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Foundations of identifying individual chromosomes by imaging flow cytometry with applications in radiation biodosimetry

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ABSTRACT

Biodosimetry is an important tool for triage in the case of large-scale radiological or nuclear emergencies, but traditional microscope-based methods can be tedious and prone to scorer fatigue. While the dicentric chromosome assay (DCA) has been adapted for use in triage situations, it is still time-consuming to create and score slides. Recent adaptations of traditional biodosimetry assays to imaging flow cytometry (IFC) methods have dramatically increased throughput. Additionally, recent improvements in image analysis algorithms in the IFC software have resulted in improved specificity for spot counting of small events. In the IFC method for the dicentric chromosome analysis (FDCA), lymphocytes isolated from whole blood samples are cultured with PHA and Colcemid. After incubation, lymphocytes are treated with a hypotonic solution and chromosomes are isolated in suspension, labelled with a centromere marker and stained for DNA content with DRAQ5. Stained individual chromosomes are analyzed on the ImageStream®X (EMD-Millipore, Billerica, MA) and mono- and dicentric chromosome populations are identified and enumerated using advanced image processing techniques. Both the preparation of the isolated chromosome suspensions as well as the image analysis methods were fine-tuned in order to optimize the FDCA. In this paper we describe the method to identify and score centromeres in individual chromosomes by IFC and show that the FDCA method may further improve throughput for triage biodosimetry in the case of large-scale radiological or nuclear emergencies.

1. Introduction

Radiation biodosimetry is a method for generating dose estimates following whole or partial body irradiation and can be used as confirmation of dose estimates obtained through physical dosimetry calculations or as a substitute when these calculations cannot be performed. Cytogenetic biodosimetry techniques include Fluorescence *in situ* Hybridization (FISH), Premature Chromosome Condensation (PCC), Cytokinesis-Block Micronucleus Assay (CBMN) and the accepted gold-standard dicentric chromosome assay (DCA). These biodosimetry methods convert a measure of DNA damage into a dose estimate of ionizing radiation exposure and are traditionally performed through manual microscopy [1]. While these methods are able to provide accurate dose estimates, the sample preparation techniques as well as slide creation and scoring can be time-consuming and tedious. In the case of suspected radiation exposure to one or a few individuals, these methods can be performed without difficulty. However, following a large-scale radiological or nuclear event in which suspected or actual exposure to hundreds or thousands of individuals is possible, triage versions of these biodosimetry assays are necessary to increase sample throughput and therefore, the rapidity of dose estimates. For example, the QuickScan slide scoring method is a modified version of the DCA in which the scoring criteria are relaxed. QuickScan can also be combied with triage scoring, where the required number of metaphase spreads scored to generate a dose estimate is reduced from 500–1000 to 50. This could result in a reduction in the required scoring time by a factor of about sixty while retaining a level of accuracy similar to the traditional method [2,3]. Further advances in semi-automated versions of the DCA involve the use of microscope-based imaging software [4,5]. These programs still require a slide-making process to acquire images of metaphase spreads, which can be time-consuming and labour intensive and in some cases, user-

Abbreviations: CIB, chromosome isolation buffer; DCA, dicentric chromosome assay; EDF, extended depth of field; FDCA, flow dicentric chromosome assay; FISH, Fluorescence *in situ* Hybridization; IFC, imaging flow cytometry; PNA, peptide nucleic acid.

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intervention is still required for analysis. For example, with the DCScore technique, candidate dicentrics are visually confirmed by a trained cytogeneticist [6].

Imaging flow cytometry (IFC) is a rapidly developing technique that combines the imaging analysis capabilities of microscopy with the increased throughput and statistical power of traditional flow cytometry [7]. With a potential rate of flow of 5000 events per second, the imaging flow cytometer has powerful implications to increase the throughput of traditional radiation biodosimetry techniques. In one of the earliest publications using this method, Basiji et al. demonstrated that FISH probes could be imaged and quantified in human peripheral blood lymphocytes using the ImageStream$^{®X}$ (IS^{X}) imaging flow cytometer [7]. Recently, Rodrigues et al. adapted the CBMN assay to an IFC method and generated dose estimates of blinded samples to within 0.5 Gy in the range of 0–4 Gy with a substantial reduction in processing time when compared to the traditional CBMN assay [8]. With respect to the DCA, individual chromosomes have been analyzed using traditional flow cytometry typically for the purposes of sorting [9–13] however, the identification and distinction of monocentric chromosomes from dicentric chromosomes has been challenging. Due to the small size of individual chromosomes when compared to larger intact cells, distinguishing them from cellular debris with traditional flow cytometry is difficult. Furthermore, identifying and differentiating between mono- and dicentric chromosomes as required for the DCA is even more complex. However, with the imaging capabilities of the imaging flow cytometer, it is now possible to identify and enumerate individual chromosomes and to distinguish between mono-, di- and multi-centric chromosomes. In order to improve the sensitivity and specificity of early proof-of-concept methods [14,15], it was determined that the chromosome preparation method had to be further optimized in order to generate higher numbers of scorable chromosomes. The focus of this paper is the overall improvements to the method, resulting in better and more stable chromosome suspensions which allowed for more optimized analysis. These improved methods have also enabled improved imaging of the chromosomes. In this paper, we expand on previous work by Beaton-Green et al. [14,15] to provide the foundations for adapting the DCA to an IFC technique on the IS^{X}.

2. Material and methods

Early proof of concept work for identifying and enumerating mono- and dicentric chromosomes has been previously described by Beaton-Green et al. [14,15]. These methods have been improved upon, with recent modifications described below.

2.1. Blood sample collection, irradiation and culture

Whole blood samples were collected from healthy donors between the ages of 20 and 60, with no history of ionizing radiation exposure in the previous 12 months. Donors signed informed consent forms approved by Health Canada's Research and Ethics Board. Samples were collected in 4, 6 or 10 mL lithium-heparin Vacutainer® tubes (BD Biosciences, Mississauga, ON) along with a 4 mL ethylenediaminetetraacetic acid (EDTA) Vacutainer® tube (BD Biosciences) for cell counts.

Irradiations (up to 4 Gy) were performed on the whole blood directly in Vacutainer® tubes at room temperature in a cabinet X-ray machine (XRAD 320, PXi, North Branford, CT) at 250 kVp and 12.5 mA with a 2 mm Al filter, for a dose rate of 1.7 Gy/min. All doses were calibrated using a PTW TW30010-10 ion chamber and a T10002 electrometer (PTW, Freiburg, Germany) with N_K = 48.3 mGy nC^{-1} at 250 kV, assuming air kerma to be equal to dose. Post irradiation, samples were kept at 37°C on a rocker for 2 h at the lowest speed to allow for DNA repair. Lymphocyte counts were acquired on the EDTA tubes using the A$^{c.}$ T™ 5diff CP hematology analyzer (Beckman Coulter, Mississauga, ON).

Lymphocyte extractions were performed, under stringent sterile conditions, on whole blood using the Histopaque®-1077 and 12 mL Accuspin™ tube (Sigma® Life Science, Oakville, ON) procedure [15] with PBS pH 7.4 (Teknova, Hollister, CA, cat. P0261), seeded in T25 flasks at approximately 1×10^6 lymphocytes per mL in 10 mL complete media and incubated for 48 h at 37°C, 5% CO_2. Complete media consisted of 1X RPMI 1640 medium (Gibco by Life Technologies™, Burlington, ON, Canada) containing 2 mM L-Glutamine-100U Penicillin-0.1 mg Streptomycin solution (Sigma® Life Science), 15% v/v heat inactivated fetal bovine serum (FBS) (Sigma® Life Science cat. F1051), 1.8% phytohaemagluttinin (PHA) M form (Gibco). Colcemid (Gibco) was added to the cultures at 1% (10 μg/mL) v/v of culture volume for the last 4 h, a notable modification from prior work.

2.2. Isolation of chromosomes in suspension: polyamine-based method

To minimize DNA breakage and degradation, all of the following procedures were performed using sterile, ATP/DNA/DNase/RNase free and polymerase chain reaction (PCR) inhibitor-free tested pipette tips and microfuge tubes.

Following the 48 h culture incubation, samples were transferred to sterile 15 mL centrifuge tubes, pelleted at 350×*g* and the supernatant was removed. Each sample had approximately 15,000–20,000 mitotic lymphocytes assuming 80% efficiency with Histopaque®-1077 and 5% mitotic cells. Based on these numbers, 5 mL of hypotonic solution (55 mM KCl (BioUltra, Sigma® Life Science, cat. 60128), 20 mM HEPES (Sigma® Life Science, cat. H0887)), was added to each sample and incubated at room temperature for 20 min to swell the metaphase cells. The samples were spun down at 350×*g* for 5 min, the hypotonic solution was removed, and 1 mL of ice cold chromosome isolation buffer (CIB) was added to each sample. The CIB was made with the following steps [9]:

1. Start with 10% v/v each of 20 mM EDTA-Na_2 (Sigma® Life Science, cat. E5134) in double-distilled water (ddH_2O), 5 mM ethylene glycol bis-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma-Aldrich, cat. E-4378) in ddH_2O and 150 mM Tris (hydroxymethyl aminomethane)-HCl (Trizma®, Bioperformance, Sigma® Life Science, cat. T5941), 800 mM KCl (BioUltra, Sigma® Life Science) and 200 mM NaCl (Fisher) in ddH_2O.
2. Adjust pH to 7.2.
3. Add 0.1% v/v each of 2-mercaptoethanol (BioUltra Sigma® Life Science, cat. 63689) and Triton™ X-100 (Bio Ultra, Sigma® Life Science, cat. 93443).
4. Cover and stir for 15 min.
5. Filter sterilization with a 0.2 μm mesh (Thermo Scientific™ Nalgene™ Rapid Flow™ 50 mL disposable filter units with PES membrane, cat. 564-0020).
6. Add 0.05% each of 0.4 M spermine (BioUltra, Sigma® Life Science, cat. S3256) in ddH_2O (0.2 mM final) and 1.0 M spermidine (BioUltra, Sigma® Life Science, cat. 85558) in ddH_2O (0.5 mM final).
7. Keep on ice.

As the CIB was added to the samples, they were transferred immediately to ATP/DNA/DNase/RNase free and (PCR) inhibitor-free, 1.5 mL microfuge tubes, incubated on ice for a minimum of 15 min and then vortexed vigorously for 75 s to liberate the chromosomes from swollen mitotic cells. A small sample was removed and stained with propidium iodide (PI) and checked under the