Subnuclear foci quantification using high-throughput 3D image cytometry

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ABSTRACT

Ionising radiation causes various types of DNA damages including double strand breaks (DSBs). DSBs are often recognized by DNA repair protein ATM which forms gamma-H2AX foci at the site of the DSBs that can be visualized using immunohistochemistry. However most of such experiments are of low throughput in terms of imaging and image analysis techniques. Most of the studies still use manual counting or classification. Hence they are limited to counting a low number of foci per cell (5 foci per nucleus) as the quantification process is extremely labour intensive. Therefore we have developed a high throughput instrumentation and computational pipeline specialized for gamma-H2AX foci quantification. A population of cells with highly clustered foci inside nuclei were imaged, in 3D with submicron resolution, using an in-house developed high throughput image cytometer. Imaging speeds as high as 800 cells/second in 3D were achieved by using HiLo wide-field depth resolved imaging and a remote z-scanning technique. Then the number of foci per cell nucleus were quantified using a 3D extended maxima transform based algorithm. Our results suggests that while most of the other 2D imaging and manual quantification studies can count only up to about 5 foci per nucleus our method is capable of counting more than 100. Moreover we show that 3D analysis is significantly superior compared to the 2D techniques.

Keywords: image cytometry, foci counting , gamma-h2ax, image analysis

Various agents, such as ionizing radiation and pollutants, can cause cancer by inducing DNA damage which can lead to sequence rearrangements in cells. Of many types of DNA damages, one of the most genotoxic is the double strand break (DSB). DSBs are recognized by DNA repair proteins, including ATM, which phosphorylates H2AX at serine 129 to form gamma-H2AX. Phosphorylation of H2AX stretches for tens of kb from the breakpoint, leading to a structural change that can be visualized by immunohistochemistry.[1] These foci can be easily detected by secondary antibodies that fluoresce. Thus, fluorescence based detection of the gamma-H2AX marker of DNA damage has been used broadly to study DNA damage and repair, and in particular, to study DSBs.[2] Quantification of the frequency of repair foci can be done using different criteria. The most widely used is by classifying cells as positives and negatives using a threshold for the number of foci inside the cell nucleus. This threshold is usually kept at very low values such as 5 foci per nucleus. However quantifying the number of foci per cell, rather than the number of cells with a focus frequency higher than a specified number, provides a much more accurate representation of the DSB event frequency. It is not feasible for scientists to count the number of foci per cell, since there can be over a dozen foci per cell (often overlapping), and robust experimental designs require upwards of 100 cells per condition. Therefore, what is needed is an automated counting algorithm that can function in 3D. In addition to the challenge of creating the distribution of repair foci among cells, it is also challenging to learn about the effects of DNA damaging exposures under conditions where the level of damage is

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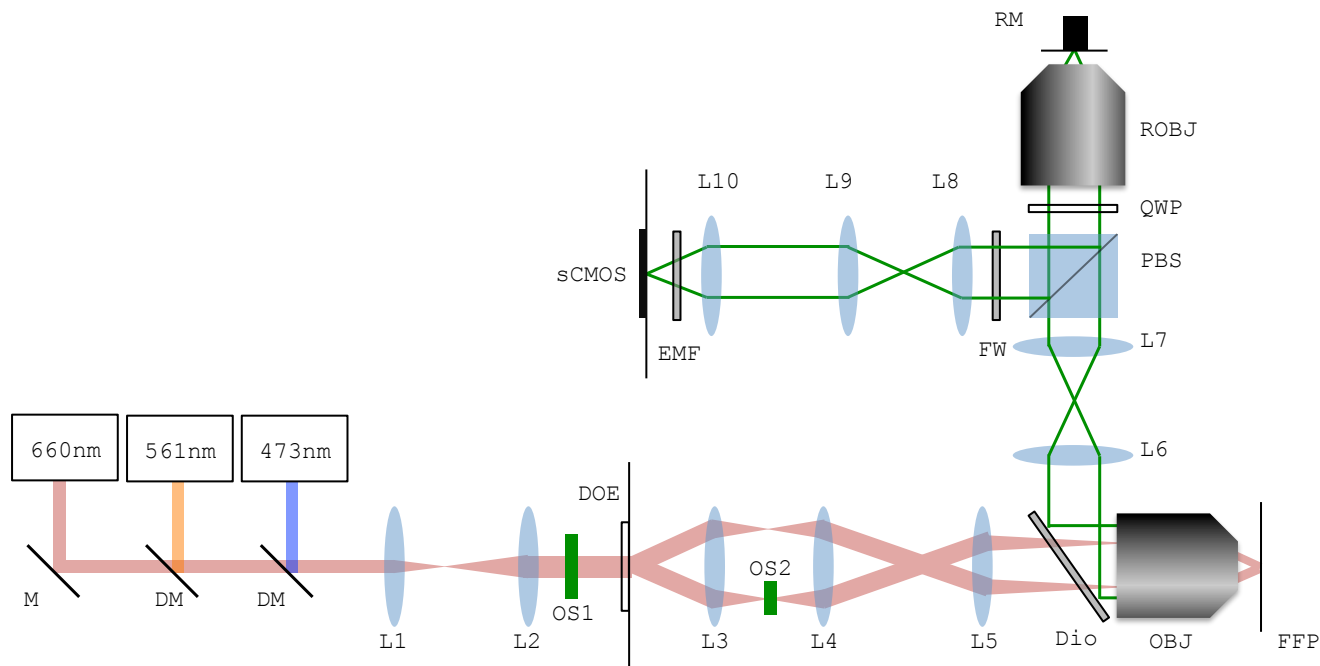Dushan N. Wadduwage: E-mail: dushan@u.nus.edu

Figure 1. Schematic diagram of the high throughput 3D image cytometer. M: mirror, DM: dichroic mirror, DOE: diffractive optical element, OS1: optical shutter for minimising the photobleaching effect, OS2: optical shutter for generating UI and SI, Dio: dichroic mirror before objective, OBJ: imaging objective (Zeiss 20x Water NA1.0), FFP: front focal plane, PBS: polarising beam splitter cube, QWP: quarter-wave plate, ROBJ: remote focusing objective (Nikon 20x Air NA0.75), RM: remote focusing mirror attached to a piezo actuator, FW: filter wheel with three band pass emission filters, EmF: emission filter, sCMOS: Scientific CMOS detector.

very low. Hence, in order to identify cells that harbor an excess of only a few events would require analysis of a large population of cells.[3] Therefore, high throughput imaging and image quantification is critical for quantifying very small differences in the frequency of repair foci.

Most of the currently available technologies are of limited throughput due to several reasons. First, the limitations in imaging speed limits either the number of cells that can be imaged per each experimental condition and there is a need for high resolution imaging. On one hand, there are high throughput flow cytometers that can study tens of thousands of cells per second but they generate only a few bits of information per cell. On the other hand, there are image cytometers that can generate few hundred or thousands of bits of information per cell, but they are limited in speed such that quantification of large numbers of cells per condition is not feasible. Moreover, most of experiments done using image cytometers are performed in two dimensional imaging mode to enhance throughput, but this limits the level of detail that can be analyzed. The second major issue is image processing. Commonly available image processing algorithms employed to mine image data vastly limits either the accuracy or the throughput. Faster algorithms are usually simpler and of linear complexities but sometimes are not accurate enough. One reason for the problem of accuracy is that it is difficult to fine tune algorithm parameters that enable analysis of a broad range of conditions. More complex algorithms, such as Level sets,[4] usually employ iterative segmentation steps and hence are very slow. Taken together, it is clear that we need to integrate fast imaging techniques seamlessly with fast image processing algorithms to enhance the speed of the entire process and eliminate any bottlenecks. To address this need, we have developed an instrumentation and computational pipeline specialized for subnuclear foci quantification for DNA damage in cell cultures.

Firstly, 0.5×10^6 HeLa cells were seeded on to gelatin coated coverslips overnight at 37C. Cells were subsequently incubated with 10uM edU in DMEM+ 10% Fetal Bovine Serum at 37C for 20 min. After replacing the edU solution with fresh media, the cells were irradiated with 10Gy from a Cesium source. The cells were
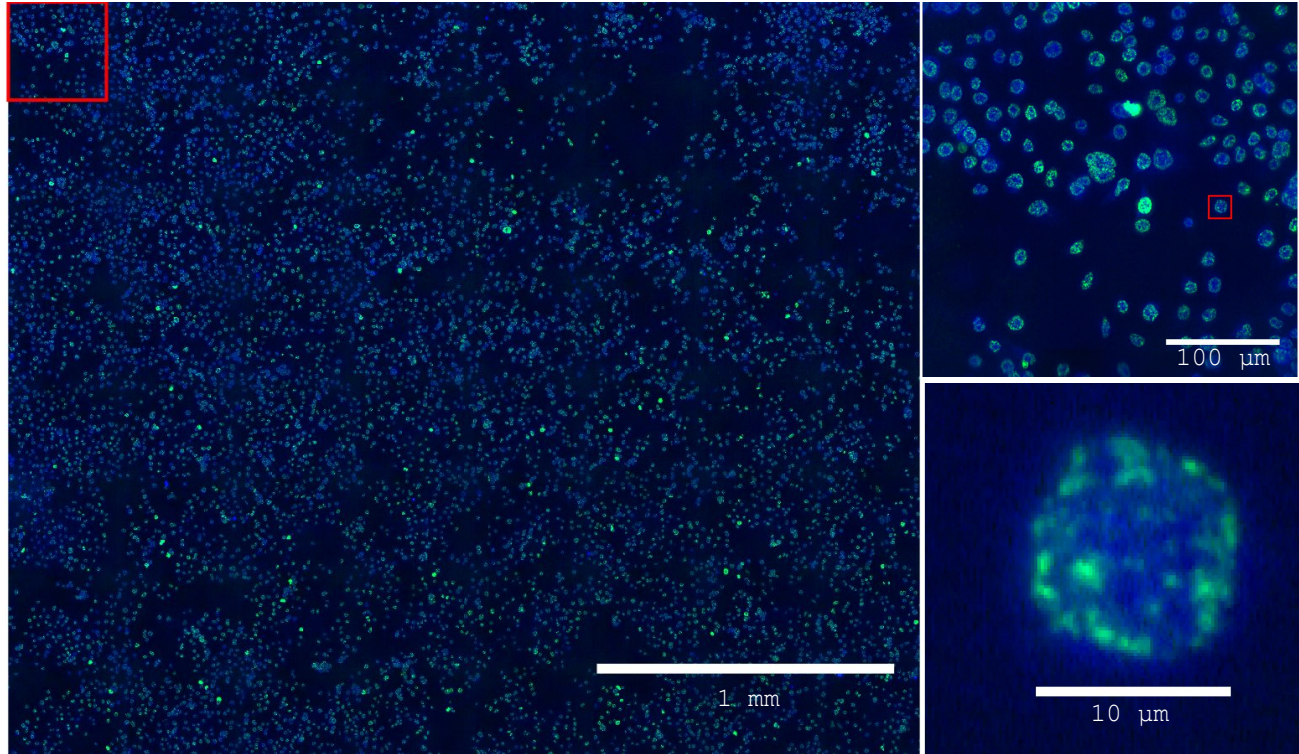
Figure 2. Depth resolved stitched image of a cell population with subnuclear foci at three scales. Blue channel shows nuclei and green shows subnuclear foci.

subsequently fixed with 4% paraformaldehyde at the indicated times. They were then permeabilized with 0.2% Triton in PBS and incubated with edU reaction cocktail according to the manufacturer. Cells were blocked with 1% BSA in TBS and treated with 1:250 Rb anti-gH2AX(Cell Signaling) overnight in blocking buffer at 4C. They were stained with 1:200 Goat anti Rabbit AlexaFluor488 and 50uM ToPro in blocking buffer. Coverslips were mounted with ProLong Gold Antifade onto glass slides.

Secondly, an in-house developed high throughput image cytometer[5] (schematic shown in figure 1) was used to image a large population of cells in 3D at a rate of 800 cells/second. This imaging speed was realized by combining few key technologies together. The structure light based HiLo[6] algorithm was used to realize depth resolved wide-field images and a high speed remote scanning[7] technique was used for depth scanning. A motorised stage was used to scan the sample laterally and the resulting frames were stitched to create a large 3D image moissac. This process was done for two channels one for nuclei and the other for gammaH2AX foci. Then these image data were computationally processed to quantify the number of foci per each nucleus.

In the nuclei channel the cell nuclei were segmented using a thresholding and a watershed based algorithm. Firstly the image was clipped with a predefined intensity cutoff and then was smoothed using a circular averaging filter. Secondly the morphological opening[8] using a circular structural element was applied on the image. These steps help preprocess the image to segment the nuclei. The image was then thresholded to segment the nuclei clusters from the background resulting a binary image. The extended maxima transform[9] was used to identify the intensity peaks, that represents the cell nuclei centers, and the resulting image and the previous binary image was used in watershed algorithm[10] to separate the clusters. The segmentation results are shown in figure 3A. Similarly In the gamma-H2AX channel the image was preprocessed and then the 3D extended maxima transform was applied to identify and segment the foci (Figure 3B). Then the segmentations from the two channels were merged to count the number of foci per nucleus.

Our results suggest that tightly clustered subnuclear foci lies on top of each other and hence the quantification using 2D imaging is considerably poor compared to the 3D imaging (figure 4). For the nucleus shown in figure 4
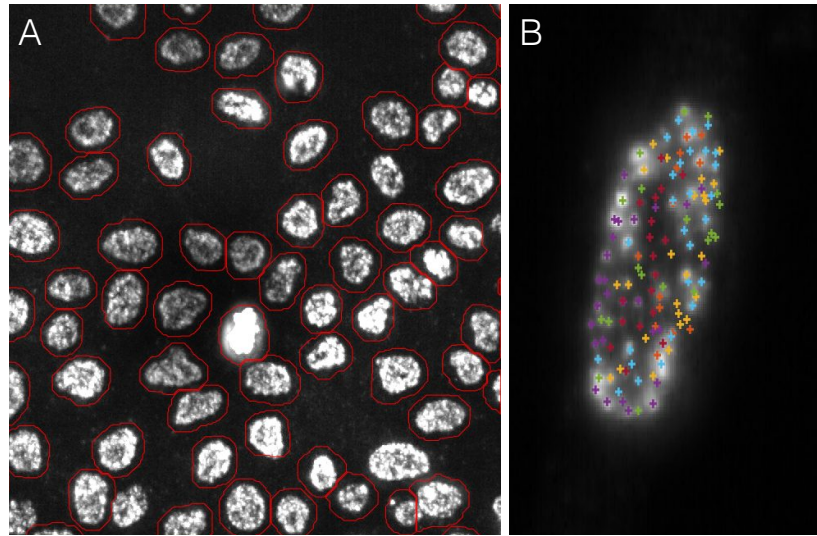
Figure 3. (A) Nuclei segmentation. Segmented boundaries are shown in red. (B) Foci counting in 3D. Different colours represent different z-depths
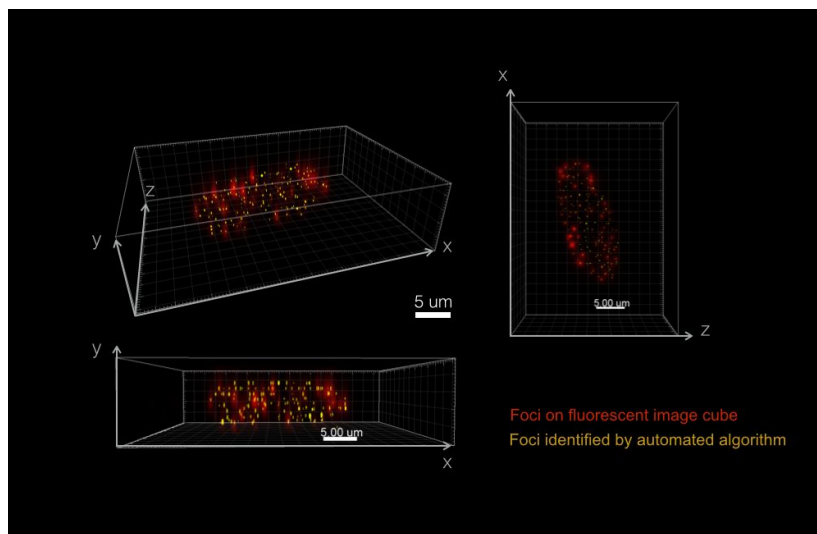

Figure 4. Representative 3D image of foci inside a cell nucleus (shown in red) and their segmentation (shown in yellow).

number of foci counted in middle z-plane (of depth resolved image stack) in 2D was 95 while the number of foci counted in 3D (on whole depth resolved image stack) was 144. Some studies used 3D imaging but used 2D quantification on maximum intensity projection.[11] For the same nucleus we counted 105 foci on the maximum intensity projection. Therefore it is clear by our results that 3D imaging followed by 3D quantification significantly enhances the quantification accuracy. While most of the current studies count only up to about 5 foci per cell nucleus we have shown that we can easily quantify cells with well above 100 subnuclear foci. This new approach will have broad applications, including basic research and studies of cancer chemotherapeutics.

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