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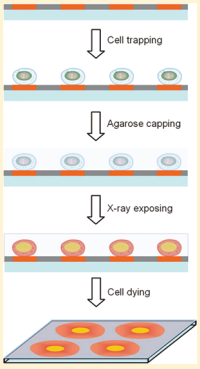
Single Cell DNA Damage/Repair Assay Using HaloChip

Yong Qiao, Chaoming Wang, Ming Su, and Liyuan Ma*

NanoScience Technology Center, University of Central Florida, Orlando, Florida 32826, United States

ABSTRACT: The molecular level damage to DNA is important due to DNA's susceptibility to free radical attacks and crucial roles in maintaining cell functions. Although a panel of techniques can be used to detect DNA damages, most of them are limited due to low sensitivity, low throughput, incompatibility for automated data analysis, and labor-intensive operations. We have developed a cell array based DNA damage assay in which mammalian cells are attached on an array of microfabricated patterns through electrostatic interactions. After trapping patterned cells inside gels, damaged DNA fragment can diffuse out of the nucleus and form a halo around each cell inside gels. The halo array can be observed fluorescently after labeling DNA with ethidium bromide. DNA damages can be determined sensitively at the single cell level, accurately due to the symmetric shape of the halo, and automatically due to the spatial registry of each cell and the nonoverlapping halos surrounding cells. The HaloChip can be used to detect DNA damages caused by chemicals and ultraviolet and X-ray irradiations with high efficiency. A major advantage of HaloChip is the ability to increase throughout by spatially encoding multiple dosing conditions on the same chip. Most importantly, the method can be used to measure variations in response to DNA damaging agents within the same cell population. Compared with halo assay or comet assay alone, this method allows automated analysis of a million cells without an overlapping issue. Compared with the microwell array based comet assay, this method can selectively capture and analyze cells, and the results can be easily analyzed to provide precise information on DNA damage. This method can be used in a broad range of clinical, epidemiological, and experimental settings.



The molecular level damage to DNA is important due to DNA's susceptibility to free radical attacks and crucial roles in maintaining cell functions.[1] DNA can be damaged in several ways including base pair deletion and insertion, cytosine oxidation, single strand break, and double strand break. Under normal conditions, DNA damages can be repaired by enzymes,[2] but when DNA is damaged too much or is too serious (i.e., double strand break) to be repaired, or damaged DNAs have to be involved in cell functions before they can be repaired, the damages will be inherited and accumulated,[3] leading to mutations that can eventually cause diseases such as cancers and central neuron system diseases, etc.[4−6] Although a panel of techniques has been used to detect DNA damages in many organisms (i.e., bacteria, cyanobacteria, phytoplankton, macroalgae, plants, animals, or humans),[7] most of these techniques lack desired sensitivity, throughput, and capability for automated data analysis and are often laborious.[8] Polymerase chain reaction (PCR) can detect gene-specific DNA damage at high sensitivity, because amplification stops at damage site,[9] but PCR has to rely on the intensity of an amplified band in electrophoresis and cannot quantify and recognize damage. Mass spectrometry can detect a wide range of DNA damaged products by combining with gas chromatography or high performance liquid chromatography,[10] but it requires extensive sampling and tends to overestimate damages. Fluorescence in situ hybridization can only detect a few types of DNA damage at one time due to spectral overlapping.[11] An electrochemical method can be used to detect chemically induced DNA damage,[12] and microfabricated electrochemical sensors can be used to detect radiation induced DNA damage.[13] However, the electrochemical method cannot detect multiple DNA damages at the same time and, thus, is not ideal for high throughput analysis.

Comet assay or single cell gel electrophoresis can provide damage distribution in a population of cells.[14] It is based on the fact that relaxed DNA loops induced by single strand break or DNA fragment migrate farther inside an agarose gel than undamaged DNA.[15] Not only can the comet assay be used to detect DNA damages at high sensitivity, but also it can be used to evaluate DNA repair and screen drugs.[16] However, the conventional comet assay is limited by low throughput and poor reproducibility between slide users and laboratories.[17] Specifically, the random distributions of cells in three dimensions inside the gel require the focus of the microscope to be changed to find sparse cells at different heights and locations. In addition, many cells form unanalyzable clumps, random debris, and overlapping comets, which can lead to loss of valuable information, especially from rare cells.[18] A comet chip based on cell array has been designed to solve the random distribution issue by trapping cells inside multiwells on the same height and at defined locations.[19] Cell spacing can be tuned to maximize the number of conditions that can be tested on each slide, and multiple conditions can be tested on the spatially encoded array. However, the physical trapping of cells through gravity and volume exclusion cannot be used to selectively capture certain cells inside microwells, and each comet has to be analyzed individually due to the complex shape of comet, which requires intensive and potentially biased user

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intervention to identify heads and tails of comets and determine their sizes and shapes. Instead of relying on an electric field to pull damaged DNA out of cells, the halo assay is dependent on self-diffusion of short DNA fragments to offer damage information.[20] The symmetric shape of each halo allows precise determination of a DNA fragment without much user intervention. However, the traditional halo assay uses cells randomly dispersed inside the agarose gel, which leads to the same issues such as random distribution, unanalyzable clumps, overlapping halos, and random debris. If halo assay could be achieved at well-defined locations (i.e., height and positions), the halo of each cell can be identified and analyzed quickly in an automated manner. However, the method used in comet chip cannot be directly used for the arrayed halo assay, because there are certain spaces between the cell and wall of the microwell, which will lead to preaccumulation of DNA fragments in the free space and failure of the halo assay.

This paper describes a single cell array based highly efficient DNA damage assay (HaloChip). The cell array has been generated on silicon substrate by combining laser microfabrication, surface modification, and site specific cell attachment. The arrayed cells are then embedded inside thin films of agarose gel, followed by exposure to a variety of conditions (i.e., ultraviolet light, X-ray radiation, and hydrogen peroxide), which can generate free radicals to damage DNAs of cells. A fast alkaline halo assay is performed to quantify the level of DNA damage (Figure 1). The main advantages of HaloChip are as follows: (1) The assay is performed at single cell level, thus needs only a small number of cells, has single cell sensitivity, and does not have an overlapping issue; (2) Live-cell assay can be performed, which allows monitoring DNA damage induced by chemical or radiation; (3) The spatial encoded halos enable simultaneous study of multiple experimental conditions; (4) The cells are located at the same height, which save much effort in changing the focus of the microscope; (5) The boundary between nuclei and halos is well-defined, thus allowing accurate and objective determination of the halo area; (6) The HaloChip allows automated imaging analysis and robust statistical analysis and requires minimal user intervention or special tools; (7) This method can be made compatible with existing high throughput techniques (24 or 96 wells), which allows its use in drug screening. This work is the first that combines the halo assay with chip idea, so that each cell is positioned at a precise 2D position and at the same height as others, and the symmetric shape of the halo allows easy determination of DNA damages.

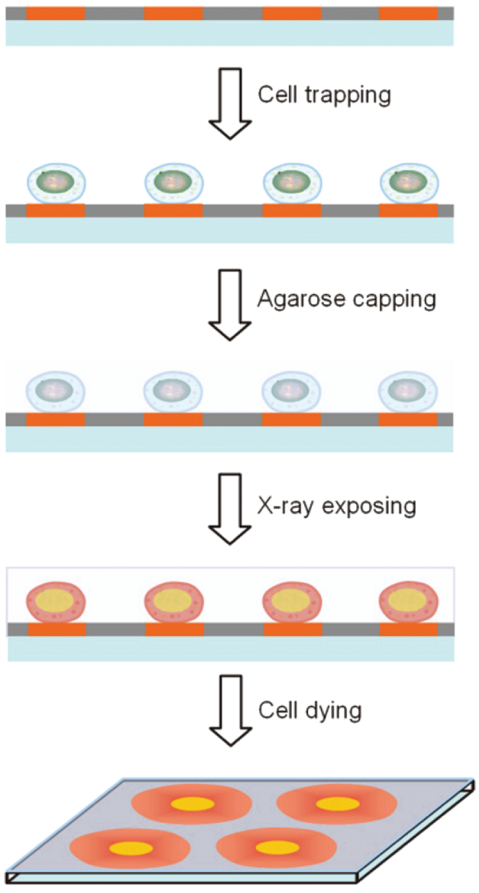

Figure 1. Single cell array based DNA damage assay with HaloChip.

EXPERIMENTAL SETUP AND METHODOLOGY

Polyethylene glycol (PEG) terminated silane (PEG-silane, 472−604 g/mol), methoxypolyethylenoxy propyl trichlorosilane, is from Gelest (Tullytown, PA). Aminopropyltriethoxy silane (APTES), triethylamine, hydrogen peroxide, RPMI-1640 medium, 1% (v/v) penicillin/streptomycin, polyvinyl alcohol, toluene, and ethanol are from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (10% (v/v)) and Ethd-1 (Live/Dead viability/cytotoxicity kit) are from Invitrogen (Carlsbad, CA). Trypsin/EDTA solution is purchased from Cell Applications (San Diego, CA). Trypan Blue, phosphate buffer saline (PBS), sodium hydroxide, methanol, hydrogen chloride, sulfuric acid, and agarose are from VWR (West Chester, PA). Silicon substrates (p-type, boron-doped, resistivity of 8−25 Ωcm^{-2}) are from UniversityWafer (South Boston, MA). Ethidium bromide (EB) is from Alfa Aesar (Ward Hill, MA). All chemicals are used without further purification. Three human cancer cell lines, MG-63 (osteosarcoma), HeLa (cervical cancer), and LNCaP (prostate cancer), are obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in standard conditions (5% CO_2 in air at 37 °C) in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. The cells are trypsinized with 0.25% trypsin/0.53 mM EDTA solution. Cell viability is determined by staining with Trypan Blue, and cell number is counted with a hemacytometer from Hausser Scientific (Horsham, PA).

Silicon substrates are cleaned by immersing in a 1:1 mixture of methanol and hydrogen chloride for 1.5 h, rinsed three times using deionized water, and transferred into concentrated sulfuric acid for 1.5 h, which generates a thin oxide film on silicon. After washing with water, the substrates are boiled in deionized water for 1 h and placed in an 80 °C oven for 3 h. The cleaned substrates are modified with polyethylene glycol terminated silane (PEG-silane) by incubating with 3 mM of PEG−silane in toluene, which contains 1% triethylamine (v/v), for 2.5 h at 60 °C under nitrogen environment. After removing unbound PEG−silane by sonication in toluene and ethanol, the substrate is then washed with water and dried in a gentle flow of nitrogen. A thin film of polyvinyl alcohol (PVA) is deposited on PEG−silane modified silicon substrate by spinning 2% (w/v) aqueous solution of PVA at 2500 rpm for 20s, followed by natural drying at ambient condition. The PVA covered silicon substrate is patterned with deep ultraviolet laser lithography using 193 nm ArF excimer laser from Lambda Physik (Santa

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Clara, CA). The intensity of laser is homogenized by passing beam through an in-line homogenizer.[21] Micropatterns are written on quartz plates in dark-field polarity, where patterned areas are transparent to laser and remaining areas covered with chromium are opaque. The PVA covered substrates are exposed at an intensity of 200 mM/pulse and a frequency of 10 Hz for 70 s. After exposure, the silicon substrates are backfilled with APTES by incubating with 1% APTES (v/v) in anhydrous toluene solution for 30 min at room temperature, followed by washing with toluene and deionized water three times, respectively. Finally, PVA is removed by sonication in deionized water, and the substrate is dried with a gentle flow of nitrogen.

RESULTS AND DISCUSSION

Laser lithography can remove PVA and underlying PEG from silicon substrate completely. High density patterns with feature size of 7 μm have been made on PVA covered silicon substrate. The PVA covered area and uncovered area have shown large optical contrast due to different light reflection capability of PVA and silicon (image not shown). The patterned areas are modified with APTES to have amine terminated positively charged groups that can attract cells through electrostatic attractions.[22] MG-63 cells are seeded on the patterned substrate at an area density of 100−3000 cells/mm^2 depending on the pattern density. After incubation for 1 h, unattached cells are rinsed away using PBS. In certain cases, the patterned areas are empty, which is due to low probability of cell interaction with these areas and a repelling force arising when the cells get too close with each other. In rare cases, two cells are attracted on the same patterned area, because cells are not well-separated in solution prior to contacting substrates. A key to have one cell on one feature is to avoid aggregates in suspension by carefully pipetting up and down at least 30 times to separate the cells into a single-cell suspension. Figure 2A shows a high density single cell array formed on a patterned silicon substrate. The PEG covered areas can effectively prevent cell attachment, making this method highly ideal for single cell based assays. The variation of cell sizes is due to the fact that cells are in different stages of growth. For instance, cells in differentiation stage tend to have larger volume. In order to confirm the attachment of cells and their integrity, the patterned cells are tested for live/dead assay using Ethd-1 (Live/Dead viability/cytotoxicity kit). Figure 2B is a fluorescent microscope image of cell array after live/dead assay, in which red color and green color are from dead cells and living cells, respectively. From this image, over 99% of patterned cells are still alive even after 1 h, indicating that it is possible to carry out living cell assay on microarray. In order to test the effect of feature size on cell attachment, a series of patterns with different feature sizes (7, 10, 15, and 20 μm) have been generated. Although a large feature can provide high affinity to the cell due to large contact area, it is possible that multiple cells can be attracted on one feature. The feature size of 15 μm is selected which allows good attachment of cells that have diameter between 10 and 20 μm, and the multiple cell attachment is not an issue as well. The spacing between features determines the surface density of cells and the total number of patterned cells at a given area. Although smaller spacing can provide higher density, the spacing used in this work is larger to avoid overlapping of halos from adjacent cells. Figure 2C is an optical image of an array of cells attached on patterns with feature size and spacing of 15 and 100 μm, respectively. Similarly, the cells attached on low

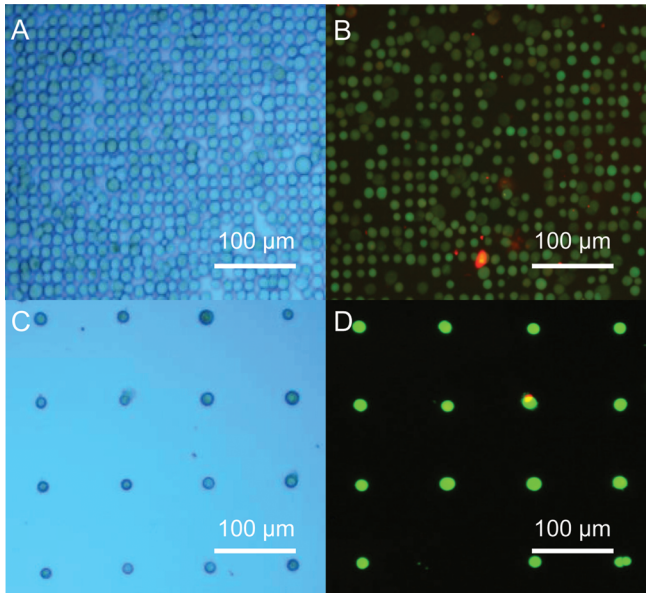

Figure 2. Optical image (A) and fluorescence image (B) of MG-63 cells attached onto a high density pattern that has feature size and spacing of 7 and 15 μm, respectively; an optical image (C) and fluorescence image (D) of MG-63 cells attached onto a low density pattern that has feature size and spacing of 15 and 100 μm, respectively. Both fluorescence images are collected after live/dead analysis, in which green and red colors correspond to live and dead cell, respectively.

density patterns have been tested using live/dead assay as described above. The fluorescence image (Figure 2D) shows that the patterned cells are alive, round, and separated from each other.

In order to perform halo assay, the surface attached cells should be embedded inside porous gels, which will provide an interconnected channel network for DNA molecule to diffuse at rates that depend on molecular weights or lengths of DNA. When molten gel is applied on silicon substrate, the moving liquid−solid interface can produce a large shear force on cells attached on substrate. The key to form an undistorted cell array is that shear force from molten gel during the spreading process is as minimal as possible through several strategies as follows. (1) Low molecular weight agarose gel that has low melting point (65 °C) and low viscosity is used to embed arrayed cells. The low processing temperature ensures that >98% of cells are alive after solidification of molten gel. (2) Since the viscosity of molten gel depends on the concentration of agarose gel, the concentration of gel is kept as low as possible. It turns out that gel concentration as low as 0.5% (w/v) can be used to make gel bed that is good for DNA diffusion and halo assay. (3) The molten gel is applied onto cell array slowly and gently to avoid variation of shear force. (4) The amount of gel deposited first is just sufficient to cover cell array to form an ultrathin film of 50−100 μm thick. After solidification of first layer, more molten gel is used to make the thick gel bed. After trial and error testing, a series of empirical parameters have been derived to make an undistorted array with minimally deformed cells as follows: melting temperature of gel, 65 °C; volume, 100 μL; concentration, 1% (w/v); and surface area, 15 × 15 mm^2. After applying gel on cell array, the substrate is left at room temperature for 30 min to allow the gel to solidify. The cell array embedded in gel cannot be seen directly under an optical microscope due to similar refraction indexes of cell and gel.

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Ethidium bromide (EB) that can be inserted into a base pair of double stranded DNA is used as a contrast agent to determine the quality of gel-embedded array by staining DNA of cells. It is found that cell array is not distorted and individual cells are not deformed (figure not shown).

After embedding cell array inside the thin film of agarose gel, cells are first exposed to X-ray radiation, which can generate free radicals from water radiolysis. The free radicals (hydroxyl radicals) damage DNA by extracting electrons from atoms contained in DNA, which causes DNA to break into segments.[23] The low dose X-ray radiation is generated from a mini-X-ray generator (Amptek, Bedford, MA) operated at 40 kV and 100 μA with silver target. The X-ray dose rate is calibrated with a radiation meter and can be evenly distributed over an area of 6 × 6 cm^2. At a vertical distance of 5 cm, there is a linear relation between X-ray dose and irradiation time. X-ray irradiation for 5 min will provide a 1.25Gy radiation dose on the whole irradiation area. Fast alkaline halo assay is carried out as follows: the sample is immersed in 0.3 M NaOH for 15 min at room temperature to allow dissociation of histones from DNA chain and rinsed twice with deionized water to remove NaOH; the sample is stained with 5 μg/mL ethidium bromide solution for 15 min to allow EB intercalation between DNA basepairs. The sample is incubated in deionized water for 5 min and imaged using a fluorescence microscope. Figure 3A shows

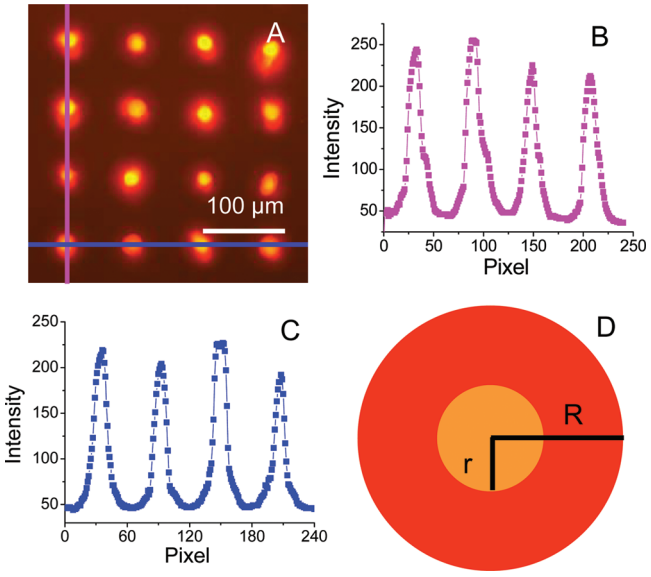

Figure 3. Fluorescence image of an ordered array of HeLa cells that are trapped inside agarose gel after X-ray irradiation for 5 min (A); the fluorescence intensity of several HeLa cells along vertical (B) and horizontal (C) lines in the fluorescence image of (A); a schematic illustration of halo around one cell (D), from which nuclear diffusion factor (NDF) can be derived.

the fluorescence image of an array of HeLa cells after X-ray irradiation for 5 min (1.25Gy dose), where well-defined circles consist of tightly core and diffusive halo. In comparison, DNA from untreated cells is entirely localized within the nucleus and appeared as a bright fluorescent circle. In order to derive the variation of halo from cell to cell, the intensities of fluorescence signals across several cells have been analyzed with image-analysis software (Scion Image). Figure 3B,C shows the fluorescence intensities of Hela cells in Figure 3A along two perpendicular lines. Both graphs show similar levels of fluorescence intensity, meaning the directionally applied agarose gel does not affect results from HaloChip. This HaloChip has also been used on other cells such as MG-63 and LNCaP cells, meaning that electrostatic attraction between patterned features and cells is a generally applicable method.

HaloChip is used to quantify DNA damages in three types of cells (HeLa, MG-63, and LNCaP) caused by ultraviolet light, X-ray radiation, and hydrogen peroxide. The intensities of fluorescence signals are proportional to the amounts of ethidium bromide inserted into DNA and also proportional to the amount of DNA (because DNA is stained randomly with ethidium bromide). The level of DNA damage can be quantified with nuclear diffusion factor (NDF), which can be derived from surface areas of halo and nucleus as follows:

$$\mathrm{NDF} = \frac{R^2 - r^2}{r^2}$$

where R and r are the radiuses of large circle and small circle (Figure 3D), respectively. As X-ray exposure time increases from 1 to 5 min, halos become larger due to more DNA damage. Meanwhile, the fluorescence intensity of the core decreases, suggesting that residual nuclei decrease progressively. The NDF values of 50−75 randomly selected cell halos show a linear relation between the level of DNA strand scission and X-ray exposure time (Figure 4A). A small variation in NDF

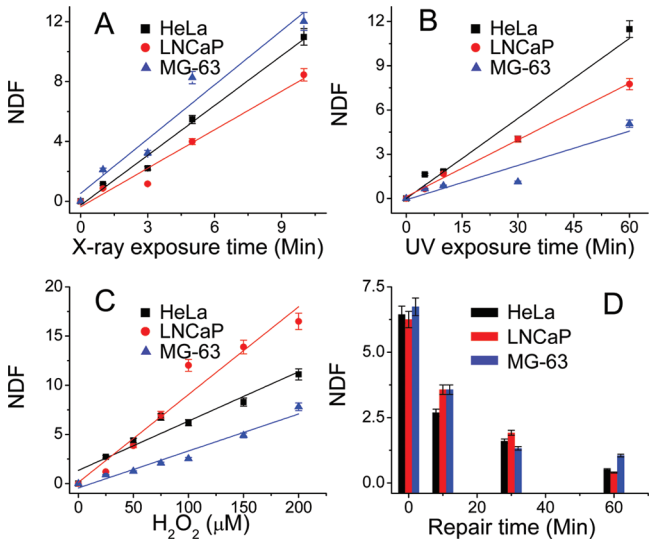

Figure 4. NDF values of three types of cells (A) as functions of X-ray radiation time for 0, 1, 3, 5, and 10 min; (B) as functions of ultraviolet irradiation time for 0, 5, 10, 30, and 60 min; (C) after 10 min treatment with hydrogen peroxide at concentration of 0, 25, 50, 75, 100, 150, and 200 μM; (D) after repairing for 0, 10, 30, and 60 min, where cells have been damaged with X-ray irradiation for 5 min.

(<15%) is observed which can be attributed to the fact that different cells are in different stages of cell cycles. It has been found that, after exposure to low radiations, the most radiosensitive cell stages are mitosis and the G1/S interface.[24] The slope of each line reflects radiosensitivity of cell. The linear relationship has also been found for DNA damages caused by ultraviolet light (364 nm, 100 mJ/m^2) generated using a hand-held UV lamp (Blak-Ray, Upland, CA) and by aqueous hydrogen peroxide solutions at different concentrations (Figure 4B,C). Linear regression has been done for cell damages after exposing to UV, X-ray, and H_2O_2 and the correlation

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coefficient, R^2, for all the regressions is more than 0.92. Although direct impingement of DNA with incoming X-ray can cause DNA damage, DNA damage is mostly induced by free radicals (i.e., hydroxyl radicals) generated from water radiolysis (X-ray radiation) and UV-lysis or decomposition of hydroxyl peroxide. Normally, cells can repair damaged DNA, but if damages are too much to be repaired, they will accumulate and cause breakage of the DNA chain which cannot be repaired. The damaging effect of ultraviolet light is found to be much lower than that of X-ray even if photon flux of ultraviolet light is much higher than that of X-ray photon. This can be explained as the result of high efficiency of water radiolysis with low energy X-ray. LNCaP cells have the highest resistance (smallest slope in NDF−dose curve), while MG-63 cells are the most sensitive to X-ray radiation, meaning that LNCaP cells can repair DNA damage more efficiently than MG-63 cells (providing that X-ray radiation can cause the same amounts of DNA damage to both cells due to a similar level of X-ray absorption). In addition, HeLa cells are the most sensitive to ultraviolet radiation compared to MG-63 cells and LNCaP cells, probably because HeLa cells can absorb ultraviolet light much stronger than the other two cells, thus producing more free radicals for DNA damages. Whatever the mechanism is, these results indicate that this method allows precise quantification of DNA damages by measuring diameters of halos. At last, the capacities of three cells to repair X-ray induced DNA damage have been evaluated. The cells are embedded into gel and irradiated with X-ray for 5 min. Instead of staining their DNAs, the irradiated cells are put back into the incubator to allow DNA repair at 37 °C for different times. Figure 4D shows the results of three types of cells after repairing for 0, 10, 30, and 60 min, where all damages have been repaired in 60 min, and LNCaP shows slightly higher repair capacity than HeLa and MG-63 cells, which is in accordance with results shown in Figure 4A.

CONCLUSIONS

Single cell DNA damage and repair can be detected using HaloChip, in which an ordered array of single cells with defined feature size and spacing is embedded inside an agarose gel. After exposing to DNA damaging agents and staining, the well-defined halo from each cell at arbitrary location and the same height allows DNA damage/repair to be detected without much user intervention or special equipment and simultaneous study of multiple experimental conditions in one run. This would bring critical information about DNA damage and repair to researchers and clinicians in a range of fields. This work is the first that combines the halo assay with the chip idea, so that each cell is positioned at a precise 2D position and at the same height as others, and the symmetric shape of halo allows easy determination of DNA damages. Owing to its ability to detect DNA damages at high sensitivity and high level of simplicity, the HaloChip has the potential to be made compatible with existing high throughput techniques (24 or 94 wells) and become a new standard in DNA damage analysis and will find a variety of biological and clinical applications such as genotoxicity studies, biomonitoring, ecological testing, and in the study of human disease.

AUTHOR INFORMATION

Corresponding Author

*E-mail: liyuanma@mail.ucf.edu.

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