is a reduced breadth of knowledge among cancer researchers. There are few people who can see the big picture, from the activated oncogene, to the patient on the treatment couch of a linear accelerator. Physicists tend to see a geometrical problem; a target that must be hit. An increase in dose means a different color on the treatment planning system, and perhaps a more favorable dose volume histogram. Biologists know that dose means DNA damage, but rarely understand how this is used to treat the patient. To them, a Gray is something that is punched into the x-ray machine in order to observe cell proteins or broken DNA.

The goal of this project, undertaken by a physicist who ventured into a biology laboratory, is to help provide physicists with the modern radiobiology techniques that use the fluorescent microscope. These techniques allow researchers to see inside the cell nucleus and quantify the level of DNA damage sustained by an individual cell. This opens the door to improving radiation treatment by tailoring a plan specifically to a

patient's biology by assessing his or her radiation tolerance and testing tumor cells for heterogeneities such as radioresistance or hypoxia.

This thesis is written largely as an introductory laboratory manual for those unfamiliar with biology research. It begins with Chapter 2, on equipment pieces that are commonly used in the biology lab. Chapter 3 focuses on the growth of cells in culture dishes, a crucial task in biology research. Chapter 4 details the modern technique of immunostaining, or labeling cell proteins with specific antibodies so the distribution of proteins can be observed with a fluorescent microscope. For the physicist, one exciting application of this process is visualizing the damage caused by high-LET and low-LET radiation. After staining, the tracks of the high-LET particles can be clearly seen, and contrasted with the random distribution of damage caused by low-LET x-ray irradiation. Chapter 5 explains the procedure known as the comet assay. This technique allows one to observe DNA damage of a single cell by using an electric field to force broken DNA fragments out of the cell volume. Chapter 6 describes the use of plasmid vectors to modify cell DNA, causing the synthesis of fluorescent proteins by living cells. Each chapter includes some experimental results that were obtained by the author.

CHAPTER 2

BASIC LAB EQUIPMENT AND PROCEDURES

One of the first and most intimidating obstacles facing a physicist who has little background in biology research is the vast array of unfamiliar tools and equipment that is present in the biology laboratory. Some of this equipment is used for only one specific task or procedure, and thus is not immediately necessary to learn. Much of it however, is used every day for almost any procedure, so the first task for a beginner in a radiobiology laboratory is to become familiar with these tools and their proper usage.

Laboratory Notebook

A crucial tool that biology researchers use every day is the laboratory notebook. A detailed record should be kept for all procedures, calculations, and results. When a new protocol is attempted, it is a good idea for the researcher to write his or her own summary of the protocol, along with the challenges encountered, lessons learned, and even reminders such as where to find a certain reagent. Detailed records of what stainings or dilutions were used in a certain experiment will prove invaluable when the results of an experiment are reviewed months after the fact. Even if electronic results such as digital images or spreadsheets have been saved, it is helpful to write down important observations or key results in the lab notebook for future reference.

Pipettes

Almost all biology lab procedures will involve the handling of various liquid solutions. The quantities to be used may range from fractions of a microliter, to multiple

liters. Manipulation of small volumes (less than 1 milliliter) is a common task, and for this the most appropriate tool is the pipette. The pipette consists of a handle attached to a shaft which extends axially in the direction of the handle. A disposable plastic tip can be easily fixed to the end of the shaft. Inside the handle is a sealed piston and cylinder, which can be adjusted to vary the displacement of the piston, and thus the amount of liquid which will be pipetted.

The controls on the pipette are straightforward. The piston is operated by a protrusion from the top of the handle. This piston is depressed before inserting the pipette tip into the liquid, and then released to suck the liquid into the pipette tip. To eject the liquid from the pipette tip, the piston is depressed again. It should be noted that the piston has two mechanical "stops". The first stop corresponds to the desired quantity of liquid, and this stop should be used for uptake of the liquid. For ejecting the liquid, it is possible to push the piston past the first stop, until it is fully depressed onto the top of the handle. This is to facilitate the ejection of all the fluid in the pipette tip.

Other controls consist of the volume adjustment and a mechanism to eject the pipette tip itself. The volume is adjusted by twisting the piston protruding from the top of the handle. A window on the front of the handle displays the volume which is currently set. Most pipettes also have a mechanism to lock the piston of the desired volume, thus it is necessary to unlock the piston before adjusting the selected volume. Also situated on top of the handle and operated by the thumb is the pipette tip ejector. When one is done using the current pipette tip, it can be conveniently ejected using this feature.

These pipettes come in various sizes for different applications. Each size is useful for a range of volumes corresponding to the adjustability of the piston. Commonly used sizes are: 0.1-2 µL, 2-20 µL, 10-100 µL, 20-200 µL, and 100-1000 µL. The different sized pipettes are designed to use different sized tips. The tips come in boxes which are color coded to show with which size pipette they should be used. The pipettes and tips are shown in Figures 1 and 2. For best accuracy, the pipettes should not be used at the

extreme low end of their volume range, i.e. to pipette 100 µL it would be better to use the 10-100 or 20-200 µL pipettes versus the 100-1000 µL pipette.

For best results, the piston should be operated smoothly, avoiding quick motions. Jerky movement of the piston may result in bubbles forming in the pipette tip which will affect the volume. When pipetting fluid into the tip, avoid pressing the tip against the bottom of the container, as this can affect flow into the tip and result in inaccurate volumes.

Figure 1 - Three different sized pipettes. The volume adjustment can be seen protruding from the top.

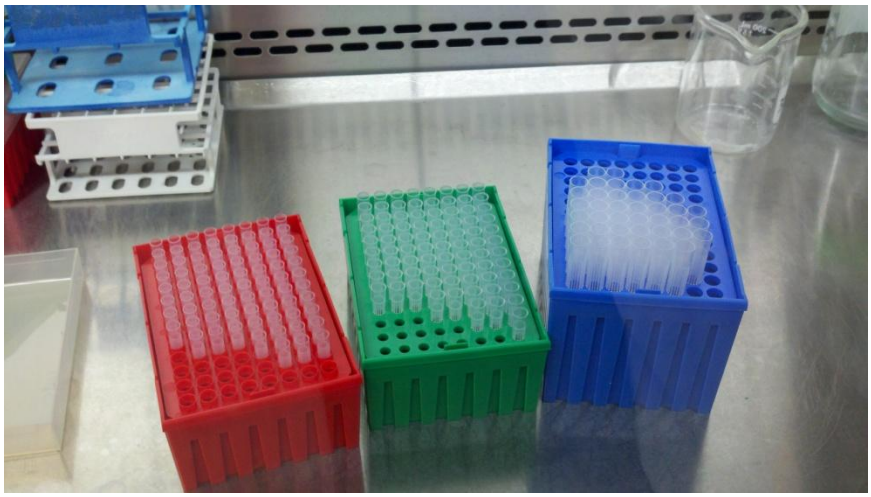

Figure 2 - Pipette tips of three sizes in their containers.

Pipettor

For dealing with volumes of liquid larger than 1 milliliter, a pipettor is the best choice. The pipettor consists of a vacuum pump which can be sealed to a glass pipette tube. There are buttons on the handle of the pipettor to uptake fluid or dispense fluid. These can be battery operated or connected to AC power. In the battery operated units, an electric motor built into the handle drives the vacuum pump. In AC powered units, the vacuum pump is housed in a separate benchtop box, which is connected to the handle via two flexible hoses which provide vacuum and pressure to the handle. The AC powered units typically have more powerful suction and pressure, and have no vibration in the handle since the pump is not in the handle. Common controls on pipettor include the ability to vary the strength of the vacuum for faster or slower pipetting, and the option for gravity or pressurized dispensing.

The glass pipette tubes that are used with the pipettor come in multiple sizes, which all work with the standard pipettor. Standard sizes are 2 mL, 5 mL, 10 mL, and 25 mL. The pipettor and glass tubes are shown in Figures 3 and 4. The tubes are graduated with both ascending and descending numbers for convenience, and the markings extend into the negative numbers, meaning the pipette tubes are slightly larger than their rated capacity. For example, the 25 mL tube is graduated to -5, so it will actually hold as much as 30 mL. The pipette tubes come in individually sealed, sterile packages, so when opening them, it is good practice to refrain from touching the actual glass tube, as this may be inserted into a sterile container to uptake solution. It is easy to hold the tube by its plastic wrapping, and peel back the wrapping without touching the tube. The tube is then

inserted into the nozzle of the pipettor, and there is no need to ever touch the tube. Details of sterile technique will be covered in detail in a subsequent section.

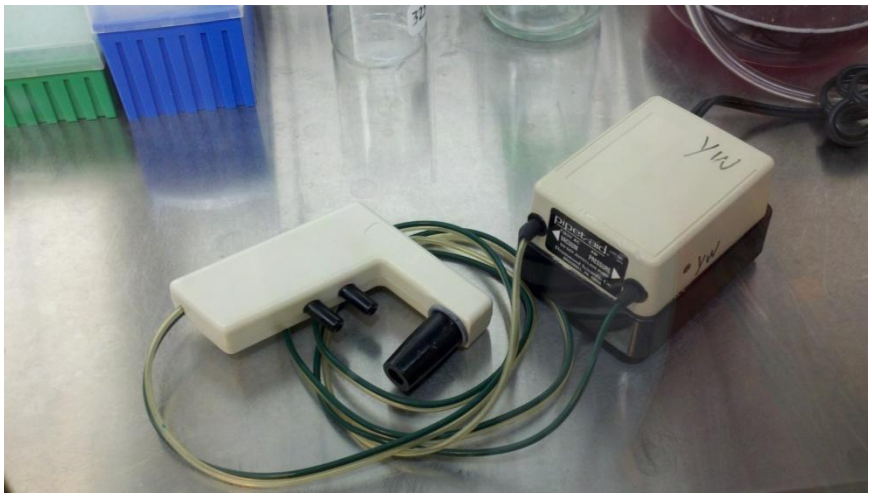

Figure 3 - A pipettor with a separate vacuum pump.

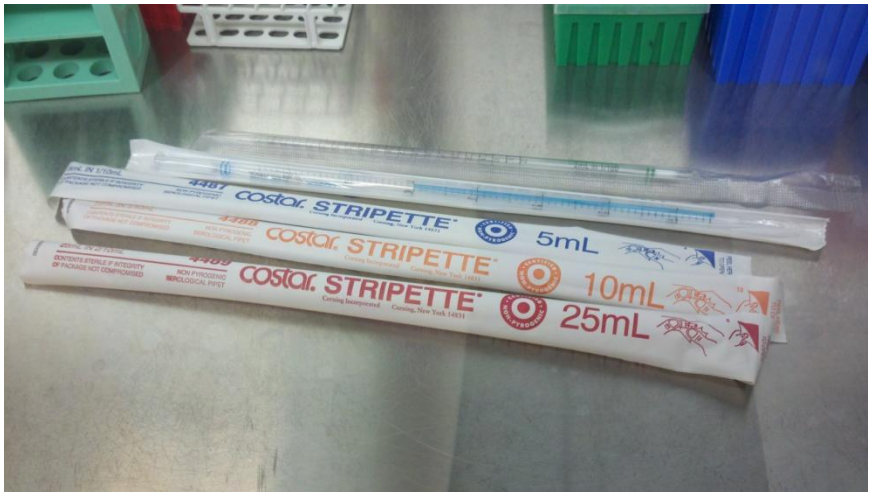

Figure 4 - Glass pipette tubes in various sizes.

Inverted Light Microscope

An everyday task in a cell biology laboratory is monitoring the growth and condition of cell cultures. Cells are typically grown in clear plastic culture dishes. Cells can be conveniently observed in their dishes using an inverted light microscope. The term

‘inverted’ indicates that the objectives look up from below the microscope stage, the platform where the sample is located. The culture dish is placed on the stage, and the objectives focus on the bottom of the dish through an opening in the stage. This is particularly convenient for adherent cells, because the cells are already attached to the bottom of the dish. The dish itself can then be moved by hand to observe various areas on the dish. To perform the same basic task using an upright microscope would require the cells to be plated onto a microscope slide, and covered with a cover slip before observation.

An inverted microscope may have multiple objectives mounted on the revolving nosepiece. The lowest power magnification is likely most suitable for routine monitoring of the concentration of cells, which is the most common task. Higher power objectives can be useful for seeing details of the cells or for comparison between cell types. Care should be taken when switching objectives not to allow any objective to contact the stage.

The two main controls on an inverted light microscope are the brightness of the light source and the focus. The light source is located above the stage, and should typically be kept quite dim, as it does not require much light to penetrate a thin monolayer of cells on a culture dish.

The focus knob is by far the most used control on the microscope. The focus control consists of a fine adjustment knob inside a larger course adjustment knob. Observing cell cultures is usually a two-handed job. One hand moves the dish while the other adjusts the focus. The bottom of a plastic culture dish is not a precisely flat surface, so as the dish is moved around on the stage, the distance from the dish to the objective will change slightly, and this will affect the focus. The image may appear perfectly

focused while looking at one point on the dish, but moving the dish even a centimeter may require a significant adjustment to the focus. With practice, it becomes natural to move the dish around with one hand, while constantly adjusting the focus back and forth to sharpen the image.

Biological Safety Cabinet

When working with growing cell cultures, it is crucial that sterile conditions be maintained. To facilitate this, all work with growing cell cultures must be performed inside a biological safety cabinet. This apparatus resembles the more familiar fume hood that many may remember from chemistry labs, but its function is different. A fume hood draws large amounts of air from the lab into the hood, up and away from the user. Its purpose is primarily the protection of the user and others in the lab from hazardous fumes. A biological safety cabinet is used in most biology labs to prevent contamination of the samples inside by any contaminants outside the cabinet. The air inside is filtered, and circulated to create a curtain of air across the opening that prevents outside air from entering.

These cabinets have a sliding glass door across the front. The door should remain closed when the cabinet is not in use, and should be opened to the specified height when ready for use. Controls for lights, fans, and ventilation are located on the front of the cabinet, and should all be turned on when the cabinet is in use. Oftentimes, these functions shut off automatically when the glass door is closed. Some cabinets are also equipped with an ultraviolet light to help maintain sterility. This may be turned on for

approximately thirty minutes when the cabinet is not in use. The UV light should be considered as secondary to frequent cleaning with antiseptics.

To maintain sterile work conditions, good aseptic technique must be used in conjunction with the biological safety cabinet. This begins with proper attire. Rubber gloves and a clean lab coat should be worn. Before beginning work, gloved hands should be lightly sprayed with 70% alcohol and rubbed dry. This is particularly necessary if the gloves have been worn for other tasks. The work surface should also be sprayed with alcohol and wiped down with a clean paper towel. As much as possible, all necessary materials should be placed inside the cabinet first, because reaching in and out of the cabinet can disturb the air barrier.

Work in the biological safety cabinet usually involves the use of pipettes and pipette tips. It is important to know when to discard these and use a new, sterile one. When pipetting a stock solution (PBS, trypsin, medium, etc.) into multiple cell dishes, the same pipette may be used, provided it is not allowed to come into contact with the cells or cell dish. As soon as a pipette or tip has touched a cell dish it may only be used with that dish or with cells of identical type, and should be discarded afterwards.

Fluorescence Microscope

Conventional light microscopes allow the observation of cells and in some cases, cell components such as chromosomes. However, observation of smaller cell components or the distribution of proteins is impossible with a conventional light microscope. Recent developments in biology research have made it possible to label, or tag, certain substances with fluorescent molecules known as fluorophores to allow them to be

observed under a microscope. This technique requires a microscope with special features known as a fluorescence microscope.

In most cases, the fluorophores do not fluoresce constantly, but must first be excited by light photons of a certain wavelength. The fluorophores then emit fluorescence light as they de-excite to the ground state. A fluorescence microscope is equipped with a light source, such as a mercury vapor lamp, which emits light in a broad spectrum of wavelengths. This excitation light is filtered to select a single wavelength, and the filtered light is directed through the objective lens and onto the cells. This wavelength is selected with a changeable filter to be the correct wavelength to excite the fluorophore present in the sample. The comparatively powerful excitation light will initiate a much weaker fluorescence emission from the fluorophore in the cells. The weak fluorescence light, as well as the powerful reflected excitation light will enter the objective. On its way to the eyepiece, the light is passed through a second filter which removes the excitation light, leaving only the fluorescence light from the sample.

Basic Operation

Fluorescence microscopes are operated in much the same way as conventional microscopes, with a few additional controls. First, to turn on the microscope, power switches on both the microscope itself and the separate excitation light source must be switched on. The lamp usually takes a minute or two to warm up. These types of microscopes have two light sources which may be used. Regular, white light is emitted from a light bulb located on the opposite side of the sample from the objective. This light may be switched on and off with a button labeled “TL” for Transmitted Light, because

this light is transmitted through the cells to reach the objective. There is also an adjustment wheel which varies the intensity of this light. This is the same type of light source found on conventional microscopes, and allows the fluorescence microscope to be used for conventional "brightfield" imaging. For most fluorescence imaging, however, this light source will need to be switched off.

The second light source is the excitation light source. It consists of a mercury vapor lamp housed in a benchtop module separate from the microscope itself. Once the light source is switched on, light is carried to the microscope itself through a fiber optic line. A dial on the front of the light source allows adjustment of the intensity of the light. It is desirable to minimize the intensity of the excitation light as well as the time of cell exposure to the light to avoid photobleaching the fluorophores in the sample. The fluorophores may be excited a finite number of times, after which they will no longer fluoresce. Photobleaching is the process of exhausting the fluorescence of the molecules so they can no longer be seen. For lengthy observation of the cells, it is good practice to set the light intensity as low as possible. The intensity may then be increased briefly to get the best image quality for photography.

With the light source powered on and warmed up, the excitation light may be switched on and off quickly with a button on the microscope body labeled "RL" for Reflected Light. This button may be used to switch the light off when not actively looking through the microscope, while keeping the lamp module powered on.

Fluorescence microscopes are often equipped with multiple sets of filters, since different wavelengths are necessary to excite different fluorophores. Each set of filters is actually two individual filters. The first selects the wavelength of the excitation light from

the white light produced by the lamp, and the second removes this wavelength from the light that enters the objective. The filters are mounted on a revolving tray inside the microscope. A selection wheel allows the operator to select which set of filters to use. Figure 5 shows some of the controls on a fluorescence microscope.

Fluorescence microscopes may be found in both inverted and upright configurations. In an upright microscope, the objective is located above the stage and looks down onto the sample. Upright microscopes are best suited for observing samples on a slide covered with a glass coverslip. The slide can be securely clipped in place on the stage. Adjustment knobs to the side of the stage then allow precise movement of the slide under the objective. Multiple objective lenses may be located on the revolving nosepiece of the microscope. Care should be taken when changing objectives, since some are longer than others, not to impact the slide below. Similarly, it is possible to run the objective into the slide during focusing. A useful strategy is to first move the objective far from the slide, and then slowly adjust the focus to bring it closer until the image is clear.

Figure 5 - Controls of fluorescence microscope, including TL/RL buttons, focus, and brightness adjustment on the front.

Oil Immersion Objectives

Some higher power objectives are known as oil immersion objectives. Due to their higher magnifying power, it is necessary to avoid having a layer of air between the lens and the sample. This is accomplished by placing a small drop of immersion oil onto the top of the slide and then lowering the objective until it contacts the oil drop. The slide can then be moved over short distances using the adjustment knobs by the stage and the oil drop will be spread around. For moving the slide over larger distances of roughly a centimeter or more, it may be necessary to apply another drop of oil, as the original drop may be spread too thin. It can be difficult to clean the oil off of the slide after using an oil objective. For this reason, if multiple objectives are to be used to observe one slide, it is best to use the oil immersion objective last. After using the oil objective, it should be wiped clean of any residual oil using clean lens paper.

Fluorescence Microscope Photography

Though cells may be observed directly through the microscope eyepiece, it is often necessary to capture the image for later analysis or publication. To that end, fluorescence microscopes are equipped with a port to attach a digital camera. The lightpath can be switched from the eyepiece to the camera, but both cannot be used at once. The camera is screwed on to the appropriate port, usually located on the side or the top of the microscope, and is then manipulated through a software program. Many software programs are available, often through the manufacturer of the microscope or camera.

Because samples often contain multiple fluorophores, each of which must be excited by a different wavelength of light, it is necessary to image the fluorophores one at a time. This process is known as multi-channel acquisition. The appropriate filter for the first fluorophore will be selected, and a digital image will be taken. This is then repeated for each fluorophore in the sample, and the images are then combined, as shown in Figure 6 below. Although each fluorophore has its own unique color, the camera captures all images in grayscale. The user must then tell the computer what color the fluorophore actually is, and the grayscale image will be rendered as varying shades of the designated color. In summary, to take a single channel image, the user selects the appropriate filter on the microscope itself, tells the computer what color to render the image, and then takes the picture.

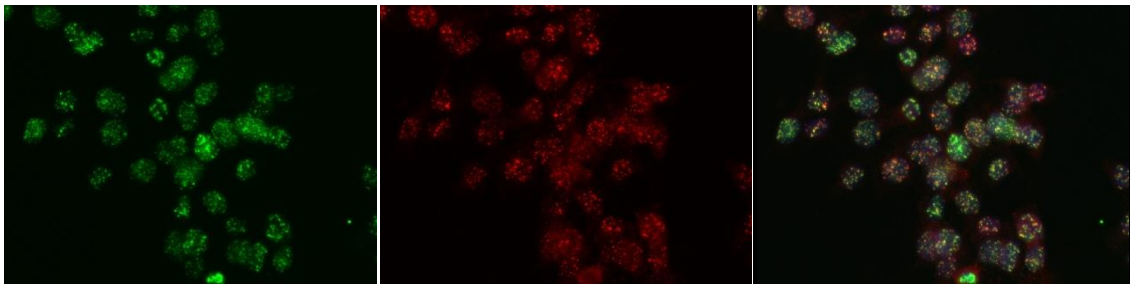

Figure 6 - Single channel images (left and center) and multichannel image (right).

To get the highest image quality, there are a few other adjustments the user may need to make. The focus may be slightly different between the eyepiece and the camera lightpaths, so once the image appears on the computer screen, it may help to adjust the focus slightly to sharpen the image. Fluorophores emit light with varying intensities depending on the individual fluorophore and the strength of the staining. The camera has a limited dynamic range, meaning that if the image is too dark it will not show up, and if it is too bright it will be saturated. The exposure time of the camera may be adjusted to

achieve a nice image in which the details of the cells are clearly visible. When adjusting the exposure time, the user should be mindful of his or her goal. If the goal is to create the best possible image quality, then any exposure time may be selected, but if the goal is to compare the distribution of fluorophores in different samples, then it may be better to select one exposure time for all images to eliminate a source of variation.

One other image parameter that may be adjusted is the response function. This is a curve that determines how bright the final image will be in relation to the intensity of the sample fluorescence. A linear response function is a good place to start. The main reason to alter the response function is to suppress the background in a low contrast image.

Hemocytometer

A hemocytometer is a specialized microscope slide designed to allow the user to determine the concentration of cells in suspension. It consists of a thick slide with a small indentation for the cell suspension which is marked with a series of grid lines. The gridlines form squares at each of the four corners of the counting area which each contain 16 smaller squares as shown in Figure 7.

To use the hemocytometer, begin with a single cell suspension. In a 1.5 mL tube, mix 100 µL of cell suspension with 100 µL of trypan blue. Cover the hemocytometer with the provided coverslip, and pipette a small amount of the mixture into the notches on the edge of the hemocytometer. The liquid will flow through the notch and into the counting chamber under the coverslip. Count the number of cells visible in each of the 16 square areas on the corners of the counting grid, and average the numbers counted in each

of the four corners. Do not count any cells which have been stained by the trypan blue, as that indicates the cell has died. Multiply the average number by two (to account for the dilution in trypan blue). Multiply this number by 10,000 to arrive at the number of cells per mL of cell suspension(Protocols and troubleshooting tips).

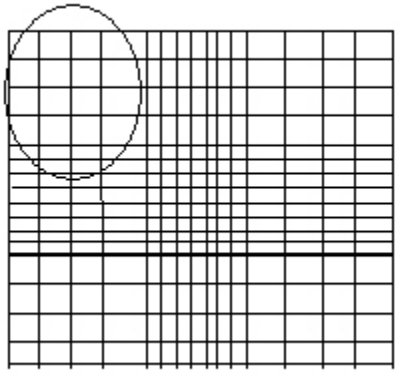

Figure 7 - A counting grid of a hemocytometer as seen through a microscope.

CHAPTER 3

GROWING AND SUBCULTURING OF CELLS

In order to study cells in the laboratory, cells must first be cultured, or grown. Labs may use new cells obtained from their own sources, or conduct research on established cell lines. Many established cell lines exist. These are proliferating cells all derived from the same source, which may be either a healthy or diseased human or animal specimen. The existence of these established cell lines allows researchers around the world to know that they are working with identical cells and to reliably compare data. One example is the HeLa cell line, derived from cervical cancer cells taken from Henrietta Lacks, who died of her cancer in 1951(Jones). HeLa cells are still commonly used in biology research. The U87 cell line is also widely used, and was derived from human glioblastoma cells.

Basics of Cell Growth

To successfully grow cell cultures, it is necessary to understand the process by which cells grow and divide. The series of events by which cells grow and divide is known as the cell cycle, while the actual process of cell division is known as mitosis. At the highest level, cells may be said to be actively dividing, in which case they are in the mitosis phase, or not actively dividing, which is known as interphase. Both interphase and mitosis can be further subdivided into smaller phases. Interphase, which accounts for over 90% of the cell cycle time, is further divided into the G1, S, and G2 phases. In the G1, or Gap 1, phase, the cell carries out its usual functions and grows. The chromatin in the nucleus is condensed in to chromosomes and replicated during the S, or Synthesis, phase. The cell prepares for division in the G2 phase (Seeley, VanPutte and Regan 91).

Mitosis itself is the only phase which may be observed in a conventional light microscope. At high magnification, it is possible to see the formation of the chromosomes in the nucleus. Even at low magnification, one may observe the cell become more round in shape as it becomes more loosely attached to the surface of the dish, and then divide into the two daughter cells. Experimental methods exist for distinction of other phases of the cell cycle. These usually involve stains which will selectively label cells in a certain phase of the cycle.

Cell Growth in the Lab

In nature, cells exist as part of tissues, which work together to form organs within an organism. To more conveniently study cells, techniques have been developed to grow cells in vitro, literally "within the glass". Cells can be grown in glass or plastic containers along with a growth medium, which is a solution containing the nutrients necessary for growth. Procedures vary slightly depending on whether the cells are adherent cells, meaning they attach themselves to the bottom of the vessel they are in, or suspension cells, which float freely in the medium.

Cells are manipulated in solution. For adherent cells this means first dislodging the cells from the surface to which they are attached. The concentration of cells in the solution can be measured by various means. Once the concentration is known, then cells may be placed, or "plated" in culture vessels as desired by converting the desired number of cells into a volume of cell solution. Cells are plated into appropriate culture vessels, which are then filled with a certain amount of growth medium and supplement. Table 1 lists the appropriate amount of growth medium for use with various sized dishes. The type of growth medium and supplement will vary depending on the type of cells. Cells are then stored in an incubator, which maintains an environment at 37°C and 5% CO_2.

For routine growth and maintenance of cell cultures, the cells are plated into the dish at a low density. Thus, initially the dish will be sparsely populated with cells. Cells then proceed to divide and begin to fill up the dish. As the cells grow and multiply they will exhaust the supply of nutrients provided by the growth medium and supplements. Therefore it is necessary to periodically change the medium by removing the old and adding fresh medium.

For most types of cells, the medium should be replaced every 48 hours. This is a simple task for adherent cell cultures. The cells are attached to the bottom of the dish, so the old medium may simply be aspirated from the dish using a pipette tip attached to a vacuum line. To avoid dislodging the cells, the pipette tip should not contact the bottom surface of the dish. Tilt the dish and place the pipette tip at the junction of the base and the wall of the dish. After all of the old medium has been aspirated, the correct volume of new medium may be added using a sterile pipette tube.

Note: When adding fresh medium to cell cultures, the supply of new medium is sterile. Take care not to contaminate the bottle of fresh medium with cells from the dish. Fill the pipette from the bottle of fresh medium, and then add the medium to the dish of cells without touching the pipette to the dish. Using this method, the same pipette may be used to replace the medium in multiple dishes, as long as the pipette does not touch any dish containing cells.

Table 1 - Culture dish sizes and appropriate volumes of growth medium.

Dish Diameter (mm) or Type	Volume of Medium
35 mm	2 mL
60 mm	5 mL
100 mm	10 mL
6-well	2 mL
96-well	200 µL

If culture dishes are initially plated at a low density, most cell cultures will grow to nearly fill the dish within 4-5 days. Cells are said to be confluent when they have filled the dish to the point where the cells are in contact with adjacent cells. Figure 8 shows a comparison between non-confluent and confluent cells. When the cell culture has reached a state of 80-90% confluence it is necessary to remove a small fraction of the cells and plate them in a new, empty dish so that they may continue to grow. This procedure is known as subculturing the cells. Cells that are left in a full dish for too long may become differentiated, meaning they cease to divide, or even die off. A detailed protocol for subculturing of adherent cells is provided below.

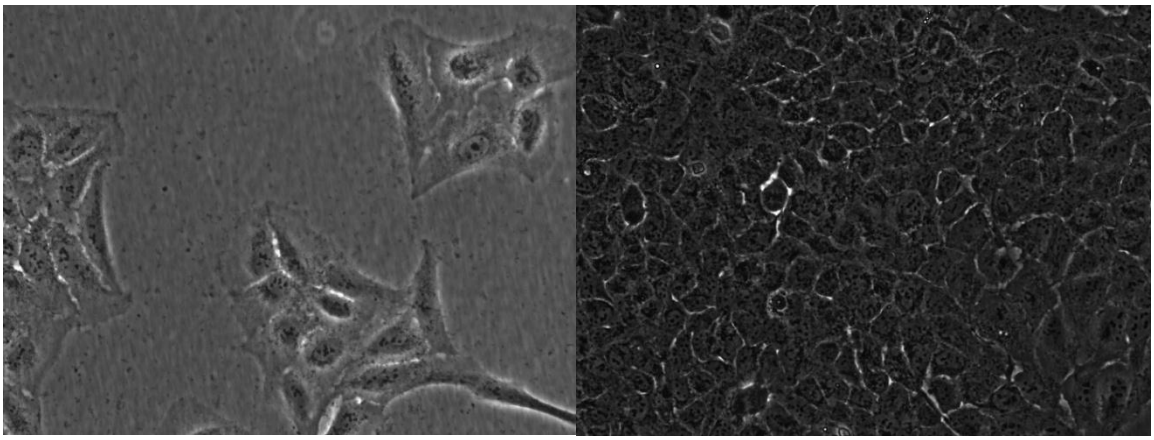

Figure 8 - Non-confluent (left) and confluent (right) cells

Protocol for Subculturing of Adherent Cells

This protocol assumes that the goal is routine maintenance of adherent cell cultures. The goal is to plate cells into new dishes which will be ready to subculture again in 4-5 days. To plate cells for an upcoming experiment, it may be necessary to increase the number plated in order for cells to be ready within a day or two.

1. Ensure that the biological safety cabinet is ready for use. Turn on the light and airflow; place the glass window at the correct height. Wipe down the surface with 70% alcohol and ensure that the workspace is clear.

2. Gather the necessary materials. Remove the PBS, trypsin, and growth medium from the refrigerator and place them in the work area. Ensure that glass pipettes and pipette tips are available and within reach. Label the new dishes to be plated with the cell type, date, and number of cells to be plated.
3. Remove 80-90% confluent cell dishes from the incubator and place them in the work area.
4. Turn on the vacuum line. Fix a sterile plastic pipette tip to the end of the vacuum line. Aspirate the medium from each dish by tilting the dish and placing the pipette tip at the corner of the dish, where the base and wall meet. Avoid touching the tip to the base of the dish, as this may dislodge the cells.

   HINT: The cell dishes have plastic covers on them. When aspirating medium from the dishes, it is convenient to hold the base of the dish between the thumb and middle finger, tilt the dish, and then lift one side of the cover using the index finger.

5. Rinse the cells by adding enough PBS to each dish to cover the base of the dish. Use a glass pipette and pipettor to add the PBS. Avoid touching the pipette to the dishes, as this could contaminate the sterile container of PBS.
6. Aspirate the PBS from the dishes using the same method used to aspirate the medium.
7. Using a glass pipette, add enough trypsin to barely coat the base of the dish (2-2.5 mL for a 100 mm dish). Allow the trypsin to remain on the dish for 10-30 seconds.
8. Aspirate nearly all of the trypsin. Use the method of tilting the dish and aspirating from the corner, leaving only a trace amount of trypsin at the junction of wall and base.

9. Place the dish in an incubator at 37°C and 5% CO_2 for 5 minutes. Alternatively, leave at room temperature for about 8 minutes.
10. Remove the dish from the incubator. The cells should look like dust particles which slide freely on the base of the dish as it is tilted. This means they have been dislodged by the trypsin.
11. Add the appropriate volume of growth medium to the dish (see table) using a glass pipette. Cells will float free from the bottom of the dish, but will be collected in clumps, which will be visible to the naked eye.
12. Tilt the dish slightly. The cells and medium will collect in the lower part of the dish. Pipette the medium up from the low part of the dish and onto the higher part repeatedly. The medium will flow down the sloping base of the dish and help to dislodge any cells still adhering to the dish. Continue pipetting the medium in this way until all visible clumps of cells have disappeared. Between 5 and 10 repetitions of this should suffice. The dish should now contain a single cell suspension, meaning that the cells are floating freely and individually in the medium.
13. A fraction of the cells will now be moved to a new dish to continue growing. One of two methods may be used. A specified number of cells may be plated into the new dish. This requires determining the concentration of cells in suspension using either a cell counter or hemocytometer. With the concentration known, the appropriate volume of single cell suspension may be calculated in order to plate the desired number of cells.

$$\frac{desired\ \#\ of\ cells\ [cells]}{cell\ concentration\ \left[\frac{cells}{mL}\right]} = volume\ cell\ suspension\ [mL]$$

The other method that may be used is to plate a specified fraction of the cells into the new dish. This is a convenient method since it does not require the cell concentration to be known. The appropriate fraction can be estimated by looking at the dish through a microscope. Table 2 provides estimates of the time required for cells to grow to confluence after splitting (Protocols and troubleshooting tips).

Table 2 - Cell culture splits and approximate time to confluence.

Split Fraction	Estimated Time to Confluence
1:2	1-2 days
1:5	2-4 days
1:10	4-6 days

Pipette the appropriate volume of cell suspension (medium) from the old dish into the new dish.

14. Add enough fresh medium to the new dish to bring the total volume to the correct amount.
15. Swirl the dish clockwise, counter-clockwise, and slide it left to right and front to back several times each to distribute the cells evenly around the dish.
16. Place the newly plated dish in the incubator.

CHAPTER 4

IMMUNOSTAINING PROCEDURES AND RESULTS

Immunohistochemistry is the process of studying the spatial distribution of specific proteins, or antigens, through the use of antibodies which bind only to those antigens. By selecting a fluorescent antibody which binds specifically to a certain protein, that protein becomes visible under a fluorescent microscope. This technique has revolutionized light microscopy and has limitless applications in biological research.

Specifically, in radiation biology research, it has always been a challenge to correlate a known physical quantity, such as absorbed dose, with a biological endpoint (Wang 6088). In the past, rather crude techniques such as cell survival curves have been used. Immunostaining techniques have allowed researchers to determine that cells synthesize certain proteins in response to DNA damage. Many of these DNA damage repair proteins are concentrated at the sites of DNA double strand breaks, forming "foci" which are visible if stained with fluorescent antibodies (Rogakou, Pilch and Orr 5858).

A commonly used variation of this technique is indirect immunofluorescence. In this method, a primary antibody binds to the target protein of interest, but this antibody is not fluorescent, and thus cannot be seen. A secondary fluorescent antibody is added subsequently, which binds to the primary antibody and allows the spatial distribution to be observed (Ramos-Vara 414). The advantages of this indirect method include greater sensitivity, because each primary antibody provides multiple binding sites for the second, fluorescent antibody, and increased flexibility. With this method, the same second

antibody can be used for many primary antibodies, which simplifies laboratory logistics. A detailed protocol for indirect immunofluorescence is included below.

Protocol for Immunostaining Irradiated Cells

1. Plate 20,000 cells into each well of an 8-well chamber slide, and grow overnight.
2. When cells are 80-90% confluent, irradiate the cells in the x-ray machine to desired dose levels. Each slide may be irradiated to a different dose if desired. Label each slide with the dose received. Leave one slide not irradiated as a control. Place all slides back in an incubator at 37°C.
3. Leave the slides in the incubator for the desired amounts of time. Different slides may be incubated for different amounts of time. Remove each slide from the incubator after its allotted amount of time, label the slide with the amount of time incubated, and proceed with Step 4.
4. Aspirate the medium from each of the eight wells. To do this, tilt the slide and gently aspirate from the corner of the well. Avoid touching the bottom of the well. Do not completely aspirate the medium, but leave a small amount in the corners of the well. This will help avoid disturbing the cells.
5. Wash the cells with Phosphate Buffered Saline (PBS) 3 times, for about 5 minutes each time. Pipette the solution onto the walls of the wells to avoid dislodging the cells. Between washes aspirate the PBS as described in Step 4.
6. Aspirate the PBS, and fix the cells with 4% Paraformaldehyde for 10 minutes. This freezes the cells and proteins in the state they are currently in.
7. Aspirate the Paraformaldehyde, and again wash the cells with PBS 3 times, for about 5 minutes each time.

   NOTE: If the goal is to fix cells at different time points, then this is a convenient stopping point until all slides have been fixed (i.e. if fixing slides at 30 minutes

and 4 hours after irradiation, then complete Steps 4-7 on one slide starting at 30 minutes, complete Steps 4-7 on the other slide starting at 4 hours, and then proceed with Step 8 on both slides together.

HINT: Once cells are fixed, they may be left in PBS at 4°C for at least a week. If time in the lab is limited, it is possible to irradiate and fix cells one day, and then come back another day to finish the staining protocol.

8. Permeabilize the cells with 0.2% Triton X-100 in PBS for 10 minutes. This will allow the antibodies to pass through the cell membrane into the cell in subsequent steps.
9. Wash the cells with PBS two times, for about 5 minutes each time.
10. Block the cells with 10% Normal Goat Serum (NGS) in 0.02% Triton X-100 diluted in PBS for 1 hour at room temperature (or overnight at 4°C). This will saturate the non-specific binding sites so that the primary antibody will bind only to its specific antigen.
11. Add the primary antibody in 2% NGS in 0.02% Triton X-100 diluted in PBS and incubate for 2 hours at room temperature (or overnight at 4°C). Oftentimes, it is useful to stain different wells of the chamber slide with different antibodies. This can become complicated, as it requires preparation of several different antibody solutions. Follow the suggested steps below to avoid confusion.
    a. Use a volume of 150 µL antibody solution per well. Multiply this by the total number of wells to be stained. This is the total volume of primary antibody solution that will be needed. Round up to the nearest milliliter (to have a little bit extra) and then double it (because there are both primary and secondary antibodies. Prepare this volume of 2% NGS in 0.02% Triton X-100 in PBS. This is the basic solvent into which the actual antibodies will be added.

b. Determine how many unique antibody solutions will be required. How many different combinations of antibodies will be used in each slide?

Ex: If there are 3 slides, and each is to have 4 different stainings (2 wells per staining), then 4 unique primary antibody solutions will be required. Each of the 4 primary antibody solutions will need to cover 6 wells.

$$6 \text{ wells x } 150 \frac{\text{uL}}{\text{well}} = 900 \text{ µL}$$

This can be rounded up to 1000 µL for simplicity.

c. Place the appropriate amount (1000 µL in this example) of the 2% NGS in 0.02% Triton X-100 solvent into (4) 1.5 mL tubes.

d. Locate all the antibodies to be used and place them on ice near the workspace. Determine what antibody dilutions will be used based on previous experiments or the manufacturer's suggestions. These are usually expressed in the form of a 1:50 or 1:1000 dilution, for example.

e. Add the appropriate amount of antibody to the solvent (2% NGS in 0.02% Triton X-100 in PBS) based on the chosen dilution.

Ex: If there is 1000 µL of solvent and a 1:50 antibody dilution is desired, then add 20 µL of antibodies.

f. Once all antibody combinations have been created, add 150 µL to each well, taking care to note which wells got which antibodies.

12. Aspirate the primary antibody solutions, and wash with PBS 3 times, for about 5 minutes each time.

13. Add the secondary antibodies in 2% NGS in 0.02% Triton X-100 in PBS and incubate at room temperature for 1 hour.

14. Aspirate the secondary antibody solutions, and wash with PBS 3 times, for about 5 minutes each time.

15. Wash with PBS twice. Aspirate the PBS.
16. The walls of the chamber slides are designed to separate from the base. A tool is provided to help separate the two pieces. Using this tool, gently separate the walls of the slide from the base, taking care not to disturb the cells on the base of the slide.
17. Using a small pipette and pipette tip, add 8 µL of mounting medium to each of the eight wells on the slide base. Take care not to allow bubbles to form.
18. Cover the slide base with a glass coverslip. Position one edge of the coverslip on the edge of the slide, and then gently drop the other side of the coverslip onto the slide.
19. Seal the edges of the coverslip/slide with clear polishing gel.

Staining of Heavy Ion Irradiated Cells

U87 Glioblastoma cells were plated in 8-well chamber slides and irradiated with 1 GeV Iron-56 ions at Brookhaven National Lab to a dose of 0.5 Gy. Slides were fixed in paraformaldehyde at 5 minutes and 30 minutes, and brought back to Emory University for staining and analysis. Each 8-well chamber slide was stained according to Table 3. The table lists which antibodies were placed in each well, followed by an (M) or (R) to indicate mouse or rabbit antibodies, respectively, and the antibody dilution. The bottom row of each slide was treated with a PARP inhibitor (ABT888, 5 µM) 1 hour prior to irradiation. After staining, a mounting medium with DAPI was applied to stain the cell nuclei a blue color under UV light. The second antibodies used were Alexa Fluor anti-Mouse 488 and anti-Rabbit 555 with 1:500 dilutions.

Table 3 - Antibodies and dilutions used in staining of heavy ion irradiated slides.

ATM (M) 1:500 53BP1 (R) 1:500	ATM (M) 1:500 PARP (R) 1:1000	ATM (M) 1:500 Mre11 (R) 1:1000	PAR (M) 1:1000 PARP (R) 1:2000	←Control
ATM (M) 1:500 53BP1 (R) 1:500	ATM (M) 1:500 PARP (R) 1:1000	ATM (M) 1:500 Mre11 (R) 1:1000	PAR (M) 1:1000 PARP (R) 1:2000	←PARP Inhibitor

Heavy ions such as iron ions are high Linear Energy Transfer (LET) particles and therefore deposit their energy in a straight, compact track, rather than evenly dispersed around the cell nucleus, as is the case with x-rays. Figures 9, 10, and 11 show the single channel and merged images of high LET particle tracks in cells. Additional images are included in Appendix C.

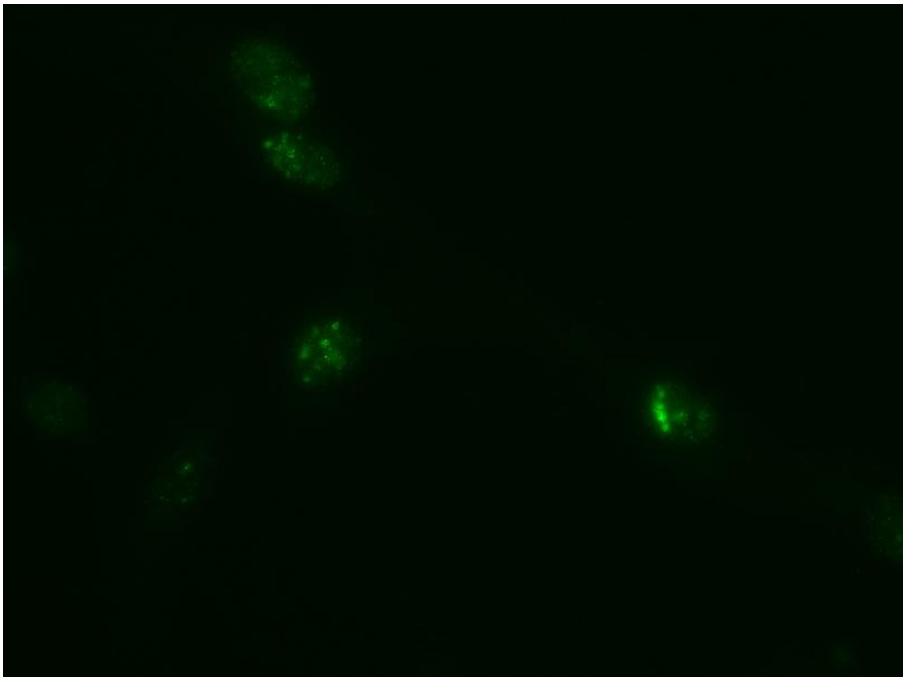

Figure 9 - ATM staining

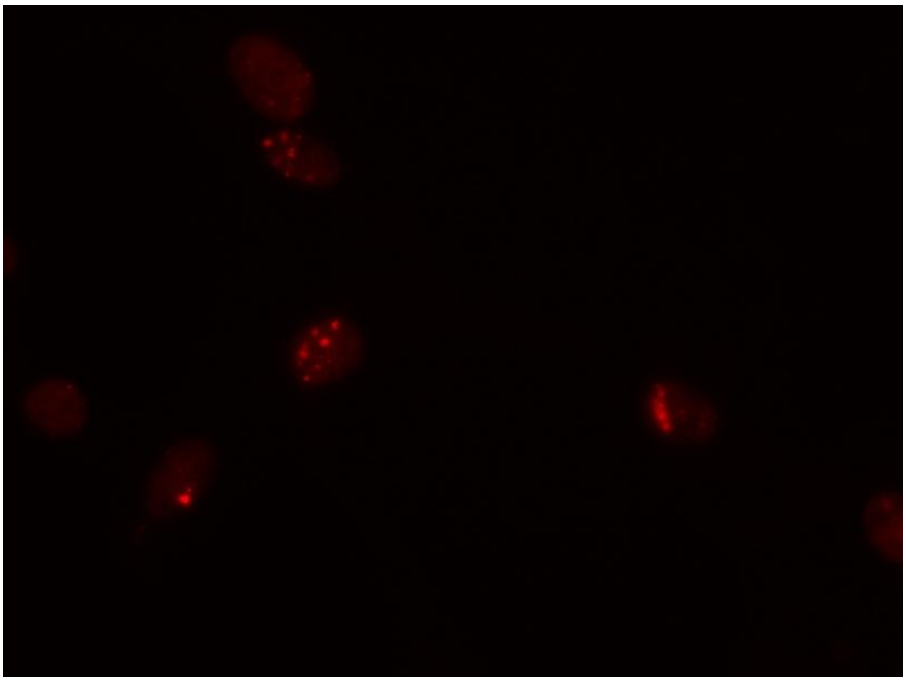

Figure 10 - Mre11 staining

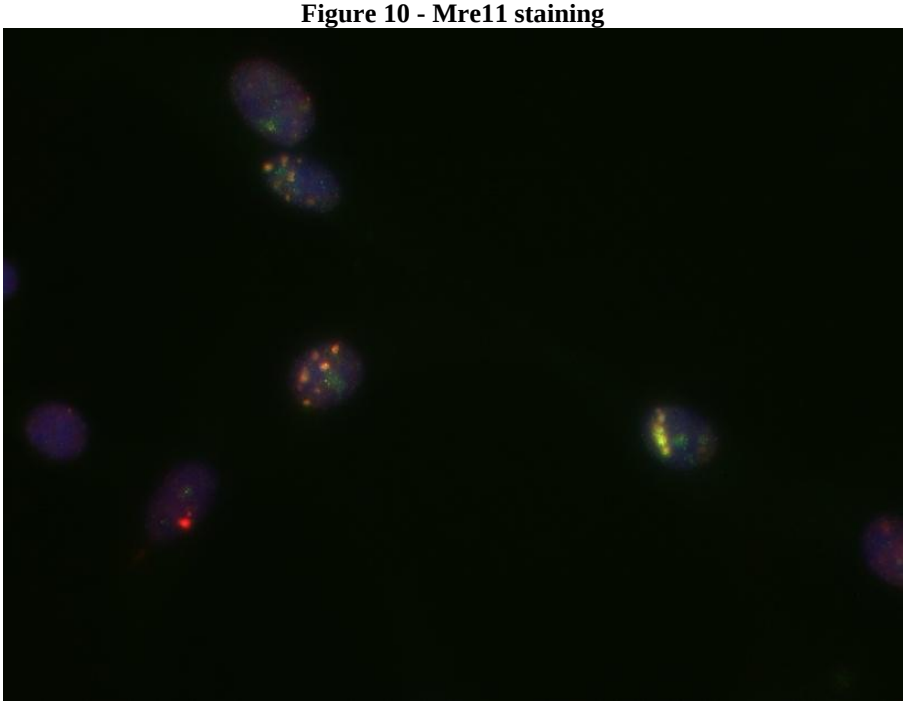

Figure 11 - Merged ATM (green), Mre11 (red), and DAPI (blue).

Staining of X-Ray Irradiated Cells

Five 8-well chamber slides were plated with 20,000 cells/well and allowed to grow to near confluence. The slides were then irradiated in an x-ray machine. Two slides received 0.5 Gy, two slides received 0.1 Gy, and one slide was left un-irradiated as a control. One slide at each dose level was incubated for 30 minutes and then fixed, while the other slide from each dose level was incubated for 4 hours and then fixed. The slides were stained according to the protocol in this chapter with the antibodies as shown in Table 4. All first antibody dilutions were 1:1000. The second antibodies used were Alexa Fluor anti-Mouse 488 and anti-Rabbit 555 with 1:500 dilutions.

After staining and mounting the slides, digital images were taken of each well for all slides using a Zeiss Axio Imager upright fluorescence microscope. Images were then analyzed using the FociCounter image processing program to count the number of foci of each protein present in each cell (Jucha).

Table 4 - Antibodies used in staining of x-ray irradiated slides.

Proliferating		Differentiated	
γ-H2AX (M) 53BP1 (R)	γ-H2AX (M) 53BP1 (R)	γ-H2AX (M) 53BP1 (R)	γ-H2AX (M) 53BP1 (R)
ATM (M) Mre11 (R)	ATM (M) Mre11 (R)	ATM (M) Mre11 (R)	ATM (M) Mre11 (R)
	PARP Inhibitor		PARP Inhibitor

Reliable analysis of foci numbers proved difficult. Whether using a software image analysis program or the human eye, it is extremely challenging to distinguish what is or is not to be counted as a focus considering that the size and intensity of visible dots in the nuclei can range over roughly two orders of magnitude. Also, it is sometimes

impossible to distinguish a single large focus from a group of smaller foci in close proximity. It was deemed impossible to count all foci down to the smallest visible speck in the nucleus. Therefore, the only remaining option was to designate a threshold level of size and intensity, and count all foci above this threshold. Software image processing is much more suitable for this task, as numerical parameters can be specified, and the program handles discrimination of foci and non-foci. Figure 12 shows a screenshot of the FociCounter program identifying 53BP1 foci.

For each protein to be analyzed, the control images were examined for background foci, and the irradiated slides were examined to estimate the average size of the foci present. Based on this, parameters were selected for each protein to attempt to exclude background foci or speckling, and count all radiation induced foci. Table 5 lists the values chosen for each parameter for all proteins.

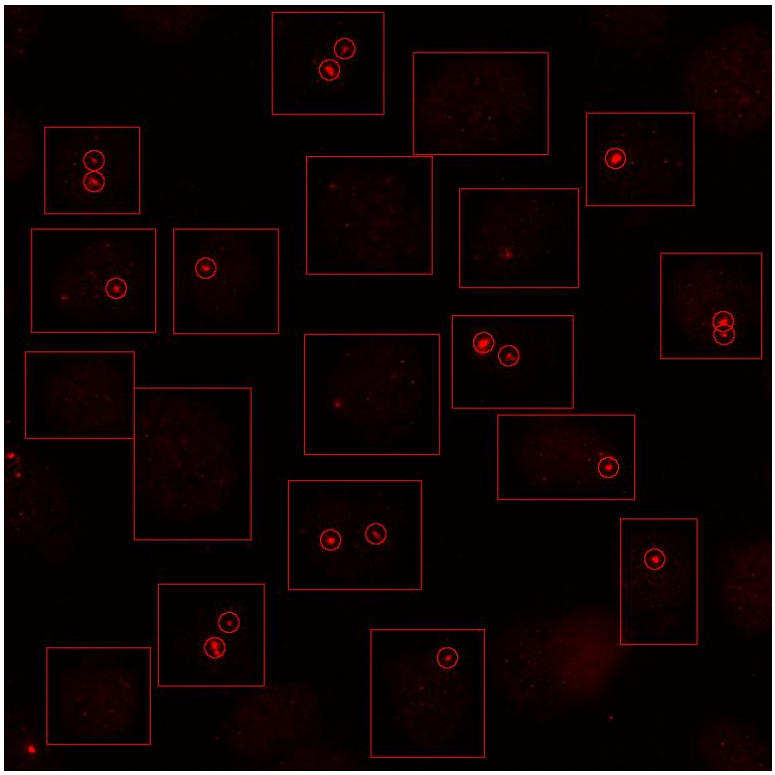

Figure 12 - Screenshot of the FociCounter software program identifying 53BP1 foci.

Table 5 - List of input parameters used for each protein for the FociCounter program.

	H2AX	53BP1	ATM	Mre11
Median Square Side	3	3	3	3
Contrast Enhancement	1.1	1.1	1.1	1.1
Crown Radius	1	2	2	1
Rim Radius	6	7	7	7
Brightness Difference	25	25	40	30

The success of this method varied among the four proteins analyzed. The most consistent results were obtained from the 53BP1 protein, which tended to have larger, more distinct foci, and a more uniform background Figures 14 and 15 show a comparison of 53BP1 and γ-H2AX foci after 0.1 Gy and 4 hours. The much greater number of small, indistinct foci in γ-H2AX can be clearly seen. The results of the 53BP1 foci analysis are presented below in Figure 13, which includes the results for proliferating and differentiated cells, with and without PARP inhibitor treatment. Following the values for proliferating cells (without PARP inhibitor) for example, one can see that the control value is low, and the highest value is reached at 0.5 Gy at 30 minutes. At 0.5 Gy at 4 hours, the value has dropped to just over the control. The values for 0.1 Gy exhibit a similar pattern. It can also be seen that, for most cases, the addition of the PARP inhibitor resulted in increased numbers of foci compared to the same cells without the PARP inhibitor, which is as expected. Results of the foci counting for all proteins are included in Appendix D.

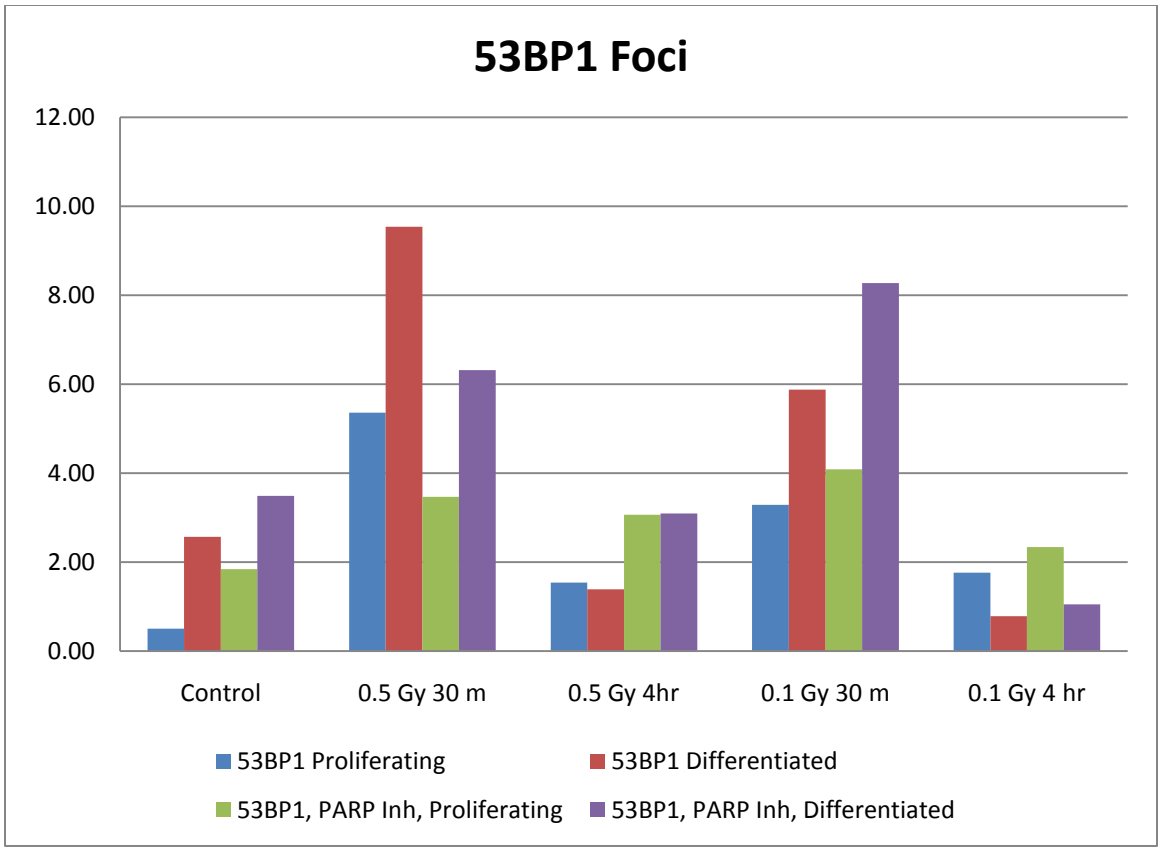

Figure 13 - Results of foci analysis for the 53BP1 protein.

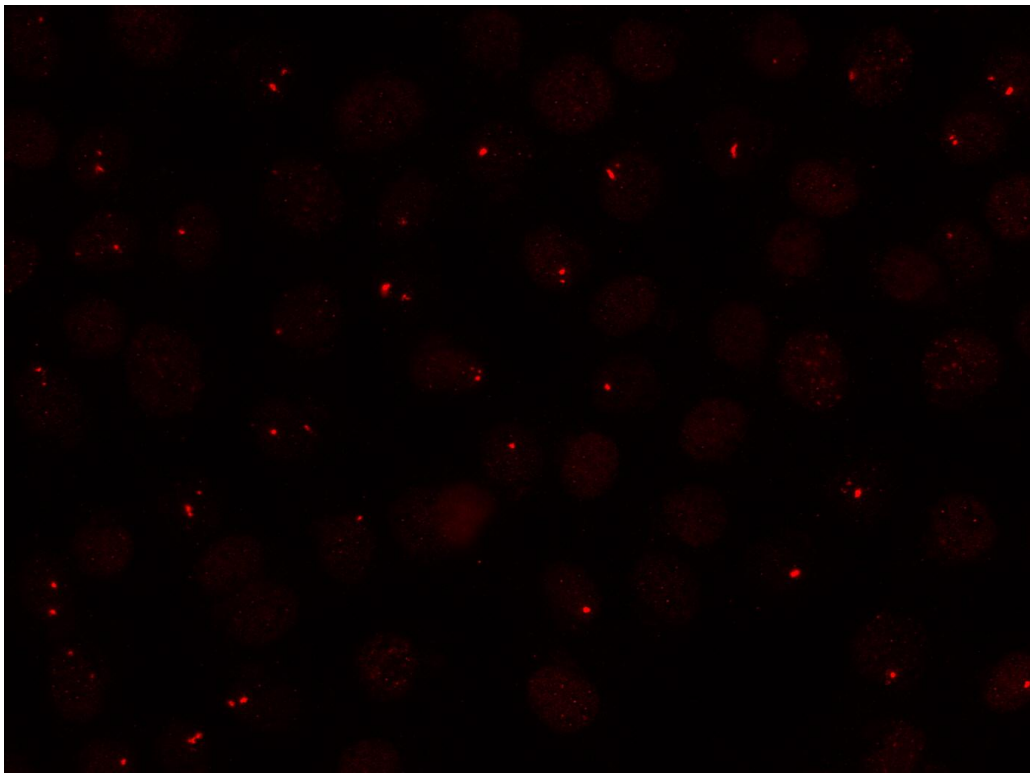

Figure 14 - 53BP1 foci from 0.1 Gy at 4 hours, showing uniform background and distinct foci.

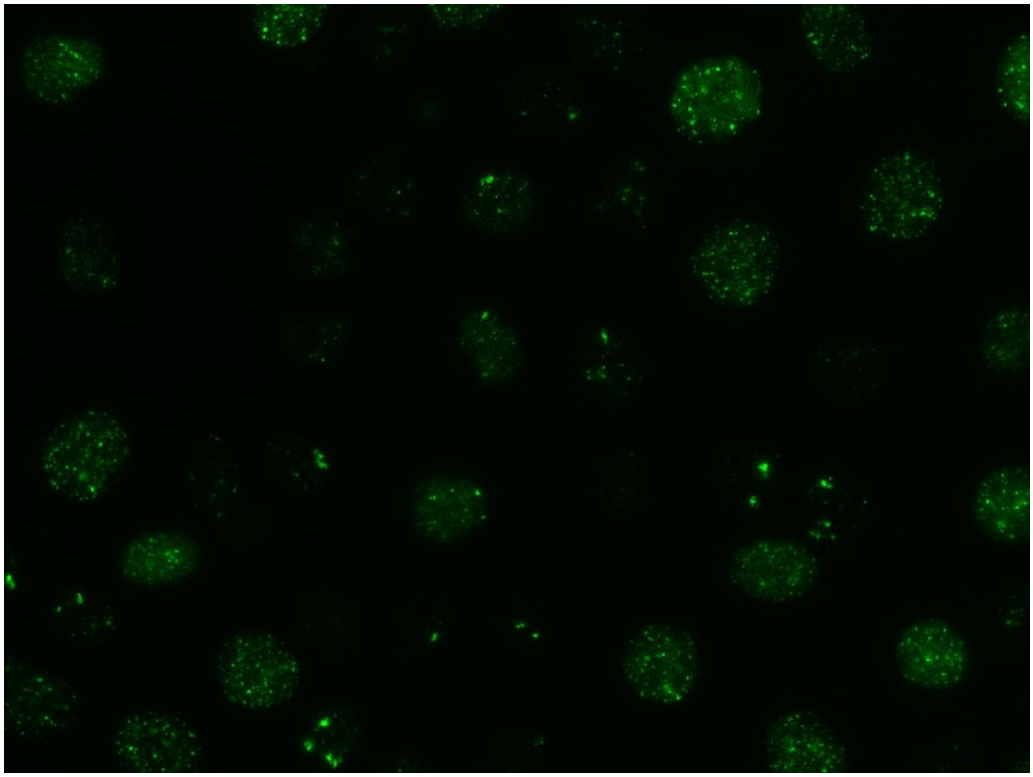

Figure 15 - H2AX foci from 0.1 Gy at 4 hours, showing many small foci.

Immunostaining Conclusions

Immunostaining of irradiated cells can provide a great deal of information on the cell's response to DNA damage. By staining for different proteins, researchers can determine which proteins are synthesized in response to gene damage. By knocking out, or deleting the genes that produce certain proteins, researchers can investigate what proteins trigger other proteins in the overall cell response to DNA damage. For instance, for proteins that usually accumulate at the site of double strand breaks, if Gene A (which synthesizes Protein A), is knocked out, and a decrease in Protein B is observed in response, it could be hypothesized that Protein A triggers the accumulation of Protein B at the site of the DNA break. Through experiments such as this, researchers are attempting to better understand the actual process by which cells repair DNA lesions.

Immunostaining provides many exciting capabilities, but in practice, it can be difficult to derive reliable, quantitative results from staining experiments. The end result of the procedure is essentially a digital image of the cells that were stained, with the proteins of interest visible as the colors of the second antibodies. In the common scenario of x-ray irradiation, processing the images into useable data usually means assessing the number of protein "foci" in each cell. This can be challenging for a number of reasons, including background staining, varying foci size, and closely clustered foci. These challenges make data from immunostaining experiments time consuming to gather, and potentially less reliable. Great care must be taken to optimize the technique used, in order to minimize the problems described.

CHAPTER 5

COMET ASSAY PROCEDURES AND RESULTS

The Comet Assay, also known as Single Cell Gel Electrophoresis is a method of evaluating the extent of DNA damage in individual cells. Though there are many variations, the basic procedure consists of imbedding cells in a agarose gel matrix, exposing the cells to a DNA damaging treatment such as ionizing radiation or chemotherapy drugs, lysing the cells (dissolving the cell membrane), and placing them in an electric field. The electric field causes the broken DNA fragments to migrate through the gel. The migration of DNA fragments out of the original cell volume creates a visible "comet tail" after the DNA is stained with a fluorescent marker. The more fragmented the DNA, the more easily it can travel through the gel.

The length and intensity of the comet tail provide the signal used to evaluate the extent of DNA damage in the cell. To be of much use, this visual signal (an image) must be processed in some way to convert it to usable numerical data. Though this can be done through manually scoring cells as damaged, undamaged, or some category in between, modern researchers have turned to digital image processing. With a digital camera attached to a fluorescent microscope, a computer can analyze cells which the operator selects. Many different measurements can be made, such as tail length, total cell fluorescence, and fraction of DNA present in the head and tail.

There is no unanimous consensus on which of these endpoints provides the most reliable relationship with dose and/or DNA damage. Tail length tends to be proportional

to dose for lower doses, but at higher doses becomes more uniform and more a function of the electrophoresis conditions (Fairbairn, Olive and O'Neill 40). An invented quantity called “Tail Moment” has been popular in recent years (Lee 130). It is defined as the length of the tail multiplied by the fraction of DNA present in the tail. This quantity has the advantage of combining a measure of the smallest fragment size (tail length) with a measure of the total amount of DNA fragments present (fraction of DNA in the tail). Two commonly used versions of this quantity are Olive Tail Moment, and Extent Tail Moment. They differ only in their definitions of tail length. The Extent Tail Moment uses the length of the tail as measured from the edge of the body to the end of the tail. The Olive Tail Moment uses the difference between the centers of gravity of the head and tail.

Variations in the Comet Assay have been developed to measure either single or double strand DNA breaks. To measure single strand breaks, the DNA double helix must be denatured, or unwound. This allows the single strand breaks to cause sufficient fragmentation for the DNA to travel through the agarose gel matrix. This is accomplished through the Alkaline Comet Assay. A protocol for this version is included below. The neutral version of the Comet Assay does not denature the DNA, and thus only double strand breaks can cause sufficient fragmentation.

The resulting damage signal from the comet assay is highly sensitive to variations in technique used. Salt concentration, pH, and lysis time can all have a large effect on the results. Therefore it is important that the solutions to be used are always mixed precisely. If excess salt from the lysis buffer remains in the gel of the slide during electrophoresis, it can slow the migration of the DNA by neutralizing the charge on the DNA molecules. Salt left in the slide would be lost faster from the top of the slide (near the gel/buffer

interface) than from deeper in the gel, so this could cause a heterogeneous distribution of salt in the gel which could lead to inconsistent comet tail formation in the slide(Fairbairn, Olive and O'Neill). The best way to avoid this problem is to rinse the slides in A2 solution before electrophoresis, a step which is included in the protocol below.

Protocol for Alkaline Comet Assay with Cells Irradiated on Slides

1. Prepare the necessary reagents. Table 6 lists the working concentration of chemicals in A1 and A2 solutions, and provides the necessary volume of each chemical to make 1L of A1/A2 from common concentrations of stock solutions. Add 0.1% by mass of sodium lauroyl sarcosinate (NLS) powder to the A1 solution on the day of the experiment.

Table 6 - Concentration of reagents in the A1 and A2 buffers, and required volume of stock solution to mix these buffers.

A1 (lysis)	Working Concentration	From Stock	Vol. in 1L solution
NaCl	1.2 M	5 M	240 mL
EDTA	0.1 M	0.5 M	200 mL
NaOH	0.26 M	10 M	26 mL
A2 (rinse/electrophoresis)			
NaOH	0.03 M	10 M	3 mL
EDTA	2 mM	0.5 M	4 mL

2. The day before the experiment, pre-coat the microscope slides with agarose gel.
    a. Place the agarose gel in a water bath to melt it, or microwave it.
    b. Clean the slides. Mark the frosted part of the slide to show which side is gel coated.
    c. Holding the slide in one hand, pipette 500 µL of liquid agarose onto the slide surface.

d. With the side of the pipette tip, spread the liquid agarose evenly across the surface of the slide. Run the pipette tip along the edges of the slide to ensure the agarose covers the whole surface.

e. Place the pre-coated slides on a tray and set aside for the gel to solidify.

3. Prior to starting the experiment, place a container of 1% low-melting agarose gel in a 37°C water bath to melt.
4. Begin with a 100 mm dish of confluent cells.
5. Detach cells from the dish by adding trypsin and incubating 5 minutes at 37°C. Re-suspend cells in serum-free medium, and pipette up and down to create a single-cell suspension.
6. Create a dilution of 0.5M cells in a 100 mm dish. This is a concentration of 50,000 cells/mL, assuming a volume of 10 mL in the dish. Keep this cell suspension on ice to minimize damage to cell DNA, which will contribute to the background signal.
7. Add 500 µL of cell suspension into a 1.5 mL centrifuge tube. Then, with a new pipette tip, add 500 µL of liquid agarose to the tube and pipette up and down a few times to mix.
8. Pipette 500 µL of this cell suspension/agarose mixture onto a pre-coated slide, and spread evenly over the surface of the slide using the side of the pipette tip.
9. Repeat Step 7 for as many slides as will be used for the experiment. After placing the cells on the slides, keep the slides on a metal tray on a bed of ice to minimize damage to the cell DNA.
10. After all slides have been prepared, place them in a cold room or refrigerator at 4°C to allow the agarose to solidify completely. This may take up to 10 minutes.
11. Bring the slides to the x-ray machine, keeping them on a metal tray on a bed of ice. Label the slides with the doses to be used.
12. Irradiate the slides to the desired doses.

13. Immerse the slides in A1 lysis buffer in plastic slide trays at 4°C. Hold the slides by the edges to prevent disturbing the gel on the surface of the slide. Lower the slides gently into the solution, taking care to keep them horizontal as they are lowered. Leave slides in the A1 lysis buffer for 1 hour. 50 mL of lysis buffer in a 6-slide plastic tray is sufficient to cover the slides.
14. Gently remove the slides from the A1 lysis buffer and lower into a tray of A2 rinse/running buffer. Leave the slides in the A2 buffer for 20 minutes to remove all traces of the A1 buffer.
15. Fill the electrophoresis tray with A2 buffer. Transfer the slides from the rinse tray to the electrophoresis tray. Ensure that they are completely submerged in the A2 buffer. Fit the cover onto the electrophoresis tray.
16. Turn on the voltage supply and set it to run at 12 volts for 20 minutes.
17. Remove the slides from the electrophoresis tray and place them in a cool, dark place to dry.
18. After the slides have dried, place them in a tray of distilled water for 10 minutes to remoisten them for staining. Remove the slides from the water and dry them by pressing an edge against a dry paper towel.
19. Pipette 70 µL of propidium iodide (PI) solution onto each slide, and cover with a coverslip.
20. View the slides under a fluorescent microscope, using a green excitation light to observe the comet tails of cell DNA.

Dose Response Comet Assay Experiment

One straightforward application of the comet assay is the observation of the effects of varying absorbed doses on cells. The greater the dose delivered, the more fragmented the cell DNA will be and the larger and more pronounced the visible “comet

tail" will appear under the microscope. Digital imaging, in conjunction with image analysis software, is used to evaluate the length of the comet tail and the fraction of DNA contained in the head and tail regions. A quantity called "Olive Tail Moment" has been defined that combines these two measurements.

$$Olive\ Tail\ Moment = Tail\ Length\ x\ Head\ DNA\ Fraction$$

To evaluate the response of the comet assay to varying doses, cell samples were prepared on slides as described in the protocol above, and irradiated to doses of 1 Gy, 2 Gy, 4 Gy, and 8 Gy, in addition to a control slide. After irradiation, lysis, electrophoresis, and staining all slides were analyzed with the Andor Komet 6 software program. For each slide, 50 cells were selected at random for software analysis. The quantities Olive Tail Moment, Extent Tail Moment, Fraction of DNA in the Tail, and Tail Length were recorded as functions of dose delivered. The numerical quantities themselves are largely arbitrary (since tail moment is an invented quantity) and their meaning is derived through the relationship to dose. Therefore, all four quantities were normalized to their maximum value (achieved at 8 Gy dose) so that they could be displayed on a single plot in Figure 16 below.

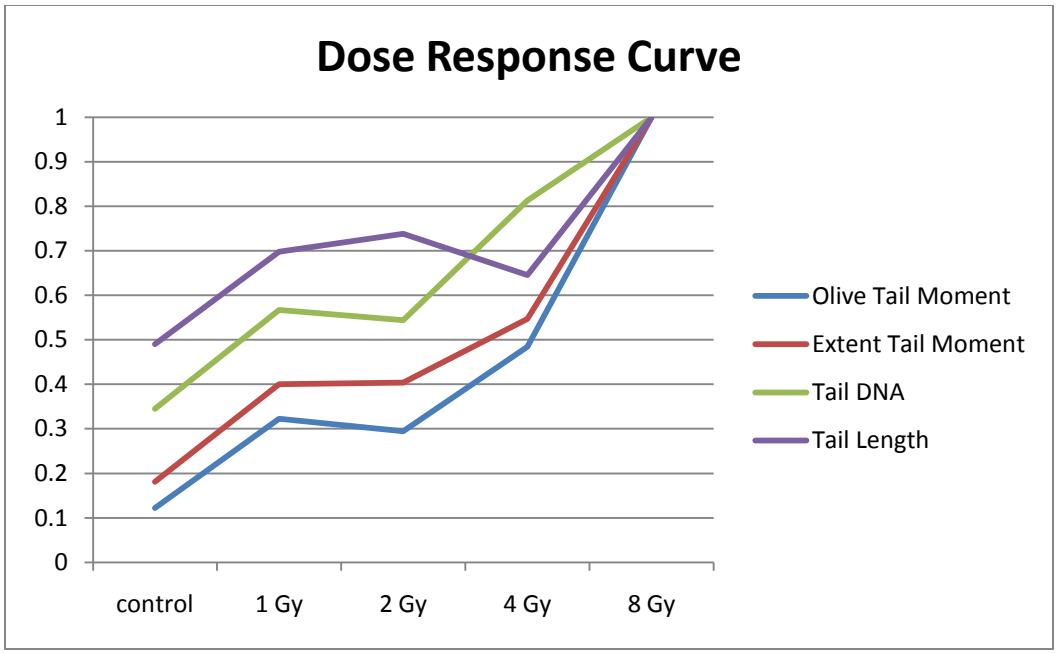

Figure 16 - Dose response curve of various measured parameters from the Comet Assay.

The resulting dose response curve was fairly consistent with the theory that DNA damage and comet tail formation is proportional to dose. The Tail Length and Tail DNA quantities showed some irregularities, but these were smoothed out significantly in both Tail Moment quantities which combine the former two measurements. Even in the tail moments, there was some inconsistency in the 1 Gy and 2 Gy measurements. These dose levels showed almost the same Extent Tail Moment, and for Olive Tail Moment, the 2 Gy value was slightly lower. This can likely be attributed to the 50 cells being selected by the user. Though random selection of cells was the goal, the natural tendency is to look for and find cells with clear, visible comet tails. At the close dose levels of 1 and 2 Gy, this user bias may have pushed the results closer together. Also, statistical variation was present in the results, and is shown in Figures 17 and 18 below, with error bars of one standard deviation. After the two Gray jump to 4 Gy, a distinct difference in signal

strength can be observed. All measured quantities showed significant and similar increases between the control and 1 Gy. Original data and cell images from this experiment are included in Appendix A.

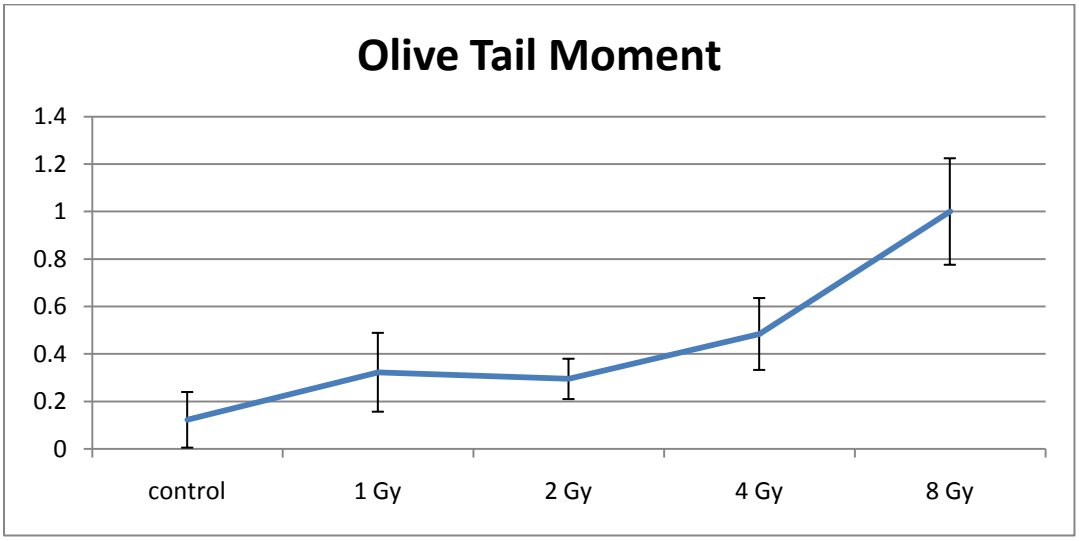

Figure 17 - Dose response of Olive Tail Moment, showing statistical variation with error bars of one standard deviation.

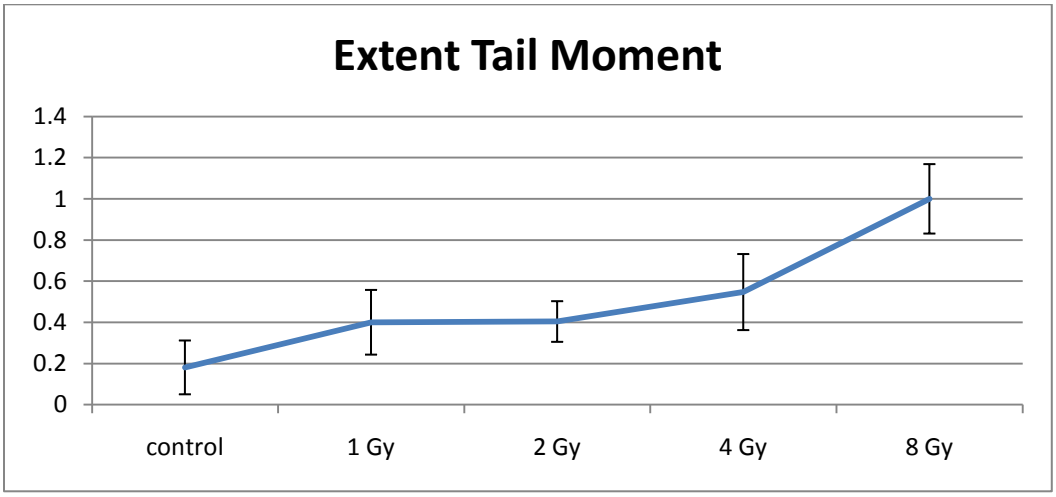

Figure 18 - Dose response of Extent Tail Moment, showing statistical variation with error bars of one standard deviation.

DNA Repair Comet Assay Experiment

The Comet Assay can also be used to observe the time course of DNA repair processes in cells. The assay provides a measure of the amount of fragmentation of the cell's DNA at the time of lysis and electrophoresis. If the cells are irradiated, and then left in growth medium in an incubator for a period of time, then the cell will begin to repair the DNA damage sustained from the ionizing radiation. The DNA will then be less fragmented at the time of lysis, and the comet tail will be less pronounced.

To perform this experiment requires a modification of the protocol above, which details the plating of cells onto gel covered slides, and irradiating the cells on these slides. Since this experiment requires the incubation of cells after irradiation, the cells must be in growth medium during and after irradiation. Plating the cells on slides in agarose gel changes the cell's environment and it cannot be predicted what effect this would have on cellular repair processes. Instead of plating cells onto slides prior to irradiation, individual 35 mm dishes were plated and allowed to grow to near confluence. The cells were irradiated in these dishes with growth medium. The dishes were placed back into the incubator after irradiation for the specified times. Then, single cell suspensions were created from each dish after the incubation time had elapsed, and the irradiated cells were plated onto slides and lysed. The modified protocol is included below.

Modification to Comet Assay Protocol for DNA Repair Measurement

1. Prepare Comet Assay reagents and precoat slides as detailed in standard protocol.
2. Plate at least one 35 mm dish for each desired time point and the control. Allow cells to grow to confluence in these dishes.

3. Irradiate the cells in these dishes to the desired dose, except the control dish. Place dishes back in the incubator for the desired DNA repair time.
4. After the desired DNA repair time has elapsed, trypsin the cells and create a single cell suspension at a concentration of 50,000 cells/mL. This would be 0.1M cells plated in 2 mL of medium in a 35 mm dish. Keep the cell suspension on ice to minimize DNA damage.
5. Continue with Step 7 of the standard protocol. Skip steps involving irradiation of the slides, as the cells have already been irradiated in their original dishes.

Results of DNA Repair Experiment

Six 35 mm dishes were plated and allowed to grow to confluence. Two were not irradiated, in order to be used as controls. The remaining four were irradiated to 4 Gy in an x-ray machine. Two were then incubated for 30 minutes, and two were incubated for 4 hours. Single cell suspensions were created and the cells were plated onto microscope slides as per the standard Comet Assay protocol. 50 cells from each slide were analyzed for Tail Length, Tail DNA, Olive Tail Moment, and Extent Tail Moment. The results of the previous dose response experiment at the 4 Gy dose level were used as a baseline level of damage at the zero time point. The various measurements at each time point are presented below in Figure 19. The results have been normalized to their highest value (the zero time point for all measurements) in order to show them all on a single plot.

The results show that the majority of DNA repair takes place in the first 30 minutes after the damage occurs. Four hours after irradiation, all measured values are at or below the level of the control. Both tail moment and tail DNA measurements exhibit very similar responses, while the tail length measurement shows a significantly slower decrease with time. The Tail DNA (fraction of total DNA present in the tail) measurement provides an indication of the total amount of DNA damage, while the tail length corresponds to the smallest fragment size. This may indicate that DNA that has

been fragmented into smaller pieces is more difficult to repair, and thus the Tail Length value does not decrease with time as rapidly as the other measured values.

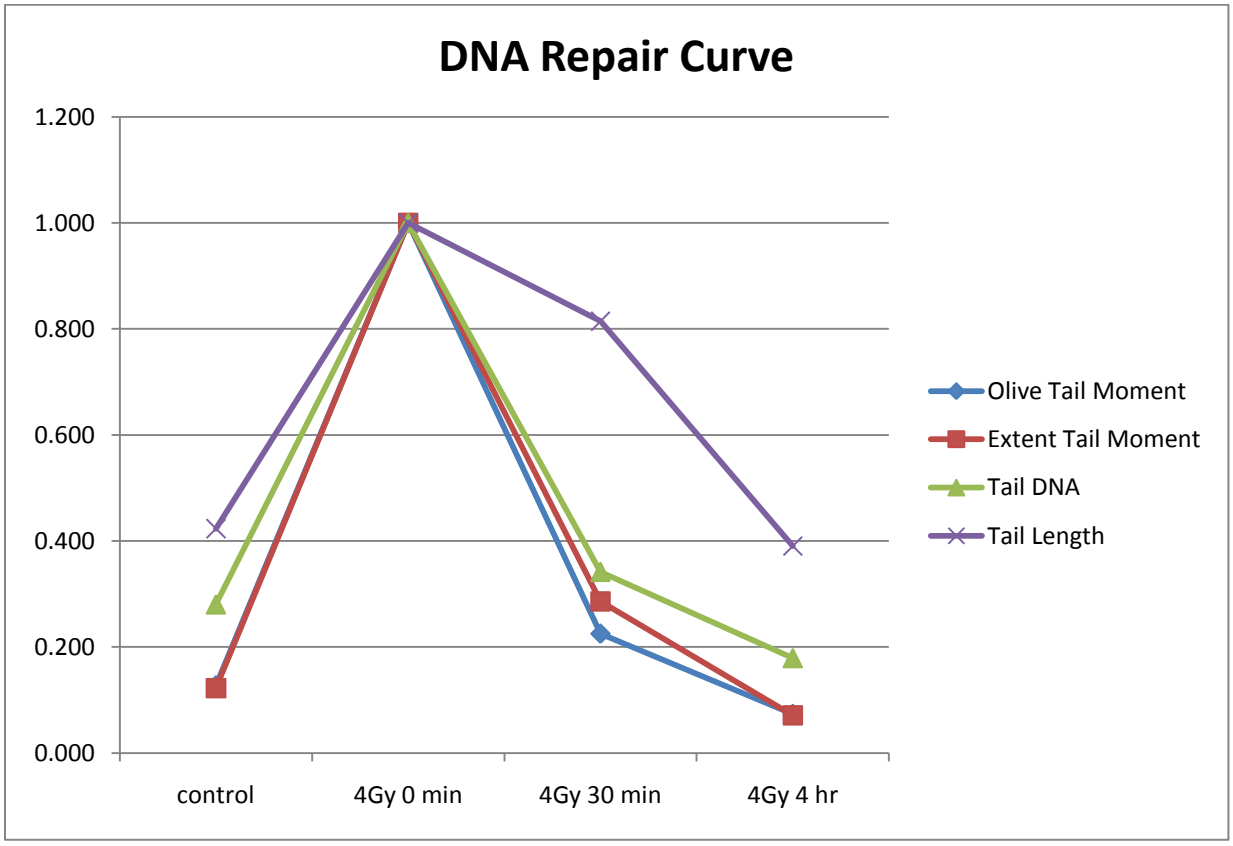

Figure 19 - Values of measured parameters after 4 Gy x-ray irradiation, as a function of repair time post-irradiation.

Comet Assay Conclusions

Compared to immunostaining, the Comet Assay is a straightforward way of measuring DNA damage in individual cells. Immunostaining is more of a chemical method, in that DNA damage is visualized through staining of proteins which the cell produces *in response* to the damage. The Comet Assay is a more physical method, because what is observed is actual fragments of DNA molecules after they are forced by the electric field into the comet tail. What the Comet Assay lacks in comparison to immunostaining is 1) the spatial distribution of the DNA damage, and 2) the ability to

study the cellular repair proteins. The spatial distribution is lost since the assay works by forcing the broken DNA to migrate, and since the DNA is stained directly, no information on cell proteins can be gained. Information on the nature of the DNA damage can be inferred through comparison of the alkaline and neutral variations of the assay, which would give an estimate of the relative abundance of single strand and double strand breaks.

The main advantage of the comet assay over immunostaining is the convenience and reliability of processing the results into numerical data. The basic quantities of tail length and fraction of DNA in the head/tail can be accurately calculated by software, and from these, the tail moments are easily calculated. This is a much more trouble-free way of assessing DNA damage than trying to distinguish foci through immunostaining.

CHAPTER 6

WORKING WITH PLASMIDS

Thus far, in order to see the cells through a fluorescent microscope, chemical staining processes have been used. Chapter 4 on immunostaining used a process of indirect staining of proteins with fluorescent antibodies, and Chapter 5 on the comet assay required the use of propidium iodide to stain the DNA molecules. These processes are simple, reliable, and can produce very good image quality if good technique is used. The disadvantage of staining cells is that the cells must first be fixed, or killed, in order to proceed with the staining. In some cases, it would be advantageous to be able to use these same fluorescence microscopy techniques with the cells still alive. This is possible, but the techniques used are somewhat more complex. It requires manipulation of the cell's DNA to cause the cell to modify certain proteins so that they become fluorescent. In this way, instead of tagging the protein of interest with a fluorescent antibody, the cell itself is made to produce that same protein with an additional fluorescent "piece" attached.

The manipulation of the cell's DNA is done through the insertion of a section of plasmid DNA into the cellular DNA. Plasmids are small, circular sections of DNA often found in bacteria in addition to the bacterial DNA. The gene to be inserted into the cell DNA is first incorporated into the plasmid, which is then known as a vector. The vector is then mixed with a chemical called Lipofectamine, which forms a membrane similar to the cell membrane around the plasmid. The membrane covered plasmid is then known as a liposome, and can be incorporated into the cell membrane, depositing the plasmid DNA

into the cell (Felgner, et al. 7414). This process is known as transfecting the new DNA into the cell.

Some of the cells present in the dish at the time of transfection will incorporate the plasmid DNA into their cellular DNA and begin to produce the modified, fluorescent form of the protein of interest. These proteins in the transfected cells will then show up under the fluorescent microscope as if they had been stained with antibodies specific to that protein, but without the need to kill the cell and go through the entire immunostaining process.

One disadvantage of this procedure is that not all of the cells will be transfected with the modified DNA. The cells in the dish will be a mixture of transfected and normal cells. The percent of cells that are transfected is known as the transfection efficiency, and can be increased through optimization of the transfection procedure. Figure 20 shows a brightfield image merged with a fluorescent image of GFP-PARP transfected cells. The cell nuclei (containing the PARP protein) can be seen superimposed over the brightfield image of the rest of the cells. Both transfected and non-transfected cells can be seen in the image. A protocol is included below that was found to produce good results.

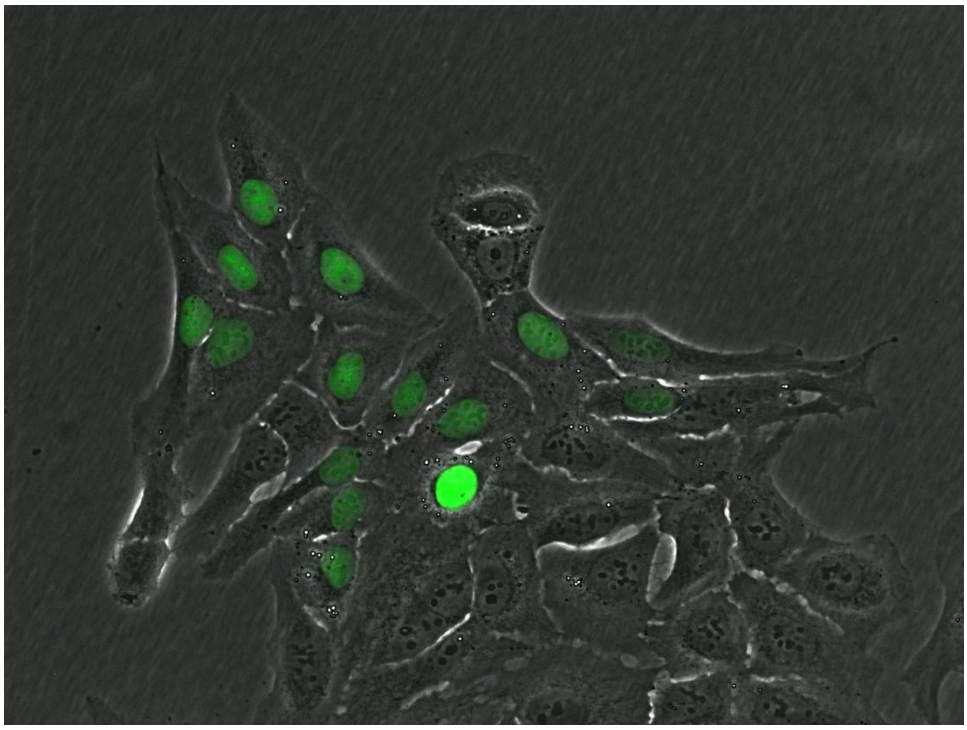

Figure 20 - Brightfield image merged with fluorescent image of cells transfected with GFP-PARP.

Even though only a fraction of the cells are initially transfected, it is possible, though challenging, to selectively grow only transfected cells. Often, the modified DNA included in the vector includes a gene that imparts an antibiotic resistance to the transfected cells. The cells may then be grown in the appropriate antibiotic, which should allow transfected cells to continue growth, while killing many of the normal cells. Over time, this method may be used to establish stable cell lines expressing the transfected gene.

Selection with antibiotics is an attractive option due to its simplicity, however it is not always a viable method. If one is trying to establish a stable cell line transfected with two different plasmids which both confer resistance to the same antibiotic, then antibiotics cannot be used to select co-transfected cells (cells which have incorporated both plasmid vectors). The reason is that in this case, successful transfection of either

vector will give the cell the necessary resistance, so cells with Vector A, Vector B, and both Vectors A and B will survive. In this situation, other selection techniques must be employed. One option is a selection by dilution. In this technique, a cell suspension is created and diluted to a very low concentration. Cells are then plated into a 96-well culture dish at a concentration such that (on average) only one or two cells will be plated in each well. If the initial cells in a given well are co-transfected with the two plasmid vectors, then all cells which grow subsequently in that well will also be co-transfected. A brief protocol for selection by dilution is included below.

Protocol for Transfecting Plasmid DNA into Cells

1. Plate cells to be transfected into 60 mm dishes. Plate one dish for each separate transfection to be performed. Allow dishes to grow to near confluence.
2. Aspirate the medium from the 60 mm dishes, and replace with Opti-MEM reduced serum medium.
3. Dilute 2 µg of the plasmid into 50 µL of Opti-MEM reduced serum medium in a 1.5 mL tube. The plasmid concentration should be known in µg/µL so the appropriate volume may be calculated as shown below. Use one 1.5 mL tube for each unique transfection.

$$volume\ [\mu L] = \frac{desired\ mass\ [\mu g]}{known\ concentration\ [{}^{\mu g}/_{\mu L}]}$$

4. In another 1.5 mL tube, combine 4 µL of Lipofectamine-2000 with 46 µL of Opti-MEM medium, for a total volume of 50 µL. Multiply this ratio (in the same tube)

by the number of transfections to be performed (i.e. for four unique transfections, use 16 µL of Lipofectamine-2000 and 184 µL of Opti-MEM medium).

5. Wait 5 minutes for the Lipofectamine-2000 and medium to mix thoroughly.
6. Add 50 µL of the Lipofectamine-2000/medium solution to each tube containing 50 µL of plasmid DNA solution, so that each tube will have 100 µL of solution. Incubate this mixture for 20 minutes at room temperature.
7. Add the 100 µL of plasmid DNA and Lipofectamine-2000 to the dish to be transfected. Swirl the dish to evenly distribute the solution.
8. Place the cell dishes back in the incubator for 18-48 hours, and then change the medium back to the usual growth medium for the cells.

Protocol for Selection by Dilution

1. Apply trypsin to the cells and resuspend in growth medium to create a single cell suspension.
2. Measure the concentration of cells in suspension. Assuming 2 cells/well is desired, dilute the cell suspension to a concentration of 10 cells/mL.
3. Pipette up and down to evenly distribute the cells. Add 200 µL of cell suspension to each well of the 96-well plate.
4. Incubate the cells at 37°C and monitor cell growth. Replace the medium every two days. When cells have grown to near confluence in the cells, check in a fluorescence microscope for co-transfected cultures.
5. Apply trypsin to any co-transfected cultures and re-plate cells in a 35 mm culture dish. Allow cells to continue growth, changing medium every two days.

Cell Transfection Conclusions

Transfecting cell lines with plasmid vectors opens the door to many new research possibilities. It allows for live cell imaging, which allows the researcher to see the immediate responses of the cell to treatments such as x-ray or high LET radiation. It is also much more conducive to studying the time course of any cellular process, such as DNA damage repair. Using fixation and immunostaining techniques would require preparing multiple samples and fixing them at various discrete timepoints (5 min, 30 min, 1 hr, for instance). Sampling at discrete timepoints can never give the detailed time resolution possible with live cell imaging, where the cells are left on the microscope, and images can be taken every second, if so desired.

With the increased capability of live cell imaging come additional challenges. There is the initial challenge of less than perfect transfection efficiency. This forces the researcher to either work will cell cultures that are only partially transfected, or go through the process of establishing stable cell lines with the transfected cells, which can be a labor intensive process. Also, live cell imaging can require additional, more costly equipment. The cells on the microscope slide are alive, so it is desirable to maintain them at as close to their normal growth conditions as possible to ensure the results are not affected. To this end, many high-end live cell microscopes are equipped with heated stages or even fully enclosed boxes kept at 37°C and 5% CO_2. When considering the use of live cell imaging techniques, the potential advantages must be weighed against the additional cost and complexity.

APPENDIX A

DATA AND IMAGES FROM COMET ASSAY DOSE RESPONSE

Table 7 - Raw data from comet assay dose response experiment.

Group	Mean Tail DNA	Tail DNA Std Dev	Mean Tail Length	Tail Length Std Dev
control	24.43	13.08	117.11	35.92
1 Gy	40.19	14.12	166.72	17.46
2 Gy	38.55	7.97	176.18	19.11
4 Gy	57.63	14.72	154.17	37.28
8 Gy	70.89	7.69	238.79	29.90

Table 8 - Raw data from comet assay dose response experiment.

Group	Mean Olive Tail Mom.	Olive Tail Mom Std Dev	Extent Tail Mom.	Extent Tail Mom. Std Dev
control	11.04	10.57	30.69	22.16
1 Gy	29.12	14.99	67.85	26.64
2 Gy	26.60	7.67	68.48	16.75
4 Gy	43.68	13.66	92.74	31.31
8 Gy	90.24	20.25	169.52	28.60

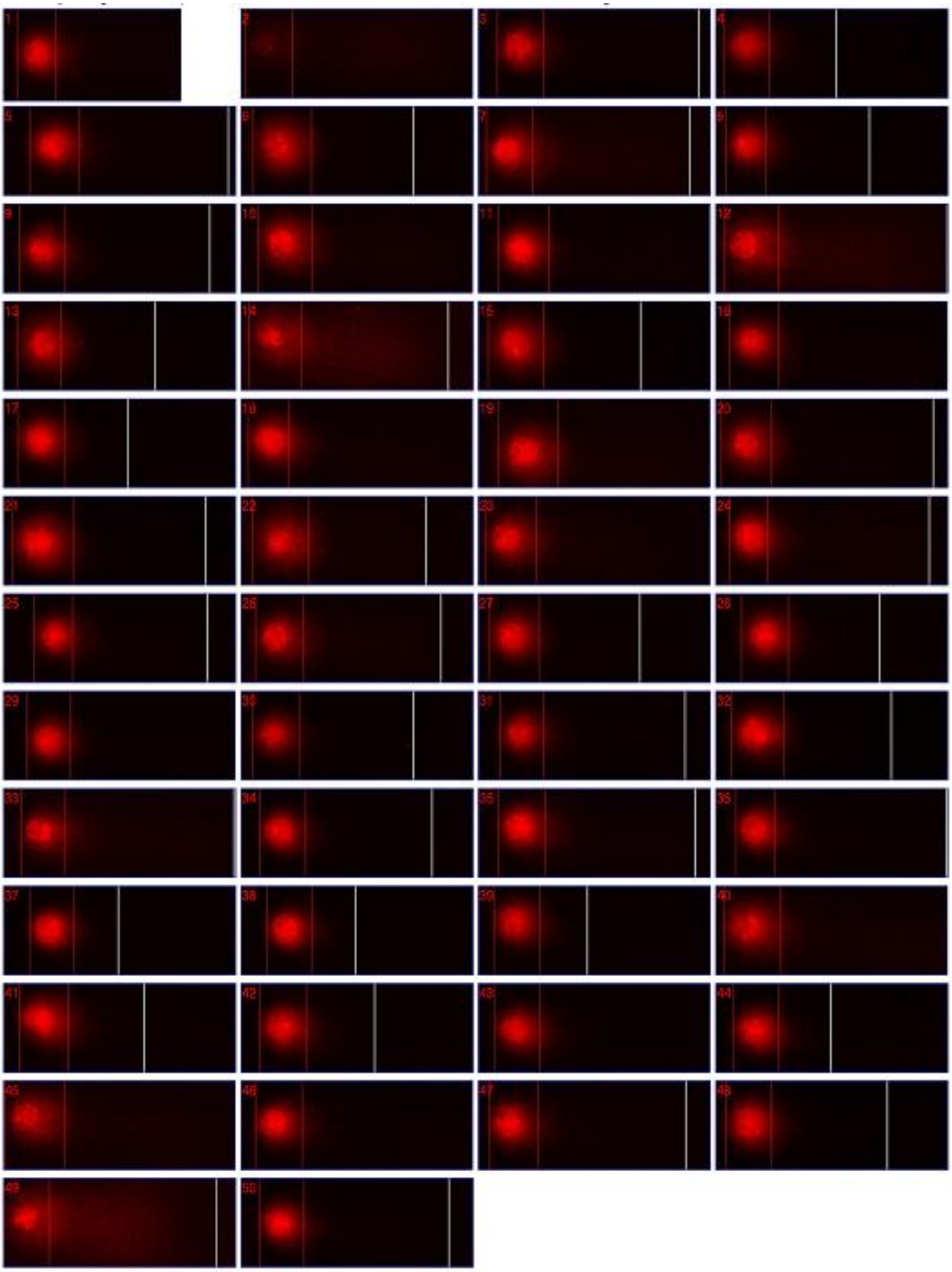

Figure 21 - Control images of cells analyzed in the comet assay dose response experiment.

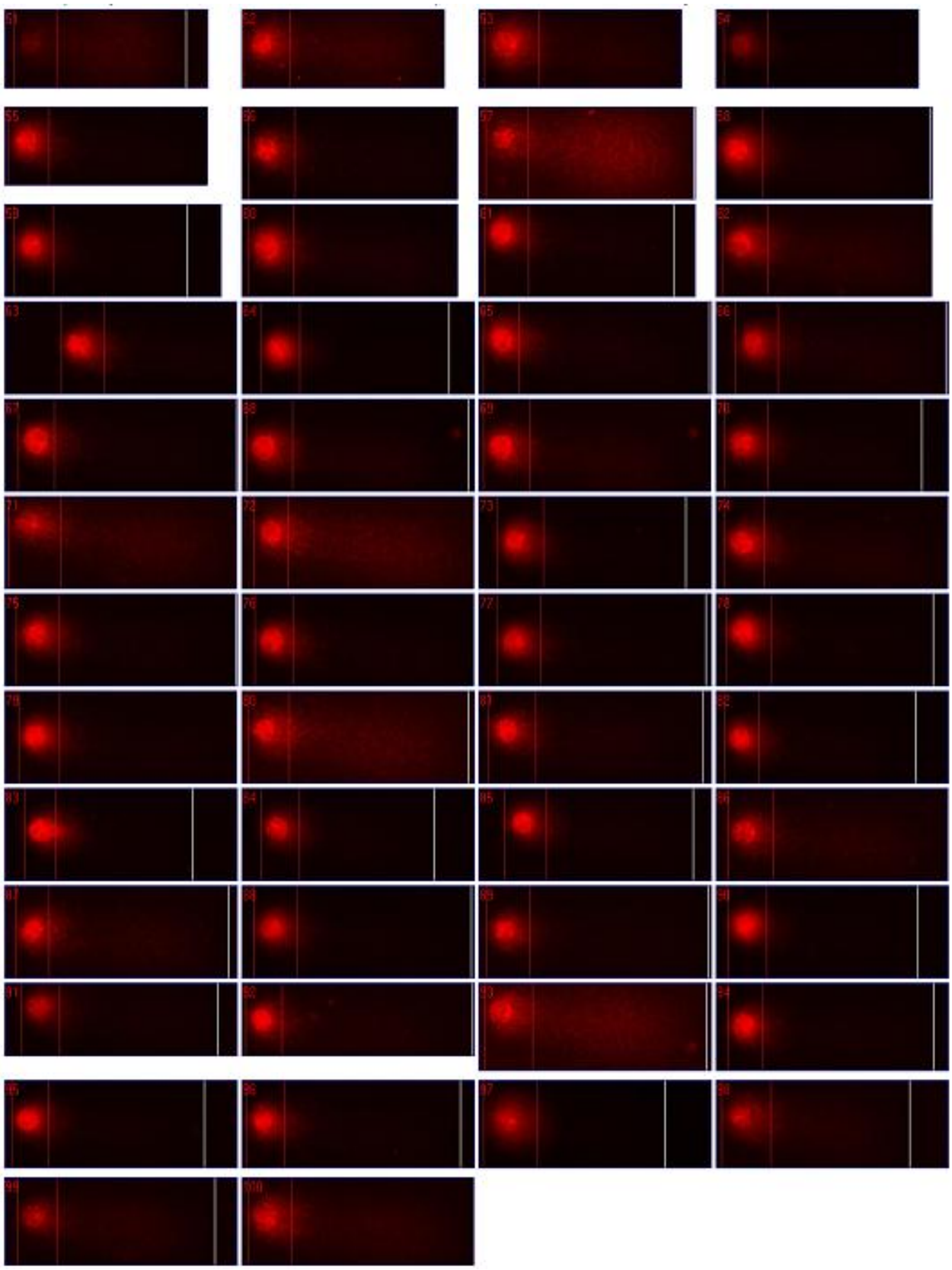

Figure 22 - 1 Gy images of cells analyzed in the comet assay dose response experiment.

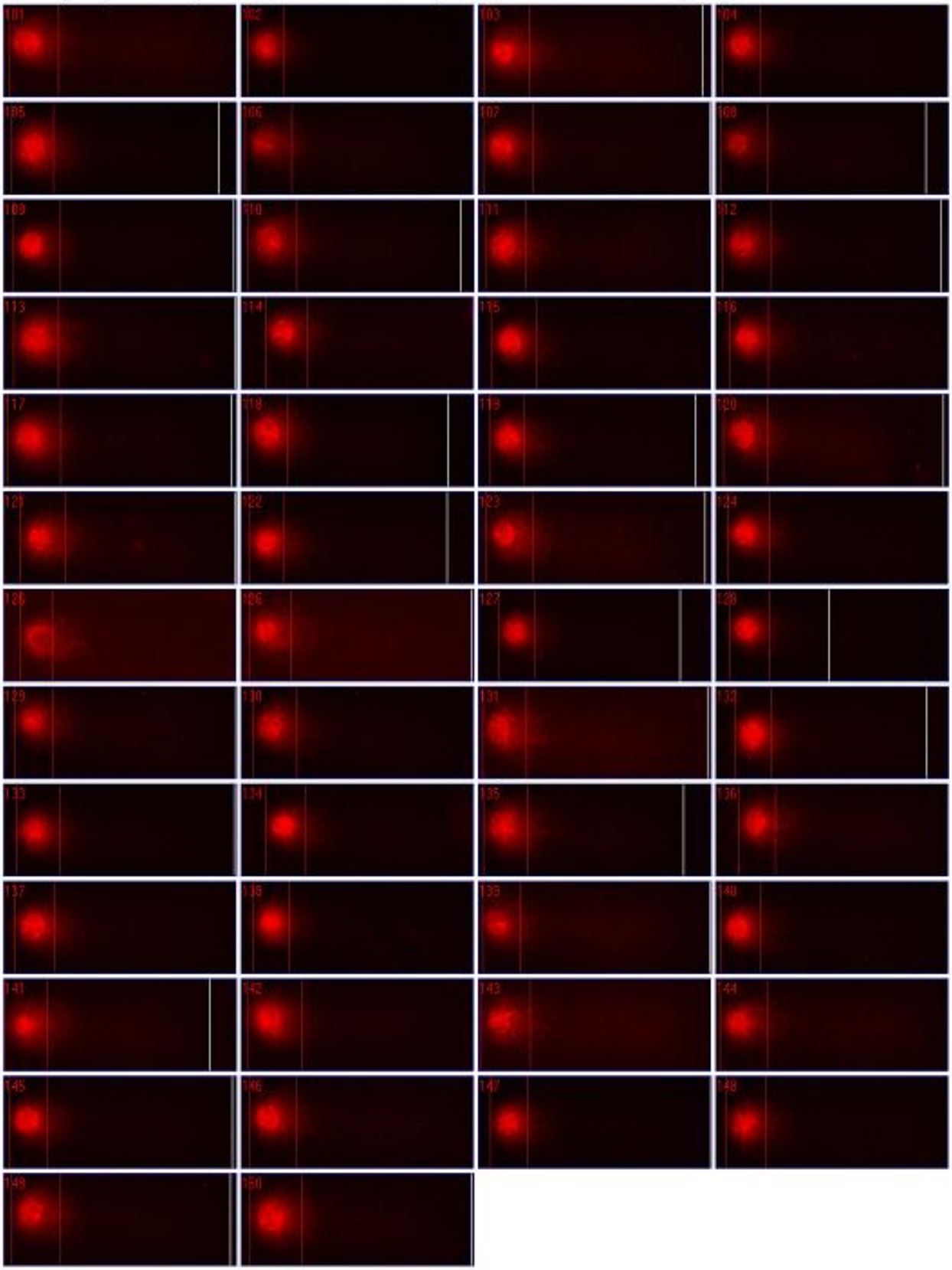

Figure 23 - 2 Gy images of cells analyzed in the comet assay dose response experiment.

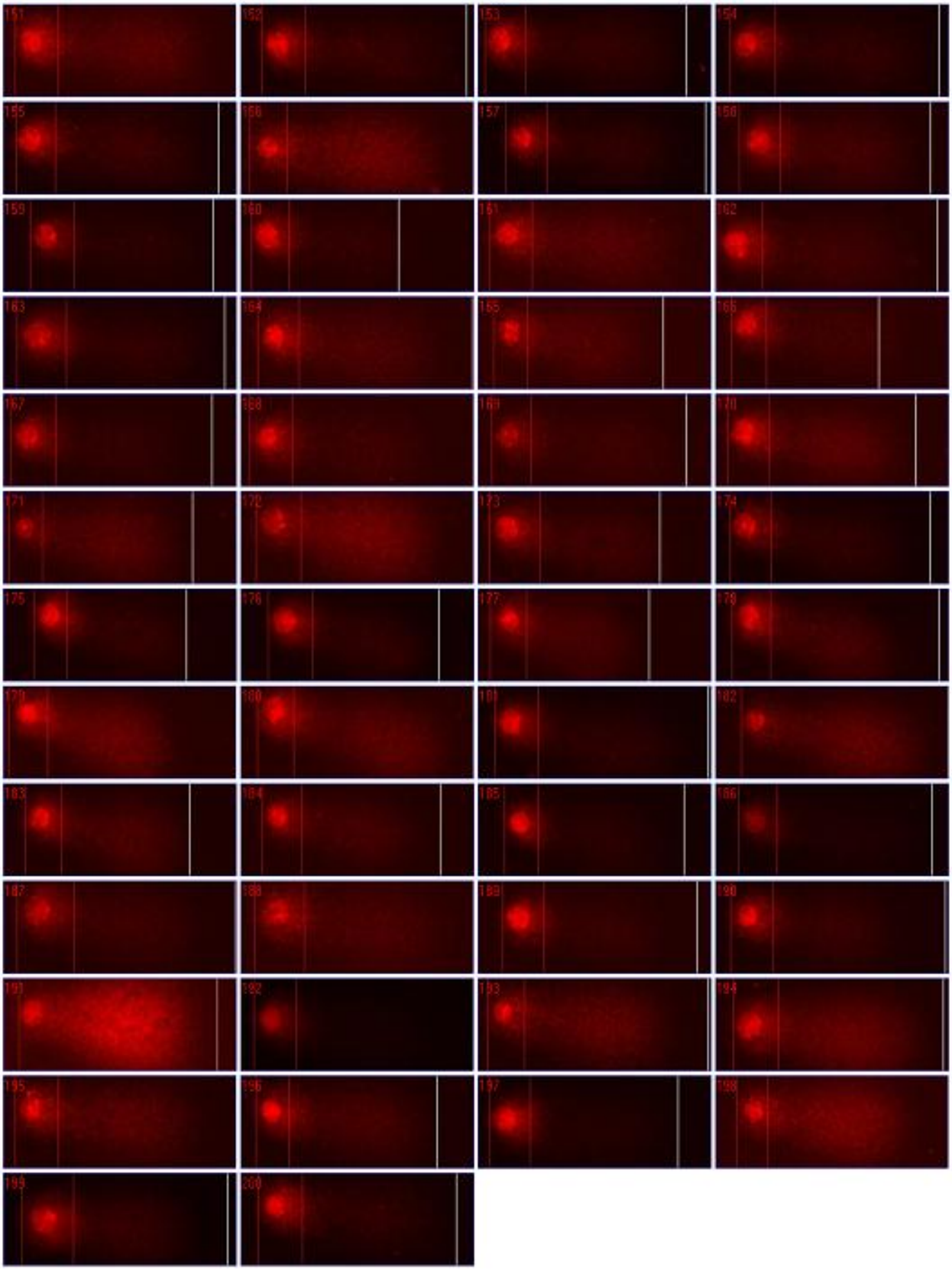

Figure 24 - 4 Gy images of cells analyzed in the comet assay dose response experiment.

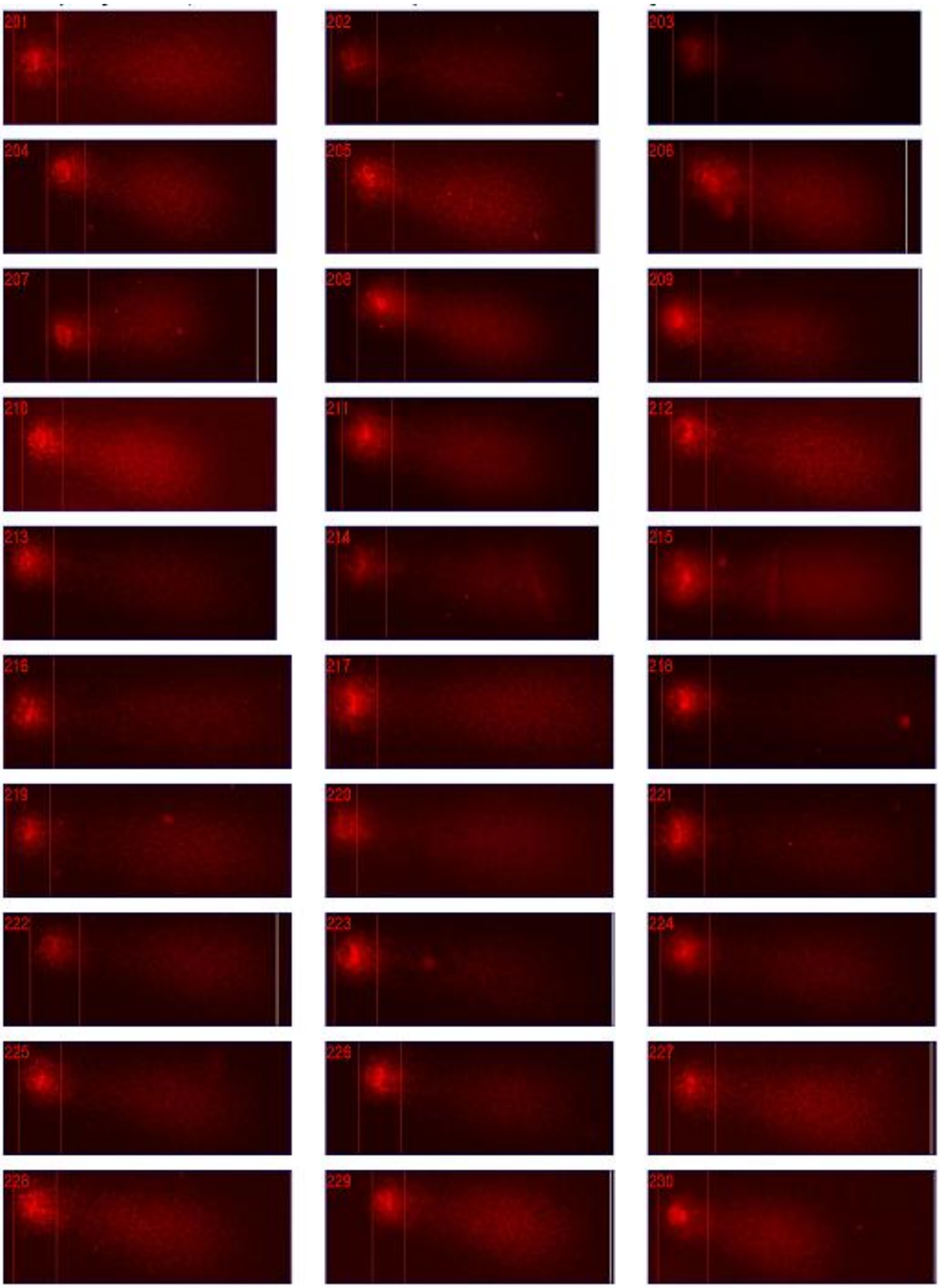

Figure 25 - 8 Gy images of cells analyzed in the comet assay dose response experiment.

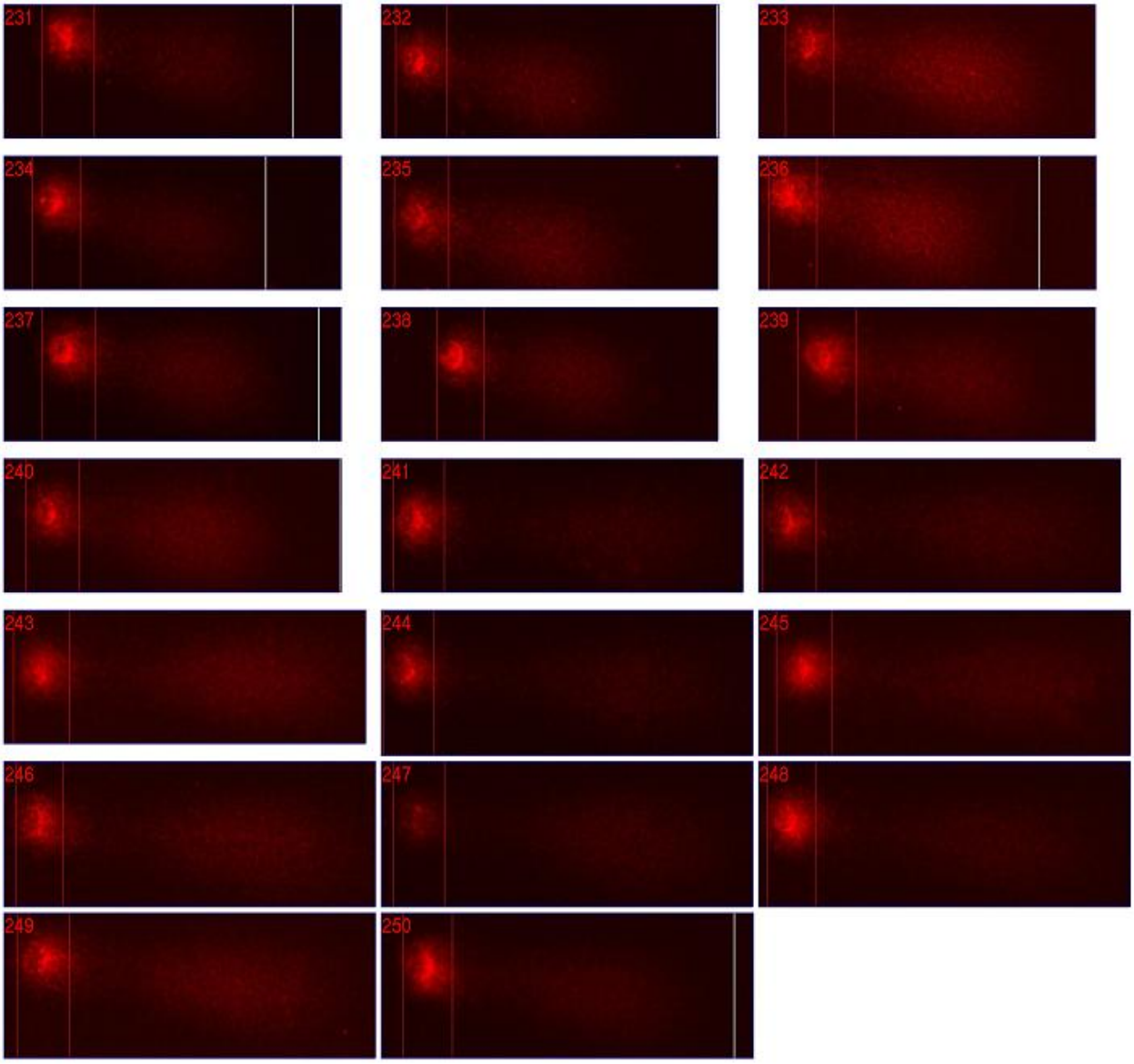

Figure 26 - 8 Gy images of cells analyzed in the comet assay dose response experiment.

APPENDIX B

DATA AND IMAGES FROM COMET ASSAY OF DNA REPAIR

Table 9 - Raw data from comet assay DNA repair experiment.

Group	Mean Tail DNA	Tail DNA Std Dev	Mean Tail Length	Tail Length Std Dev
control	16.15	7.93	65.24	18.5
4Gy 0 min	57.63	14.72	154.17	37.28
4Gy 30 min	19.69	9.32	125.56	45.41
4Gy 4 hr	10.31	7.01	60.17	30.91

Table 10 - Raw data from comet assay DNA repair experiment.

Group	Mean Olive Tail Mom.	Olive Tail Mom Std Dev	Extent Tail Mom.	Extent Tail Mom. Std Dev
control	5.55	2.73	11.33	7.26
4Gy 0 min	43.68	13.66	92.74	31.31
4Gy 30 min	9.82	4.63	26.5	18.35
4Gy 4 hr	3.2	1.91	6.6	5.81

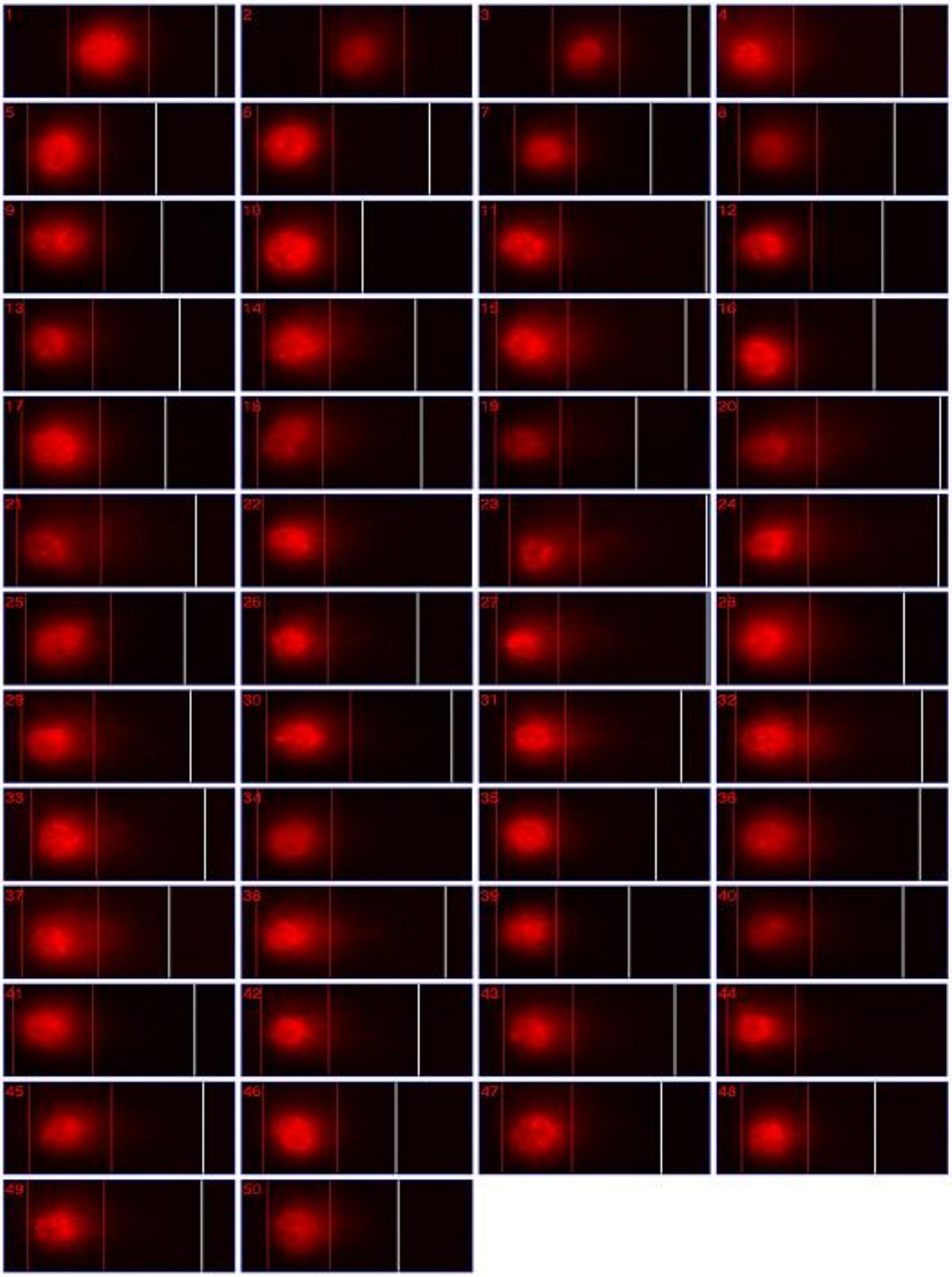

Figure 27 - Control images of cells analyzed in the comet assay DNA repair experiment.

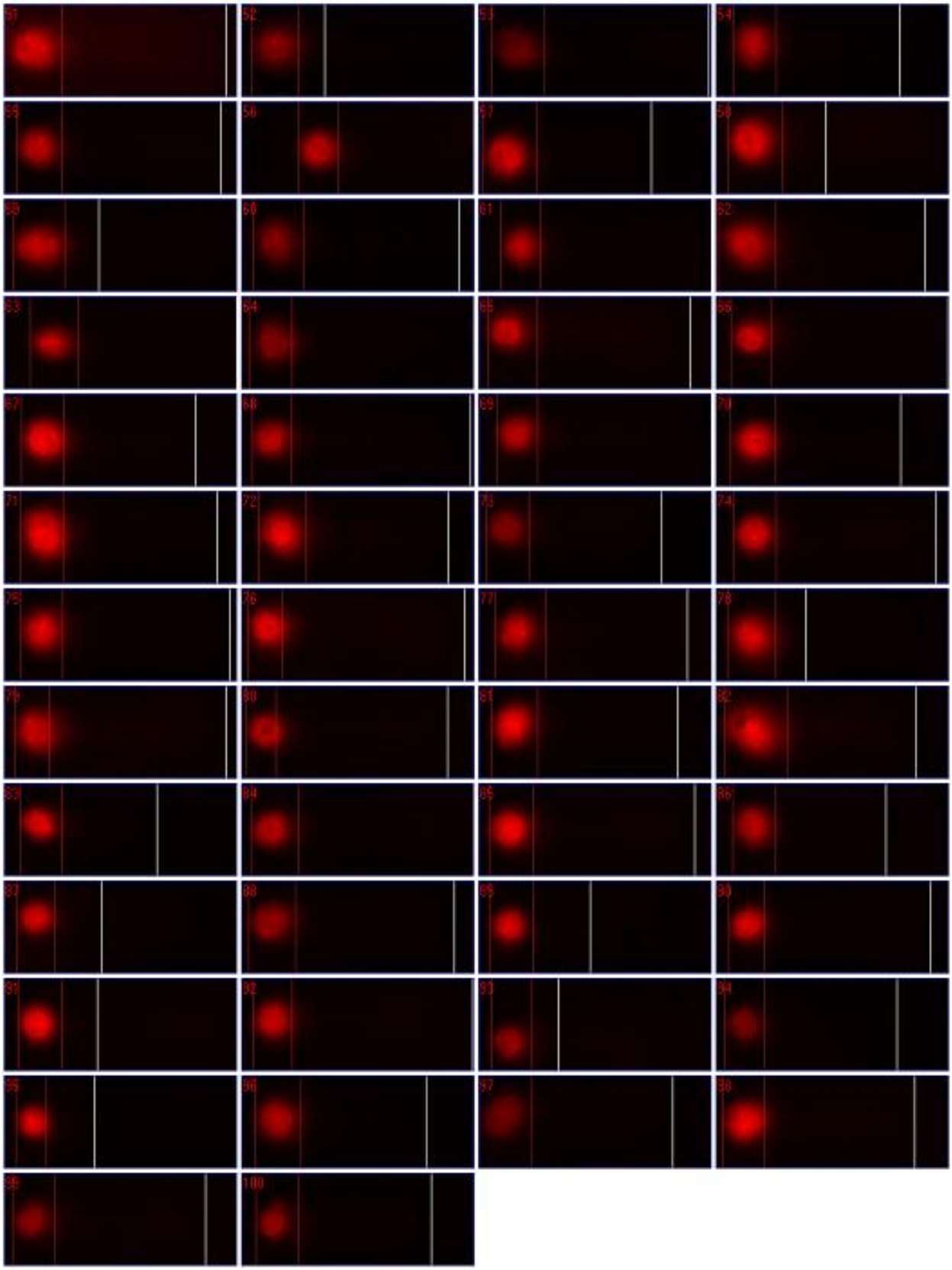

Figure 28 - Images at 4 Gy, 30 min from the comet assay DNA repair experiment.

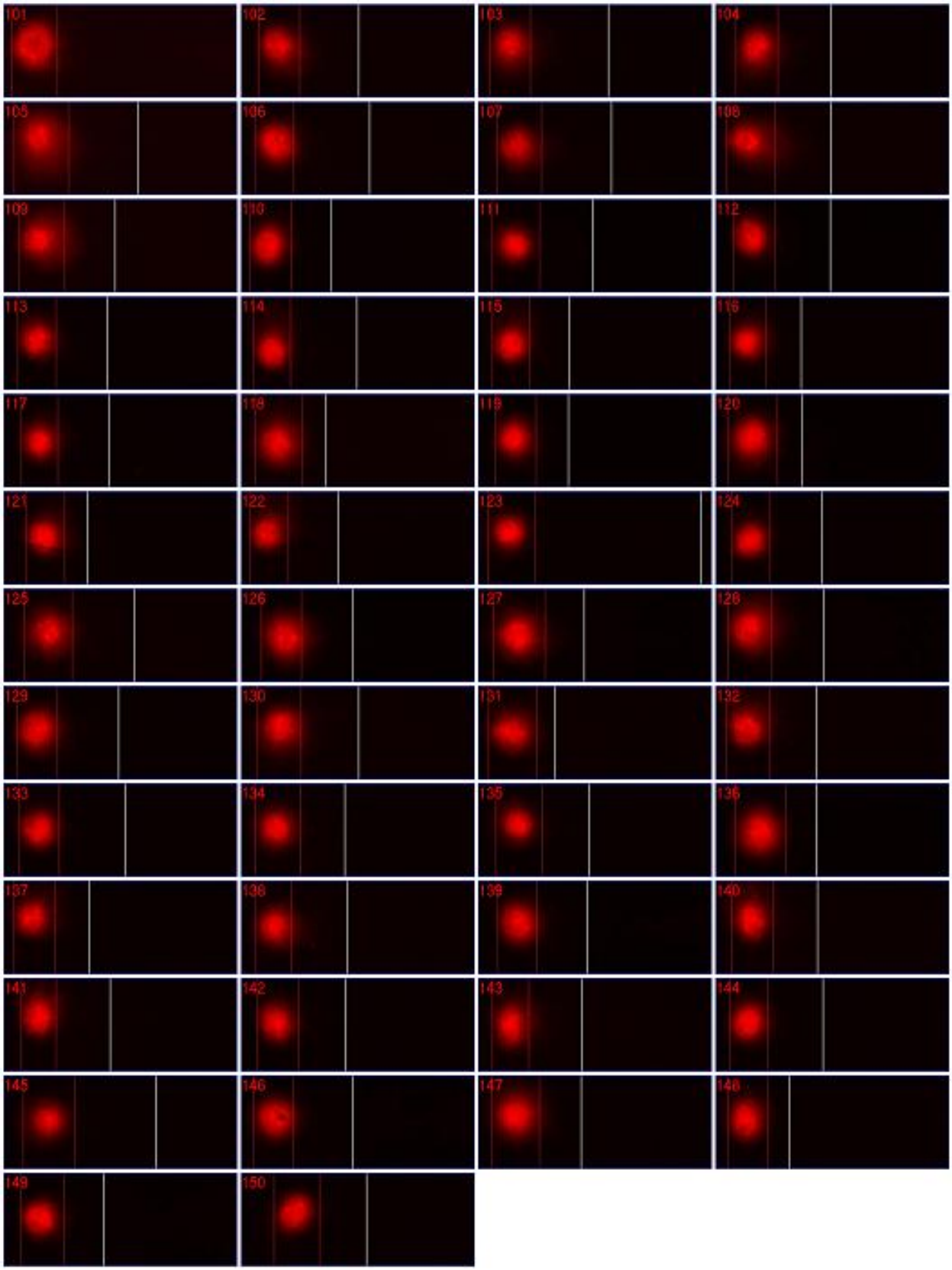

Figure 29 - Images at 4 Gy, 4 hrs from the comet assay DNA repair experiment.

APPENDIX C

HEAVY ION STAINING IMAGES

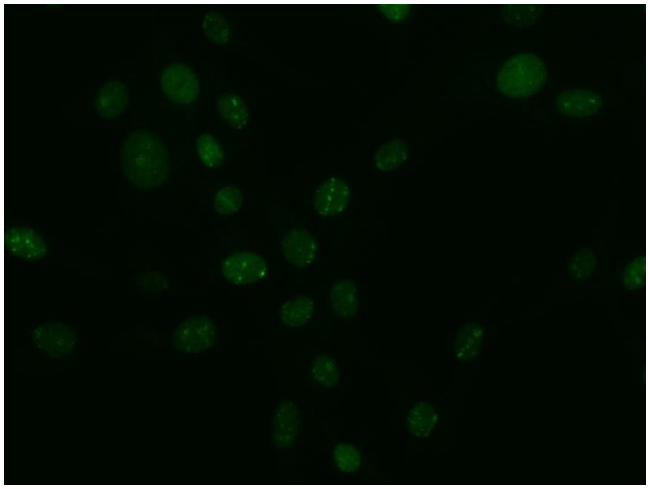

Figure 30 - ATM staining, no PARP inhibitor

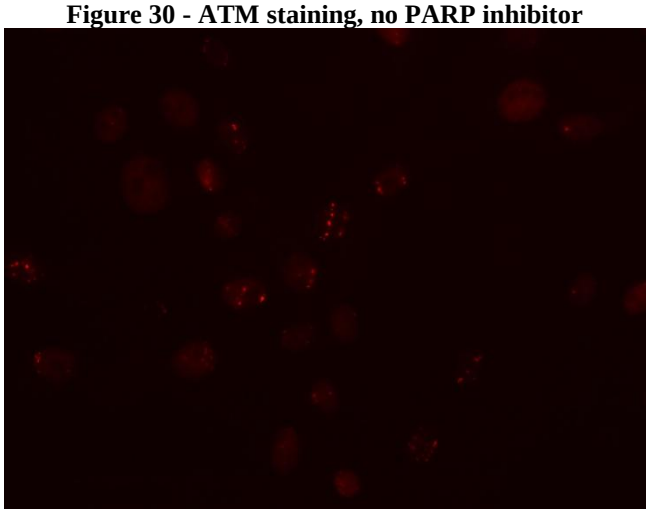

Figure 31 - 53BP1 staining, no PARP inhibitor

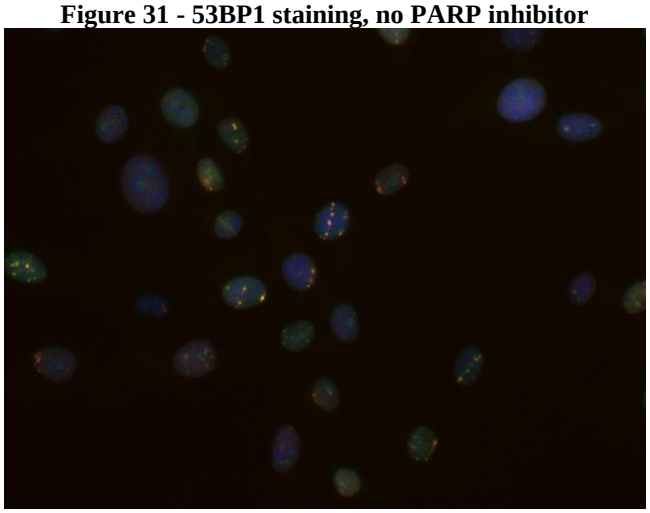

Figure 32 - Merged image of ATM (green), 53BP1 (red), and DAPI (blue), no PARP inhibitor

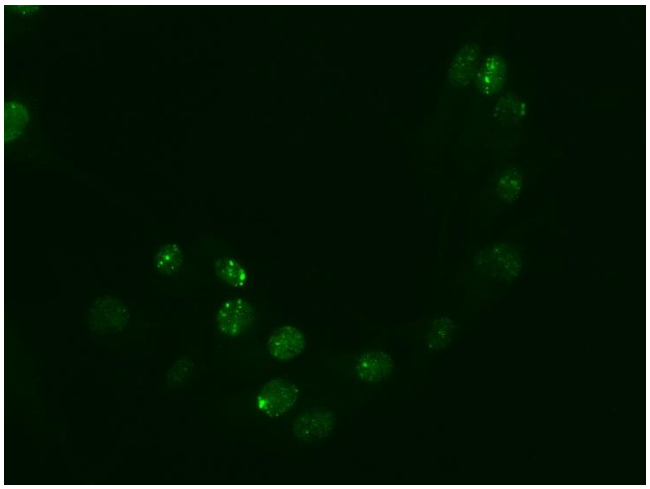

Figure 33 - ATM staining

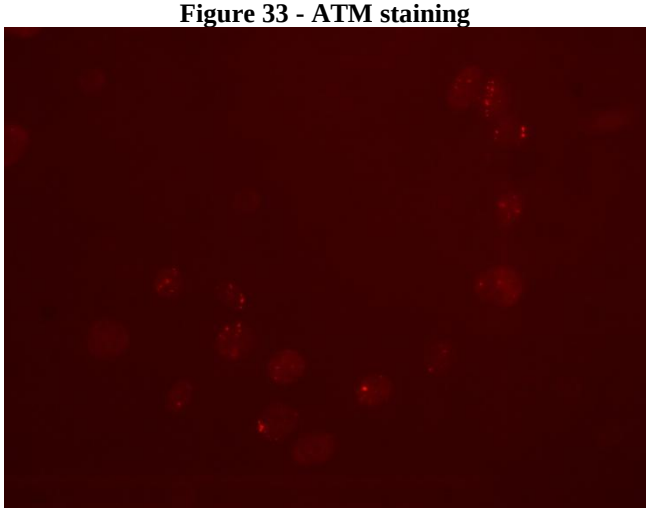

Figure 34 - Mre11 staining

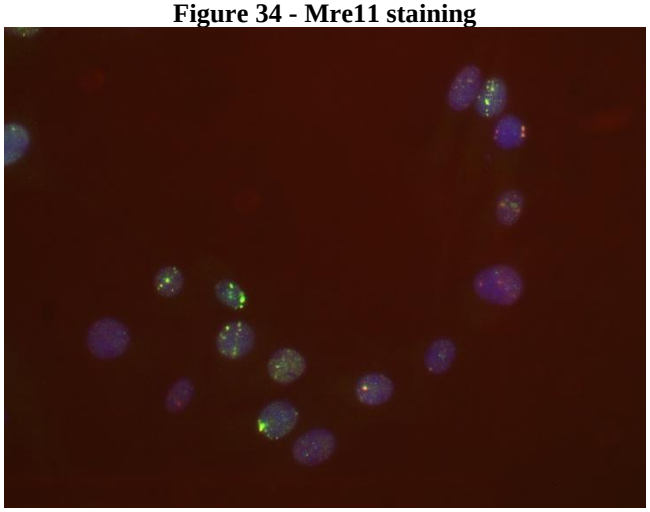

Figure 35 - Merged image of ATM (green), Mre11 (red), and DAPI (blue), no PARP inhibitor

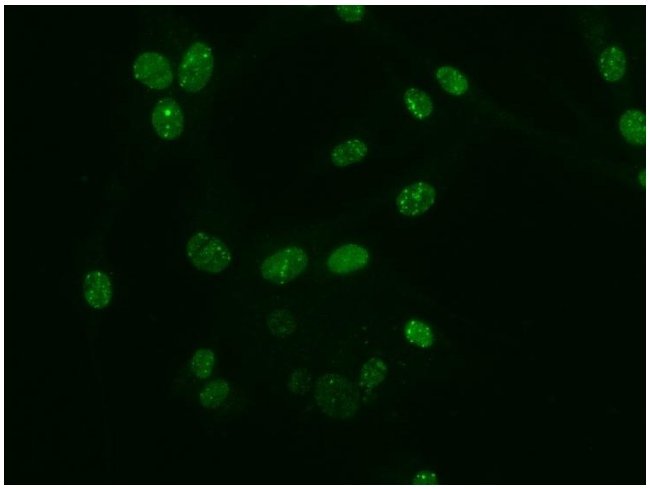

Figure 36 - ATM staining

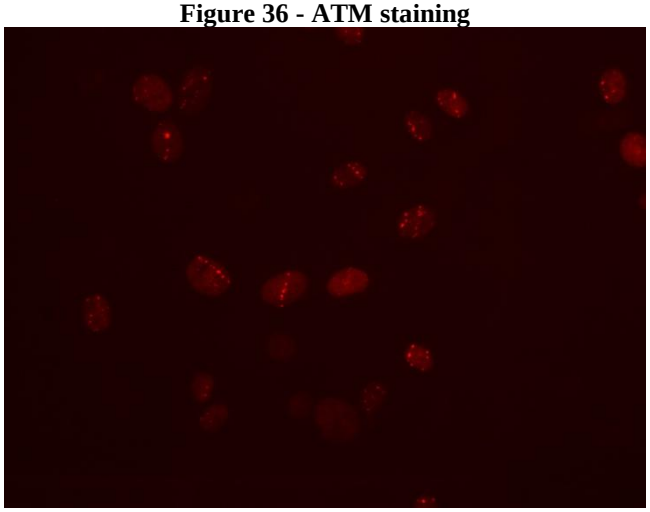

Figure 37 - PARP staining

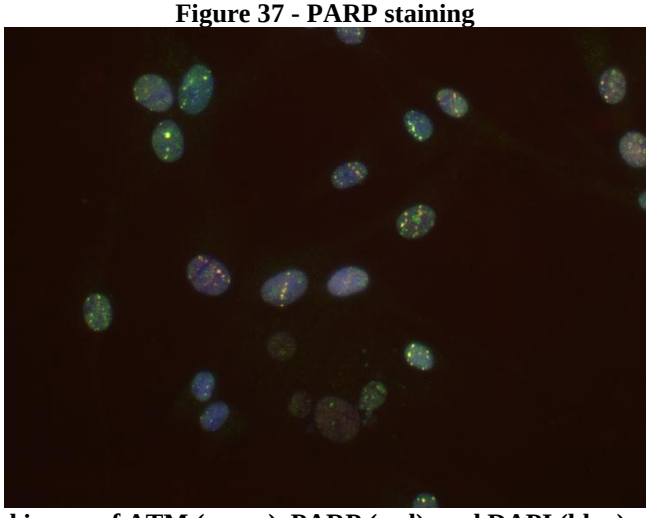

Figure 38 - Merged image of ATM (green), PARP (red), and DAPI (blue), no PARP inhibitor

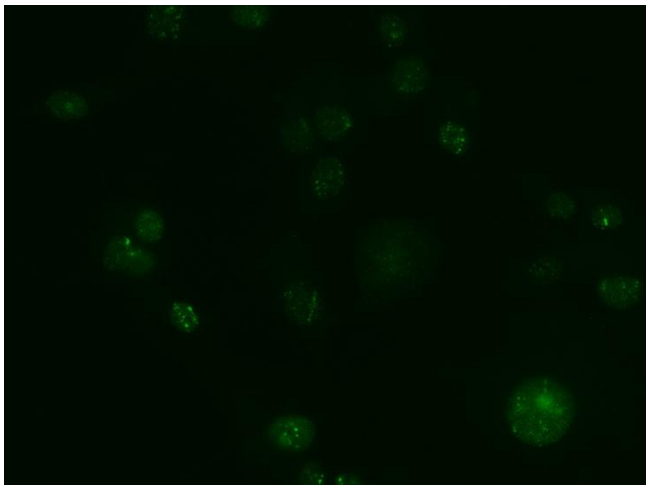

Figure 39 - PAR staining

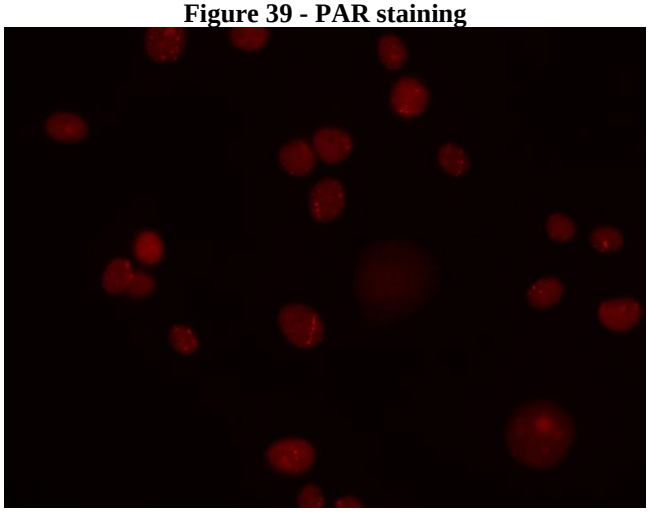

Figure 40 - PARP staining

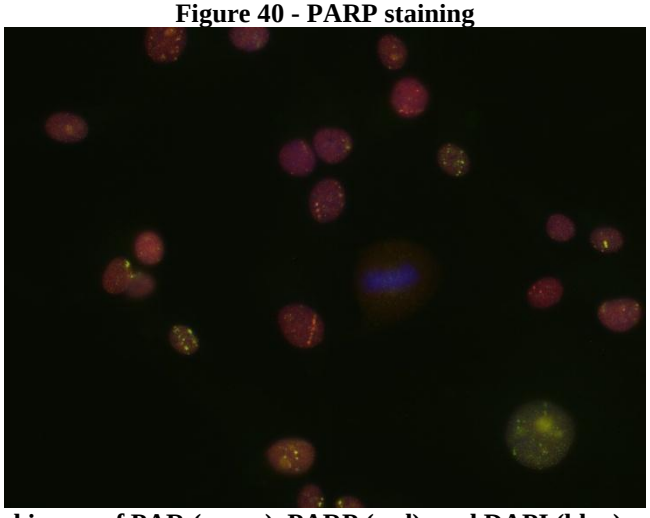

Figure 41 - Merged image of PAR (green), PARP (red), and DAPI (blue), no PARP inhibitor

APPENDIX D

RESULTS OF FOCI COUNTING IN X-RAY IRRADIATED HT22 CELLS

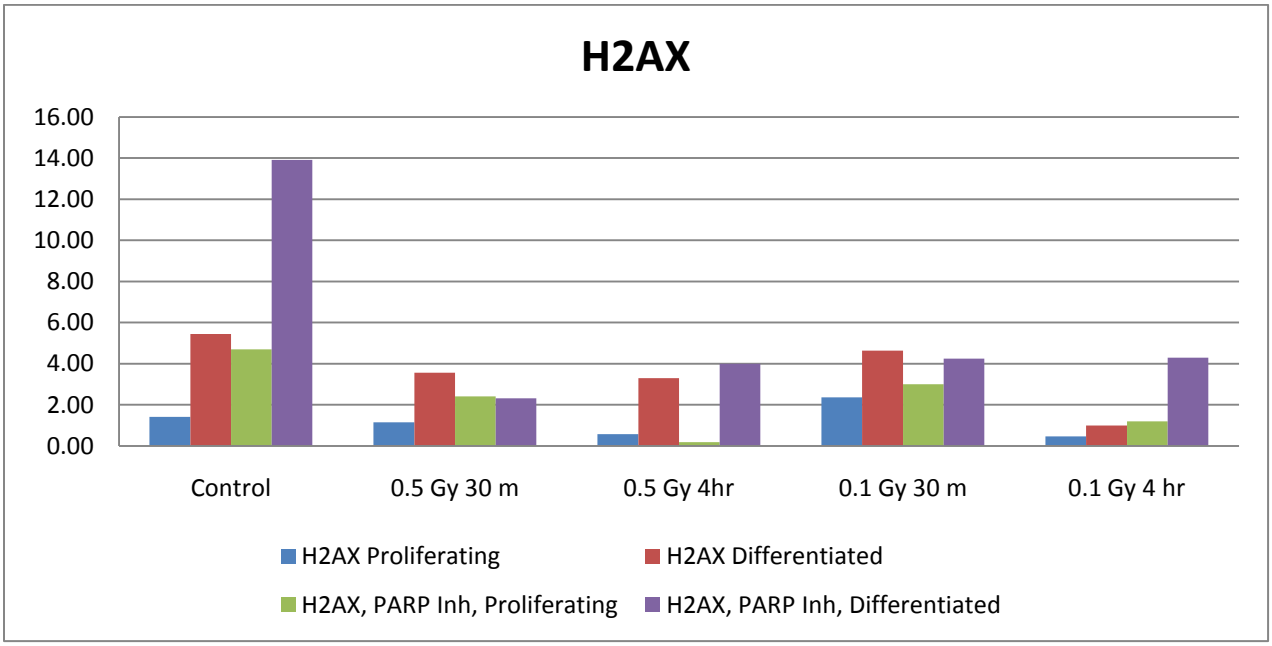

Figure 42 - Results of counting of H2AX foci.

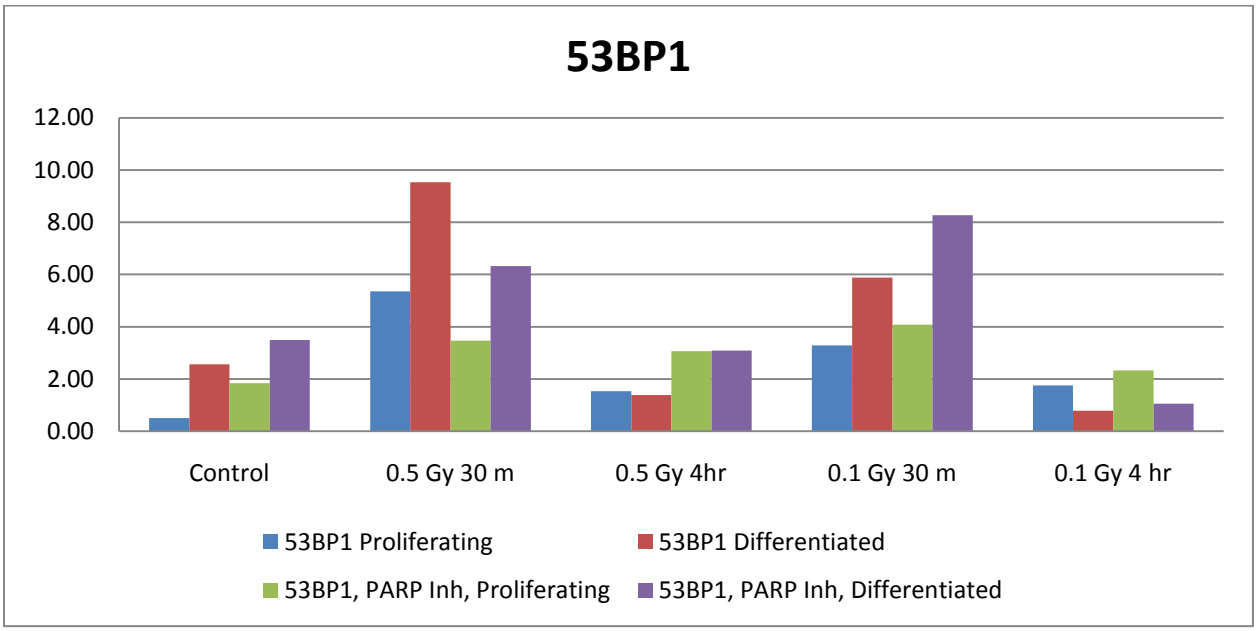

Figure 43 - Results of counting of 53BP1 foci.

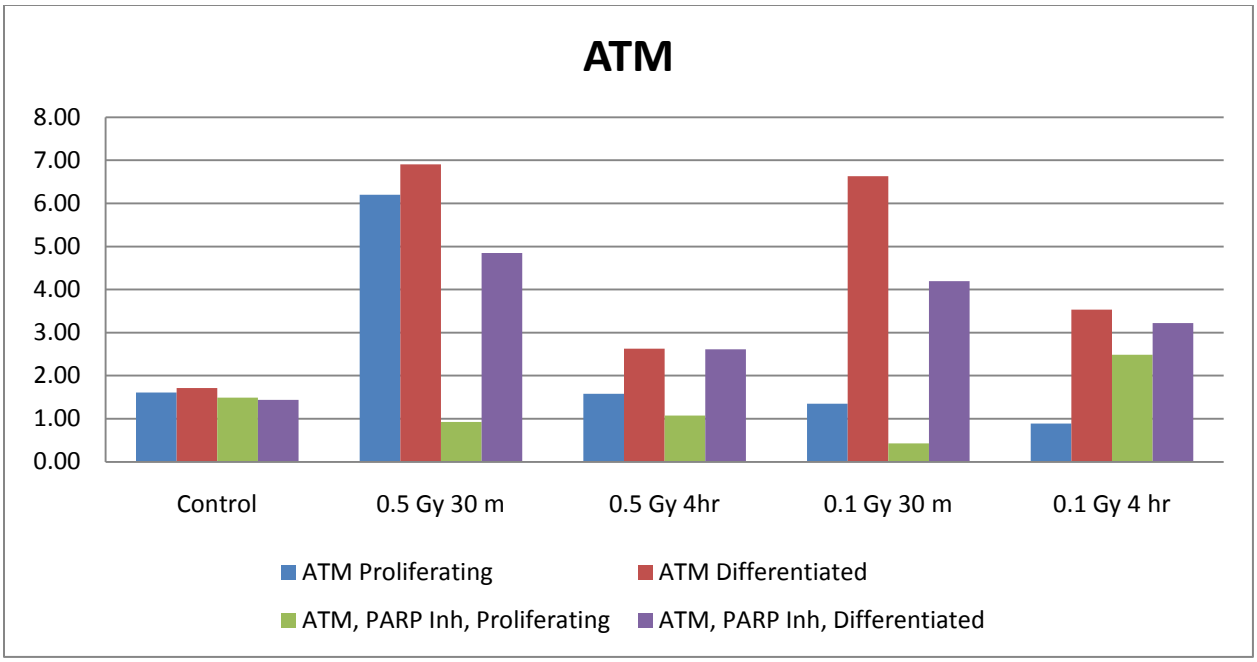

Figure 44 - Results of counting of ATM foci.

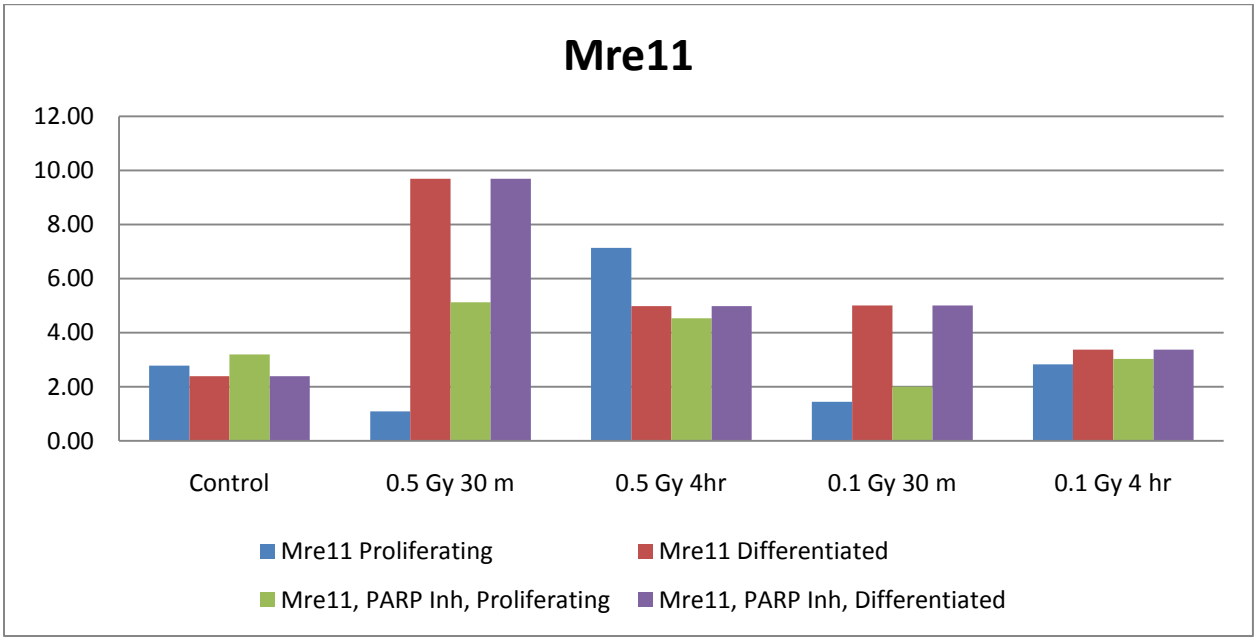

Figure 45 - Results of counting of Mre11 foci.

APPENDIX E

LIST OF CHEMICAL SOLUTIONS

A1 lysis buffer – solution used in comet assay to lyse cells, or break up the cell membrane so that other substances may move in or out

A2 rinse/running buffer – solution used in comet assay to rinse cells clean of A1 buffer, and to submerge cells for electrophoresis

G418 – antibiotic used to allow certain types of cells to continue growth, while killing other types of cells

growth medium – solution containing the nutrients necessary for cell growth

paraformaldehyde – substance used to fix, or freeze, cells in their current state for later analysis

ABT888 – PARP inhibitor

Agarose – a substance which is a gel at room temperature but liquid

DAPI – a fluorescent stain that binds to DNA

fetal bovine serum (FBS) – a cell growth supplement containing a variety of proteins

Lipofectamine 2000 – used to encapsulate plasmid DNA for transfection into cells

mounting medium – substance placed on microscope slides before the coverslip is added

normal goat serum (NGS) – used to block unspecific staining of primary antibodies in immunostaining

Opti-MEM – serum free medium used for transfections

PBS – phosphate buffered saline, used in many protocols as a wash or rinse solution

penicillin/streptomycin (PS) – antibiotic added to cell cultures to prevent infection by bacteria

propidium iodide (PI) – used to stain DNA molecules in the Comet Assay

puromycin – antibiotic used in the selection of certain transfected cells

Triton X-100 – detergent used to permeabilize cell membrane during immunostaining

trypan blue – solution used with a hemocytometer to stain dead cells

trypsin – substance used in cell subculturing to detach adherent cells from their culture dish

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