The Application of Imaging Flow Cytometry to High-Throughput Biodosimetry

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ABSTRACT

Biodosimetry methods, including the dicentric chromosome assay, the cytokinesis-block micronucleus assay and the γH2AX marker of DNA damage are used to determine the dose of ionizing radiation. These techniques are particularly useful when physical dosimetry is absent or questioned. While these assays can be very sensitive and specific, the standard methods need to be adapted to increase sample throughput in the case of a large-scale radiological/nuclear event. Recent modifications to the microscope-based assays have resulted in some increased throughput, and a number of biodosimetry networks have been, and continue to be, established and strengthened. As the imaging flow cytometer (IFC) is a technology that can automatically image and analyze processed blood samples for markers of radiation damage, the microscope-based biodosimetry techniques can be modified for the IFC for high-throughput biological dosimetry. Furthermore, the analysis templates can be easily shared between networked biodosimetry laboratories for increased capacity and improved standardization. This review describes recent advances in IFC methodology and their application to biodosimetry.

Key words: Chromosome aberration, cytokinesis-block micronucleus assay, dicentric chromosome assay, DNA, imaging flow cytometry, radiation biodosimetry

Introduction

Biodosimetry methods have been used since the 1960s for determining the amount of ionizing radiation to which an individual has been exposed. In the absence of physical dosimetry measurements or calculations, or when the physical dose estimates are in dispute, biodosimetry often provides the only measure of radiation dose. Traditionally, cytogenetic biodosimetry techniques have been used, the two most common being the dicentric chromosome assay (DCA) and the cytokinesis-block micronucleus (CBMN) assay.

The DCA was the first well-developed assay for biodosimetry. It has been standardized for biological dosimetry using manual microscopy and can generate accurate and reproducible dose estimations between 0.1 and 5 Gy.[1,2] Due to low and stable background levels in unirradiated individuals, the assay is considered to be sensitive as well as specific to ionizing radiation. The assay, however, suffers from a few disadvantages, namely that the damage is unstable (decreasing with a half-life of about 3 years[3]), cells must be cultured for 48 h prior to analysis to allow cells to cycle into mitosis where the chromosomes can be visualized, and manual scoring of dicentrics is time-consuming and labor-intensive.

The CBMN assay also measures DNA damage from exposure to ionizing radiation, with the frequency of micronuclei (MN) in binucleated cells (BNCs) being translated into a dose of ionizing radiation.[4,5] Like the DCA, the CBMN assay has also been standardized for biological dosimetry using manual microscopy and can generate accurate and reproducible dose estimations between 0.3 and 5 Gy.[6] However, this assay is less specific to ionizing radiation than the DCA, and background levels of MN are more affected by lifestyle factors such as smoking, age, diet, and gender.[4,5] In addition, lymphocytes containing unstable, radiation-induced MN decrease with a half-life of about 1 year.[7] Furthermore, cells must be cultured for 72 h as compared to 48 h for the DCA. Nevertheless, microscope-based scoring is

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easier and faster than standard DCA scoring and requires less technical expertise.

Recently γH2AX has been identified as a marker for radiation exposure. H2AX is a histone that becomes phosphorylated (γH2AX) at the site of DNA double strand breaks (DSBs), and it has been shown that γH2AX foci correspond one-to-one with DSBs,[8,9] and increase linearly with dose.[10,11] The kinetics of the response are well known, with γH2AX foci appearing within minutes following radiation-induced DNA damage in lymphocyte cells, reaching a maximum between 30 min and 2 h after exposure. As the DSBs repair, the γH2AX foci start to dephosphorylate rapidly in the first 24 h, approaching baseline values within a few days.[10,12] This rapidly changing response makes it difficult to determine the dose received with accuracy. Nevertheless, the presence of γH2AX foci is an indication of exposure to some level of radiation, and since samples can be processed within hours of exposure, the enumeration of these foci can be useful as a rapid indicator of exposure to radiation for up to 3 days.[13]

In recent years, there has been an international focus on emergency planning for mass casualty radiological/nuclear (R/N) events, in which the number of casualties would exceed the capacity for local emergency response, including that for biodosimetry.[14,15] R/N events could be caused by either accidents or malicious acts and could result in hundreds or thousands of individuals being exposed to ionizing radiation. These individuals may require biodosimetry analysis to quantify specific doses of ionizing radiation received which can aid in the guidance of medical treatment decisions. When analyzed by microscopy, cytogenetic biodosimetry is an excellent, accurate tool for providing dose estimates to small numbers of casualties. However, due to the labor-intensive nature of the assays in their traditional microscope-based form, they are ill-suited for mass casualty situations.[15] To address this shortcoming, there has been much effort put towards increasing the sample throughput of these assays for emergency response. Research on triage quality analysis has resulted in a number of improvements including a reduction in the number of scored cells for patient classification,[16,17] faster-scoring methods[18] and semi-automated microscopy for location of metaphase spreads in the DCA.[19] More recently, algorithms have been developed to enable the use of metaphase finders to identify chromosomal damage from ionizing radiation. These systems have been successfully applied to biodosimetry for the DCA,[20-23] CMBN,[21,23-26] and γH2AX.[27,28]

Networking is another critical strategy for increasing throughput for biodosimetry that can be enhanced through standardization, communication, and intercomparison. Once a network is well established, if one laboratory becomes overwhelmed, samples can be sent to other laboratories within the network with confidence that the dose estimates will be consistent and comparable.[29,30] As a complement to networking, sharing of images over the internet to increase scoring capacity is also gaining interest for increasing sample throughput. There have been several publications that describe multi-laboratory scoring of electronic images for the DCA and have demonstrated the utility of this strategy.[31-33]

All of the aforementioned biodosimetry methods still depend on microscope-based analysis, which is limited by the time required for slide preparation, scanning, and manual scoring. In the case of a large-scale R/N event, automation of sample analysis by flow cytometry can mitigate these issues.

There have been several attempts in the past to adapt these assays to traditional flow cytometry. To adapt the DCA for analysis by flow cytometry, sample preparation methods were developed several years ago in which chromosomes and centromeres were fluorescently labeled.[34,35] However, the sensitivity of traditional flow cytometers was not sufficient to reliably detect the difference between mono- and di-centric chromosomes or to distinguish dicentric chromosomes from chromosome aggregates or debris.[34,35] As early as 1984, there have been attempts to adapt the micronucleus assay to traditional flow cytometry.[36-40] While traditional flow cytometry increases the throughput of the micronucleus assay as compared to microscopy-based methods, one major drawback is that cells require lysing before the analysis to release nuclei and MN. This eliminates the ability to restrict analysis to BNCs as required for the CBMN assay to control for proliferation. As well, these methods have been plagued by difficulties in differentiating MN from debris and apoptotic bodies from dead or dying cells. Of the three assays discussed in this paper, the most easily adaptable to traditional flow cytometry is the γH2AX assay. While traditionally, fluorescently labeled γH2AX foci have been scored by microscopy, by flow cytometry, the relative fluorescence intensity of the γH2AX signal is measured instead of the number of foci scored. Although there are some limitations to γH2AX flow cytometry methods, such as reduced sensitivity (small signal changes are harder to distinguish), the relative expression of γH2AX as measured by flow cytometry has been shown to be a useful and reliable indicator of DNA damage[41,42] and measurement of γH2AX by flow cytometry has been successfully applied to biodosimetry.[13,43]

With new technologies, such as the imaging flow cytometer (IFC), it is now possible to address some of the drawbacks encountered with traditional flow cytometry and successfully adapt these assays to automated cytometry methods. This would significantly increase sample throughput to potentially hundreds of samples per day in a single laboratory and thousands across a laboratory network and would improve the applicability of biodosimetry as a casualty triage tool following an R/N emergencies. This paper describes how the recent advances in IFC technology have been applied to biodosimetry.

Imaging Flow Cytometry

The IFC combines the statistical power of traditional flow cytometry with the sensitivity and specificity of microscopy. The IFC is similar to traditional flow cytometers in that individual particles in suspension are introduced into a fluidic system where they are hydrodynamically focused into a core stream in the flow cell. Particles are then interrogated by a brightfield (BF) light-emitting diode light source and at least one laser to create transmitted and scattered light signals along with fluorescent

signals based on how each particle is labeled. The specifics of the fluorescent markers and resultant signals vary and depend on the end-point being measured. Several objective lenses collect the emitted and scattered light and fluorescence from each particle as it flows through the flow cell. With traditional flow cytometry, the outputs are a measure of fluorescent intensity and scatter signals which can be used to generate histograms or bivariate plots of the data that require further interpretation. With the IFC, the BF, transmitted, scatter, and fluorescent signals are collected by a high numerical aperture lens (20×, 40× or 60×) and decomposed into specific ranges according to their wavelengths. These wavelength ranges are then focused onto different channels of a charge-coupled device camera within the spectral range of 430–800 nm. These channels capture the sub-images, which can be viewed as individual channels or combined to observe the colocation of signals [Figure 1]. An additional function of the IFC is the extended depth of field (EDF) option, which allows light from different focal planes to be imaged on the detector plane simultaneously. The EDF option allows all structures and probes, such as chromosomes or γH2AX foci, to be focused into a single two-dimensional image that increases the ability to count spots at different focus depths within the cell. Thus, the IFC provides an image, with numerical representations of image-based features, for every particle that passes through the flow cell. The enormous benefit of the IFC over traditional flow cytometry is the ability to then use imaging analysis algorithms on high sample throughput datasets.[44] Furthermore, once established, the analysis templates can be easily shared between similar systems and therefore between laboratories for standardization within biodosimetry networks.

The imaging capabilities of the IFC provide a powerful tool which could allow for significant increases in sample throughput of existing techniques in biodosimetry that are typically analyzed by microscopy. Instead of transferring samples from suspension to slides for analysis, which can be very time-consuming, samples can be processed directly from suspension on the IFC. Particles are collected at a rate of up to 5000 events per second and saved to data files that can be analyzed at any time post-acquisition. In addition, the IFC can be enhanced with an auto-sampler that allows for unattended sample loading from 96-well plate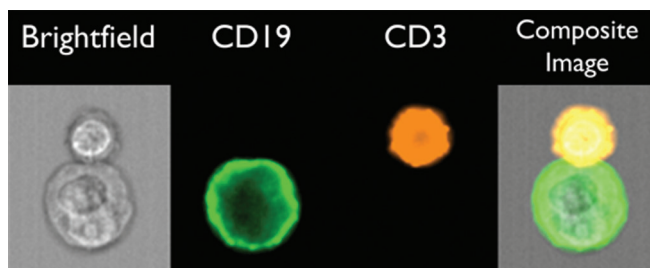s. This feature will potentially enable increased automated analysis of a large number of samples for high-throughput biodosimetry.

Applications of Imaging Flow Cytometry

Cytokinesis-block micronucleus assay

Recently, the CBMN assay has been adapted as an IFC-based method.[45-49] This significant advancement over the traditional slide-based method involves standard culture of whole blood for 72 h, lysing of red blood cells, and labeling the DNA with an intercalating DNA stain (DRAQ5). Samples can then be immediately analyzed, in suspension, on the IFC or stored for a few weeks at 4°C before analysis. This IFC method of analysis has been demonstrated to automatically image, identify, and enumerate BNCs and MN using a spot counting algorithm in IDEAS, the data analysis software that accompanies the IFC. This has resulted in a rapid, robust method that is potentially more applicable for high-throughput biodosimetry than traditional methods [Figure 2]. Recent optimization of the customized data analysis template has resulted in a dose response calibration curve that is similar in magnitude to others published in the literature using automated or semi-automated methods for scoring the CBMN assay. This optimization allows for the generation of dose estimations up to 4 Gy with an accuracy of ± 0.5 Gy,[49] similar to manual CBMN scoring in triage mode (200 cells/sample).[17] Recently, it has been demonstrated that the IFC-based method offers a significant improvement over traditional methods as dose estimates with ± 0.5 Gy accuracy can be obtained using reduced initial blood culture volumes and only 48 h of culture time [Figure 3].[45]

Dicentric chromosome assay

With respect to the DCA, imaging of individual chromosomes in suspension is challenging due to their small size when compared to larger intact cells. Distinguishing chromosomes from cellular debris using traditional flow cytometry is difficult and moreover, identifying and differentiating between mono- and di-centric chromosomes as required for the DCA is even more complex. With the imaging
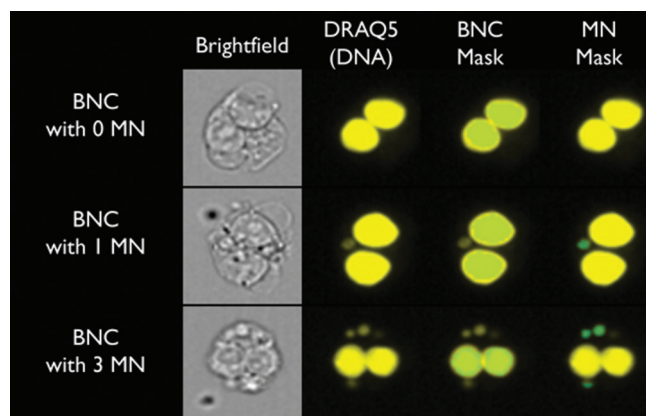

Figure 1: An example of a composite image collected on an ImageStream^X Mark II (Amnis, part of MilliporeSigma) at 60x magnification of a Raji (antigen-presenting cell) and human T cell interaction showing brightfield, CD19-Alexa Fluor 488 (green), CD3-phycoerythrin-Texas red (orange), and the merge of all three channels (modified with permission from S. Friend, Amnis, part of MilliporeSigma)

Figure 2: Representative images of binucleated cells collected on an ImageStream^X (Amnis, part of MilliporeSigma) at 40x magnification showing brightfield, DRAQ5 (DNA stain), the binucleated cell mask and the micronucleius mask for binucleated cells identified with 0, 1, and 3 micronuclei
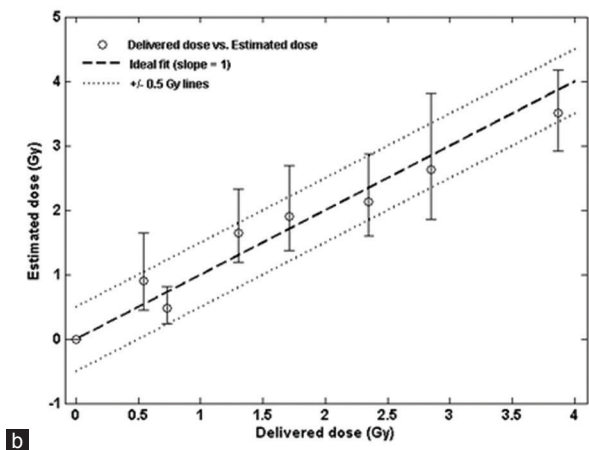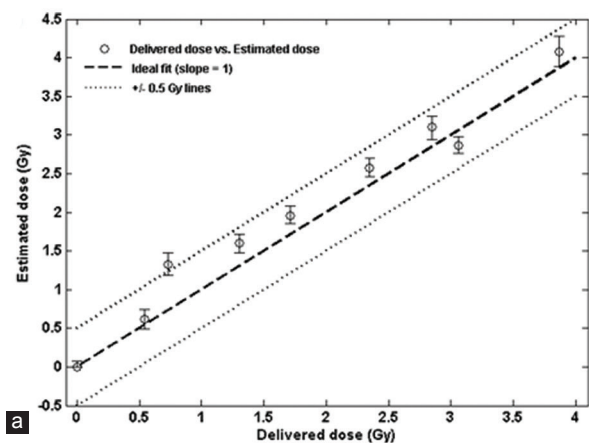
Figure 4: Representative images of individual chromosomes collected on an ImageStream^X (Amnis, a part of MilliporeSigma) at 60x magnification with extended depth of field showing brightfield, FAM (5-Carboxyfluorescein) centromere probe (green), DRAQ5 DNA stain (red), and a composite image for a monocentric and a dicentric chromosome

Figure 3: Estimated dose (Gy) versus delivered dose (Gy) determined by the imaging flow cytometry CBMN assay method for (a) nine blinded samples after 72 h of culture and (b) eight blinded samples after 48 h of culture. The dashed line represents a slope of one, and the dotted lines represent ± 0.5 Gy from the ideal slope. The error bars represent the standard error on the estimated dose

capabilities of the IFC, it is now possible to identify and enumerate individual chromosomes and to distinguish between mono-, di-, and multicentric chromosomes as demonstrated by Beaton-Green and Wilkins[50,51] [Figure 4]. In this method, chromosomes are released from the cell and labeled with a centromere probe (fluorescently labeled pan-centromere peptide nucleic acid) and stained for DNA content with an intercalating DNA stain (such as DRAQ5) allowing individual chromosomes to be identified. In addition, with the use of the EDF option, it is possible to bring all small objects in the field of view, such as chromosomes, into focus.

Once the chromosome population has been identified, the spot counting algorithm can be applied to the centromere signal. The spot count feature allows the number of centromeres on each chromosome to be counted automatically and the frequency of dicentric chromosomes to be quantified. From this, calibration curves can be generated, and dose estimates can be made for biodosimetry as with traditional microscope-based DCA.

γH2AX Assay

A method for performing the γH2AX assay using IFC has been developed, and it has been demonstrated that nuclear
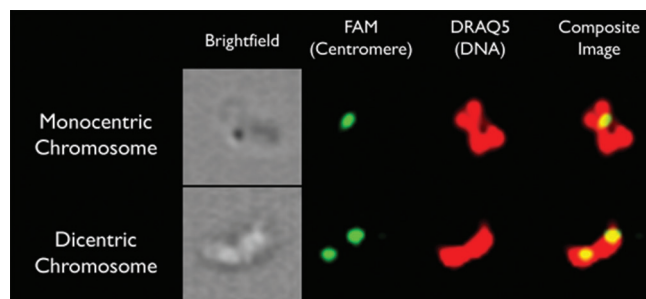
fluorescence increases with dose up to 8 Gy.[52] Bourton *et al.* compared the number of foci measured by IFC using PE to stain γH2AX foci and DRAQ5 to stain DNA content [Figure 5] with the number of foci measured using microscopy. Their results indicated that a lower number of foci were measured by the IFC, but the relative difference between the two methods was maintained. This is similar to results from the CBMN assay performed using the IFC, where the frequency of MN measured was lower than what is typically measured by microscopy.[49] For biodosimetry purposes, when calibration curves are generated for each method, these differences cease to be an issue.

Other methods with potential to be adapted to the imaging flow cytometer

In addition to the three methods discussed above, there are other methods used in biodosimetry that have the potential to be adapted to the IFC. Once the method for chromosome analysis is well established, this method could be expanded to include whole color fluorescence *in situ* hybridization probes for stable translocation analysis, which is the method of choice for retrospective dose assessment.[53] Alternatively, centromere and telomere probes could be used in conjunction with premature chromosome condensation for reduced culture time to analysis and increasing the applicable dose range[54] Furthermore, centromere probes could be added to the CBMN assay to identify MN without centromeres which have been shown to be more specific for radiation damage.[55,56]

Discussion and Conclusion

There has been much effort recently on the development of assays for high-throughput biodosimetry for application following a mass casualty R/N event. Although the treatment of casualties is mostly dependent on emergent symptoms rather than dose, biodosimetry provides additional information that can modify treatment as more accurate doses become available. The ideal method would be a field deployable point-of-care (POC) method that would provide accurate dose estimates for thousands of individuals within hours of the exposure. There are several emerging technologies including gene expression, protein, and metabolomics markers that have potential to address this gap, however, they are currently less accurate than traditional

Figure 5: A representative image of a cell collected on an ImageStream^X (Amnis, part of MilliporeSigma) showing brightfield, phycoerythrin-labeled γH2AX foci, DRAQ5 nuclear stain, γH2AX spot mask, and the nuclear morphology mask (modified with permission from C. Parris, Brunel University)

cytogenetic techniques.[57] In the absence of established rapid POC assays, fully automated cytogenetic and DNA damage assays would allow samples to be processed 24 h a day with little operator intervention reducing the delay between exposure and dose determination.

Current methods, using microscopy-based analysis, are limited by the requirement to generate slides before analysis can commence. The use of the IFC removes this step and allows samples in suspension to be analyzed on the IFC immediately after processing, saving several hours. With the adaptation and miniaturization of these assays to a 96-well plate format, IFC analysis could be conducted on multiple samples without the need for an operator to manually change samples. By reducing the amount of operator intervention, the issues of scorer bias, fatigue as well as inter-scorer variability would be largely mitigated. With the continued advancement of technology, portable IFCs will be field deployable,[58] allowing the γH2AX assay to become a POC method. In addition, the development of automated sample processing systems such as the rapid automated biodosimetry tool[59] will allow the entire assay to be performed automatically. Once all the sample data have been collected, it can be batch processed with pre-established templates for that assay. Since the analysis templates can be shared between networks with similar instrumentation, standardization across laboratories becomes more repeatable, and less dependent on the individual scorers. As well, since all of the particles are imaged and saved, any verification of the samples or reanalysis of the files is easily performed.

As is typical with automated and semi-automated methods, the amount of scored damage is less than that of manual scoring.[20,25,26,46,60] This is largely the result of a reduction in the number of allowed false positives in the automated algorithms. As long as the calibration curves for each assay are carefully prepared with the same technique as the samples, the automated analysis algorithms are capable of generating accurate dose estimates.[20,26] In addition, the imaging algorithms allow for an enormous increase in the number of measured parameters for each particle (based on features such as shape, signal, morphology, and numerous combinations thereof) which have the potential to be used for multi-parametric analysis of each assay.

The adaptation of these techniques to IFC will result in a toolbox of high-throughput methods which can be easily shared within a network and can be used together to facilitate human monitoring management during a mass casualty R/N emergency. The γH2AX IFC assay will be able to rapidly identify those who have been sampled within a day of the suspected exposure. This rapid triage capability will reduce the number of individuals who will require more accurate dose estimates with the CBMN or DCA IFC methods. Subsequently, these high-throughput CBMN and DCA methods will be able to provide rapid dose estimates to the medical community with enough accuracy to assist in planning the medical management of these individuals. Moreover, rapid identification using γH2AX IFC of those who have not been exposed will provide these individuals with assurance that will help reduce their stress during such an event. As emerging technologies are developed and validated, they could be used in combination with the γH2AX assay to improve the accuracy of dose estimates in the field. With the increased sampling capacity for cytogenetic techniques, additional biodosimetry could be performed at a later time, with more accuracy, to further assure any concerned individuals.

Conflicts of interest
There are no conflicts of interest.

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