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Report

Cytogenetic Biodosimetry in Radiation Emergency Medicine: 5. The Dicentric Chromosome and its Role in Biodosimetry

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In the case of nuclear or radiological emergencies, biodosimetry has been used to estimate radiation dose to exposed persons and provide information to physicians for clinical treatment and counselling of possible future stochastic consequences. There are currently several biological endpoints and techniques available for assessing partial or whole-body radiological exposure in peripheral blood lymphocytes. However, the use of dicentric chromosomes (Dic) in biodosimetry is still recognized as the main dose-assessment method for estimating exposure to ionizing radiation and has become a routine component of radiation protection. Dics are specific to radiation exposure and the background level is low in non-exposed individuals, making them advantageous in biodosimetry. Here, we provide a review of Dics and its role in biodosimetry as research efforts on assay optimization and high-throughput have been published since the mid-1960s. Additionally, we provide recommended technical information (e.g., colcemid addition, scoring, generating dose-response curves) needed to implement the dicentric chromosome assay (DCA) in laboratories and to allow comparable dose assessment following exposure to acute ionizing radiation. While DCA has been optimized for nuclear or radiological emergencies, increased uncertainty in dose estimation can be caused by the scoring of Dic and variation of calibration curves. Total dose, dose-rate, radiation quality, and sampling time after exposure are some of the factors that influence the results of DCA. Future consideration is also needed as no single assay is sufficient for all radiation scenarios.

Key words: dicentric, radiation, biodosimetry, assay, DNA damage

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1. Introduction

Biological dosimetry (henceforth biodosimetry) and chromosome rearrangements in human peripheral blood lymphocytes are widely used to quantify exposure to a variety of mutagenic agents, including ionizing radiation[1–4]. Cytogenetic methods, such as the dicentric chromosome assay (DCA), have seen great improvements because of the development of an initiator of mitosis in cultures of normal human leukocytes in the 1960s[5], which made metaphase chromosomes accessible for research. While the current techniques of radiation biomarkers for dose assessment are constantly improving for practical and high-throughput use in biodosimetry, the analysis of dicentric chromosomes (Dic) has become a routine procedure in radiation protection as it has the sensitivity to detect previous exposures to ionizing radiation[6]. DCA in biodosimetry has also been used in both accidental and occupational overexposures where physical dosimeter measurements were not available[2, 3, 7–10]. In this paper, we exclusively discuss the DCA as a valuable dose assessment method, especially in acute exposures, as it is paramount to understand the molecular mechanisms of Dic and its role as a radiation biomarker in biodosimetry.

1.1. Radiation-induced chromosomal rearrangements

Ionizing radiation consists of both subatomic particles and high-energy electromagnetic waves with sufficient energy to detach electrons from atoms or molecules. Examples of these subatomic particles include alpha particles, beta particles, and neutrons. Examples of high-energy electromagnetic waves include X-rays and gamma rays. Due to its high energy, ionizing radiation exposure causes cellular damage. Tissue reactions, such as radiation burns and radiation sickness, are caused by high acute doses. Lower doses over a protracted period may cause stochastic effects such as cancer. Radiation can also cause direct damage to DNA or indirect damage by the production of hydroxyl radicals, superoxides or other chemical species from ionized water molecules. If the DNA is damaged, the cell will attempt to correct the damage through DNA repair. However, if the DNA is misrepaired, chromosomal rearrangements could occur. Particularly, in a dividing cell, the misrepair of radiation-induced DNA damage can cause stable chromosome aberrations (e.g., translocations) that are inherited by daughter cells or unstable chromosome aberrations (e.g., Dic) that will eventually lead to apoptosis[11]. In this series, we will discuss the unstable Dic aberration in detail. For more information about other chromosome aberrations or mechanisms of radiation-induced DNA strand breaks, please refer to Series 3 and 4 in the special issue[11, 12].

As ionizing radiation is not detectable by human senses, instruments such as personal or pocket dosimeters (i.e., thermoluminescent dosimeter) must be used to detect and measure radiation exposure. However, dosimeters may not be readily available or exposed persons may not have been wearing dosimeters at the time of radiation exposure. In this case or in other unplanned radiation exposures, chromosomal rearrangements can be quantified in different assays to estimate the absorbed dose and provide information for medical treatment. Radiation dose can be estimated because in both acute and chronic exposures, chromosomal rearrangements are formed when radiation-induced single or DNA double strand breaks fail to repair (Fig. 1). These chromosomal rearrangements, or stable and unstable chromosome aberrations, accumulate at different frequencies per cell depending on the radiation dose and quality. As aforementioned, the most widely used unstable aberration is the Dic and it is currently regarded as one of the most validated radiation biomarker[1].

2. Dic aberrations

As previously mentioned, radiation can cause single or DNA double strand breaks. In response, the cell will undergo DNA repair to identify and correct the damage. However, mistakes can happen during DNA repair where a misrepair of two broken chromosome ends might form a Dic[11, 13], which is an unstable chromosomal rearrangement with two centromeres on the same chromosome (Fig. 1 and 2). The type of Dic, or the pattern, will depend on the two chromosomes involved in the chromosomal rearrangement (Fig. 2), i.e., the pattern will change if the chromosomes are metacentric, submetacentric, or acrocentric. An acentric fragment, or a chromosome with no centromere, will also be among the products produced by the misrepair. During mitosis, the unstable Dic forms an anaphase bridge, causing chromosome breakage and apoptosis. Fragments are unable to migrate to either cell pole and will disappear during anaphase.

In general, background Dic frequency is about 1 in 1000 metaphase cells[14, 15]. At least 80% of this background Dic level in the human population can be attributed to environmental and medical radiation[6]. Therefore, Dic is a biomarker which is highly specific for ionizing radiation and can be studied to assess exposed subjects. Both acute and chronic radiation exposures increase the probability of Dic formation, and higher radiation doses produce a higher Dic frequency. Absorbed dose to irradiated subjects can be estimated by the frequency of Dic in peripheral blood lymphocytes.

If chromosomal rearrangements, such as Dics, are present during cell division, cell-cycle arrest at various cell-cycle checkpoints will occur until DNA damage is repaired. If damaged chromosomes bypass these

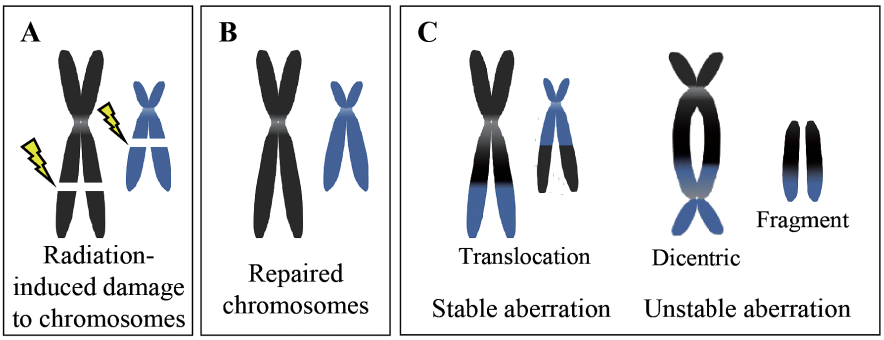

Fig. 1. (A) Ionizing radiation can cause breaks in DNA which may (B) successfully repair or (C) fail to repair, inducing stable and unstable chromosomal aberrations. Misrepair can cause stable chromosome aberrations (e.g., translocations) that are inherited by daughter cells and unstable chromosome aberrations (e.g., dicentric) that result in apoptosis.

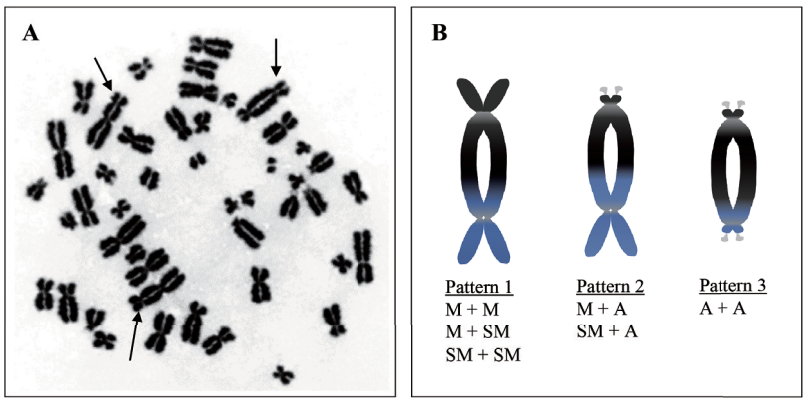

Fig. 2. (A) Dic in human cell and (B) different Dic patterns in humans. M = metacentric chromosome; SM = submetacentric chromosome; A = acrocentric chromosome.

checkpoints, the genetic information can be transmitted to daughter cells after cell division. However, Dics are unlikely to be inherited by daughter cells because cells with Dics are highly likely to undergo apoptosis because of their inability to progress to anaphase and telophase. Thus, Dic is also known as an unstable aberration and is typically only scored for subjects irradiated by an acute and recent radiation exposure.

While spontaneous Dic can be induced at low frequencies (about 1 in 1000 cells), acute radiation will cause a greater Dic frequency as doses increase. However, the number of Dic is expected to decline after the single radiation event if no other radiation events occur as cells with Dic and other unstable aberrations are not able to proliferate and die[16]. The ability to observe lymphocytes with Dic is generally limited to the lifespan of circulating G_0 lymphocytes as human peripheral blood is used for DCA in biodosimetry. Generally, the stimulated cells by phytohemagglutinin (PHA) that enter cell division are typically T lymphocytes[17] which have an average half-life of about three years in healthy humans[18, 19]. However, the true lymphocyte lifetime is still only estimated, and its lifespan could also be influenced because of radiation-induced cell damage and death. Studies have shown that the mean lymphocyte lifetime in patients who received radiotherapy for cervical cancer was about 1.5 years[20], while a mean of 4.3 years was observed in patients who received radiotherapy for ankylosing spondylitis[18, 21]. In either case, abnormalities in circulating lymphocytes in peripheral blood with a lifespan of 3-4 days would be difficult to detect if months or years have elapsed after exposure.

The half-life of Dic (i.e., a reduction of examined Dic frequency to half of the initial Dic frequency) can be generally assumed to be three years based on a single exponential decline with a time factor correction observed in Chernobyl subjects[22]. When the study compared biological doses estimated from Dic frequency to physical

dosimetry, the time interval between irradiation and blood sampling needs to be considered, or it could lead to dose underestimation. A similar methodology which evaluated occupational exposure to ionizing radiation in nuclear power plant workers suggested a longer half-life of seven years might be more realistic[23, 24]. Further debate arises as some researchers suggest that the decline is delayed about a year after exposure[21, 25]. Dics in lymphocytes several years after the initial irradiation are likely derived from long-life stem cells or progenitor cells with radiation-induced chromosomal instabilities or caused by protracted radiogenic environmental stressors. Thus, it has been well established that Dic is best suited to evaluate recent acute exposures, while delayed sampling will result in an increased uncertainty of dose estimation.

3. DCA in biodosimetry

Circulating lymphocytes in the G_0 stage of the cell cycle can be stimulated with a mitogenic agent to start the process of DNA replication and enter cell division. The metaphase lymphocytes can then be observed for chromosome aberrations, such as Dic. Based on the principle that Dic is primarily attributed to the misrepair of radiation-induced DNA double strand breaks, an assay quantifying Dic frequency in lymphocytes can be performed to assess prior radiation exposures, i.e., the DCA. Dic frequency in peripheral blood lymphocytes in DCA can give a good approximation of radiation dose for as low as 0.1 Gy[26], especially if blood sampling has not been delayed. Manual Dic scoring in DCA is a time-consuming process that necessitates highly trained staff. Additionally, many factors will influence the estimated dose of the DCA, including total dose, dose-rate, percentage of body irradiated, radiation quality, and sampling time after exposure. Human error or discrepancies in Dic identification and mis-scoring are also known to influence results. Thus, it is important to understand these contributing factors when interpreting Dic frequency for precise radiation dose estimation (see section 3.8 for more details).

3.1. Modifications of DCA

Dic frequency has been used as a dose estimation tool for biological dosimetry since the 1960s. The information in this section about DCA was adopted from IAEA 2011[1] and is currently the international guideline for DCA in routine assessment. However, from the period prior to, and after, the IAEA 2011 publication, there have been few changes worth mentioning while some parts are still relatively debated. In general, the measured Dic frequency is robust against culture and reagent protocol variations[27, 28]. However, modifications to the protocol could cause uncertainties in dose estimation during laboratory inter-comparisons[29]. Nonetheless, DCA still remains the gold standard for radiation assessment and further research will continue to allow the progression or modification of the assay to extend the assay's utility to multiple radiation scenarios.

One of the main modifications to DCA analysis is the type of aberration used to estimate dose. Originally, the assessment required both Dic and ring chromosomes (rings) for dose estimation. The total yield of Dic and rings are referenced to an appropriate dose-response curve to estimate dose (i.e., Dic + ring per cell curves). However, Giemsa-stained rings are difficult to identify and require significantly higher scorer expertise. In the IAEA 2011 publication, and as recommended by the authors, only Dic frequency in cells should be used to estimate dose. In either case, cross-checking and validation of scoring results is required, and scoring should be performed by experienced personnel.

The other variable discussed in the IAEA 2011 publication is the appropriate length of time to add demecolcine (colcemid) to the lymphocyte culture. Colcemid, a synthetic analogue of colchicine, consists of alkaloid compounds that inhibit spindle formation during mitosis[30]. Once added to a culture, the cells are arrested during metaphase and cannot progress to anaphase. As IAEA 2011 publication states, colcemid (0.05-0.10 μg/mL) is typically added 2 or 3 hours before cultures are completed, i.e., at 45-46 hours. However, the authors and other researchers recommend adding a lower concentration of colcemid (0.05 μg/mL) either at the start of culture[31–33] or at 24 hours[1, 34, 35]. Even though a longer colcemid treatment could affect mitotic indices and the quality of metaphase chromosomes observed, i.e., too condensed or too elongated, earlier colcemid addition will prevent cells from progressing beyond the first metaphase and avoid any analysis of second-division metaphase cells. Please refer to section 3.3 for more details on the importance of first-division metaphase analysis.

3.2. DCA protocol

DCA follows the general procedures given in the IAEA 2011 publication, which have been adopted by cytogenetic laboratories to assess human peripheral blood samples (full methodology details used by authors are provided in Appendix A and B). Briefly, heparinized peripheral blood is obtained from individuals potentially exposed to radiation following the recommended blood sampling procedures and management discussed in Series 1 and 2[36, 37]. Then, whole blood or isolated mononuclear cells are stimulated by PHA to proliferate in culture (incubated at 37 °C , 5% CO_2 for 48 hours). Cells are then harvested and fixed, and first-division metaphases are spread on glass slides. Metaphase chromosomes are stained and

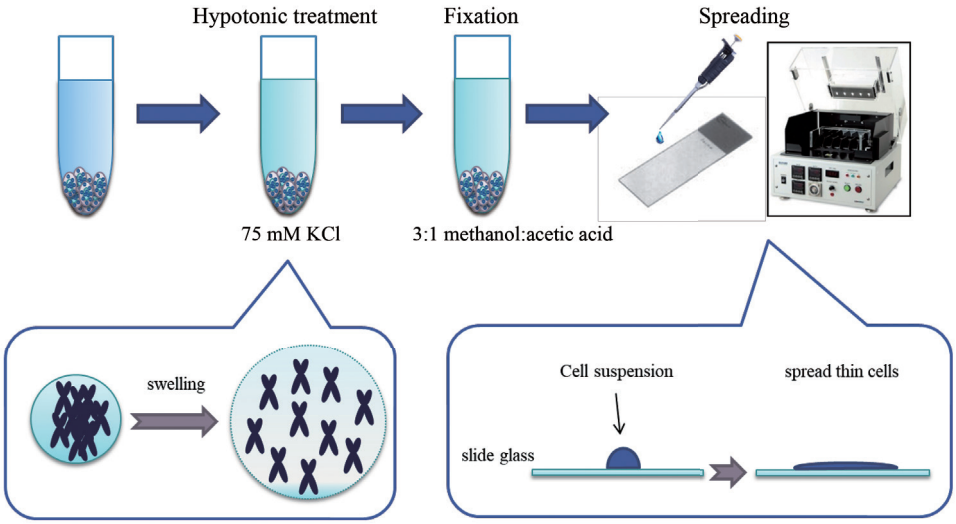

Fig. 3. Hypotonic solution of 75 mM potassium chloride is added for cell swelling and to reduce metaphase chromosome overlap. During slide preparation, fixative is slowly pipetted on the slide to ensure cells are dispersed into a uniform suspension.

analyzed for chromosome aberrations. After Dic scoring, dose estimates are evaluated with Dic frequency from the sample with calibration curves of similar radiation quality.

3.3. Mitotic arrest and significance of first-division metaphases

As mentioned previously, DCA requires a culturing method to ensure that first-division metaphases are scored. This is achieved by adding colcemid to synchronize and arrest cell division to mid-M phase. In other words, colcemid inhibits the anaphase movement of chromosomes and halts other stages of the mitotic cycle. As recommended by the authors, colcemid is added from the start of culture or 24 h after the start of culture to ascertain first-division metaphase analysis. If colcemid timing and concentration are not considered, the first and second metaphases must be distinguished using Fluorescence Plus Giemsa (FPG) staining[38].

First-division metaphases (46-48 h after initiation of the culture) must be analyzed because Dic is an unstable chromosome aberration, and cells with these aberrant chromosomes often fail to divide normally and die during mitosis. If the cell-cycle progresses, cells with Dics may die, decreasing the overall Dic frequency and ultimately underestimating radiation dose. It was previously shown that the Dic frequency of second-division cells decreased by 46% as compared to first-division cells[39]. In other words, about half of the cells containing unstable aberrations will be eliminated after each mitosis. Therefore, it is necessary to arrest dividing cells in the beginning or the middle of the first cycle of mitosis after PHA stimulation to ensure acute biodosimetry evaluation.

3.4. Cell spreading and cell suspension improvement

After blood culture, cells are first treated with hypotonic solution (75 mM potassium chloride) for cell swelling and fixed in 3:1 methanol:acetic acid. After fixation, cells are spread onto a pre-cleaned slide glass (Fig. 3). It should be noted that the quality of metaphase chromosomes can be affected by humidity, temperature and cell concentration (Fig. 4). Lack of consideration for optimized cell swelling may make it difficult to distinguish chromosomes (see section 3.8 for Dic identification). It is also recommended to check the metaphase quality on a test slide before preparing multiple slides for DCA evaluation. Laboratories can also use automatic sample preparation systems, such as the HANABI-PVI Metaphase Spreader (ADSTEC, Japan), to control environmental conditions for optimized metaphase spreading on slides.

3.5. Slide staining

Generally, slides are stained with Giemsa in Gurr buffer (pH 6.8) in DCA because of its reduced cost and time for slide preparation. However, depending on the type of microscopes (e.g., bright-field, fluorescence microscopes) available in the lab and other endpoints analyzed (e.g., centromere identification), slides may be stained with other reagents such as 4′,6-diamidino-2-phenylindole (DAPI). Other techniques can also be used to visualize and score Dic, such as those that highlight centromeres (C-banding) or use of fluorescent probes to detect and localize specific DNA sequences on chromosomes (fluorescence *in situ* hybridization using peptide nucleic acid probes; PNA-FISH). In this case, C-banding techniques will typically be adapted from protocols

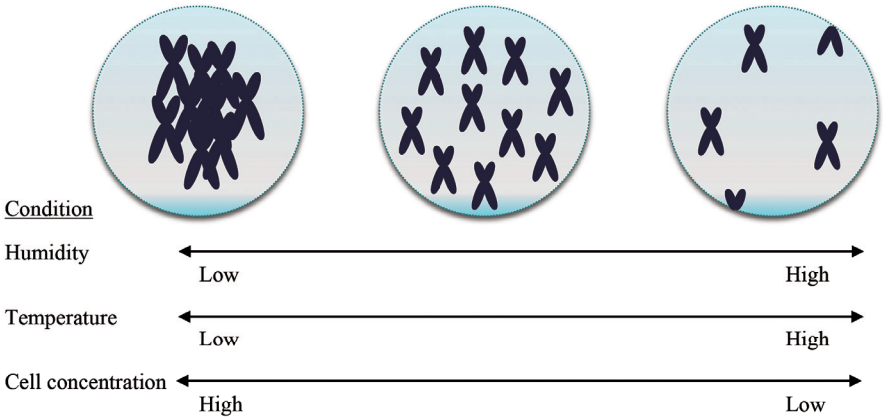

Fig. 4. Humidity, temperature and cell concentration can affect the quality of metaphase spreads on slides. Before preparing multiple slides for DCA analysis, it is a good habit to check how the metaphase chromosomes spread on a test slide. If the test slide shows poor metaphase quality, adjust the above conditions again until optimal metaphases are obtained.

Table 1. Typical DCA assessment of the reference, triage and QuickScan methods.

Method	Number of metaphase spreads or Dic needed	Centromere count required	ISO*
Reference[1, 45]	1000 metaphases or 100 Dic	Yes	Yes
Triage[46, 47]	50 metaphases or 30 Dic	Yes	Yes
QuickScan[48]	50 metaphases	No	No

*Found in ISO documents and consensus-based International Standards

referenced from Sumner *et al.*[40] and Prosser *et al.*[41], while PNA-FISH can be adapted from Finnon *et al.*[42] and Shi *et al.*[43]. For routine DCA with Giemsa, adequate distinction of centromeres and chromosome arms are required to reduce misidentifications. Slide staining with C-banding or PNA-FISH may assist in resolving the ambiguous images and provide a better identification of suspected Dic.

3.6. Metaphase imaging

Stained slides are typically observed using an upright optical microscope. High objective oil lens of 63× (60×) or 100× are used to observe chromosome structures. Metaphase spreads should first be located using a 10× magnification objective lens, and then imaged with a 63× or 100× lens for manual chromosome aberration analysis. A metaphase location software[44] can also be used more efficient metaphase imaging as several thousand metaphases can be captured automatically, which can then be saved as digital images and accessed for future scorer analysis.

3.7. Dic scoring and number of cells for analysis

It is recommended to score at least 500 to 1000 metaphases for accurate dose estimation as increasing the number of scored cells will improve resolution, support initial assessments, and provide evidence of inhomogeneous exposure[45]. It is not necessary to score beyond 100 Dic if the aberration yield is high or if there exists a high abundance of Dic in a few metaphase spreads. In the low dose range or when assessing radiation doses years after exposure, thousands of metaphases may need to be scored to confirm an exposure. The number of scored metaphases should be decided based on the radiation scenario and assay's objective. In emergency triage, the number of metaphases analyzed need to be accurate enough to provide an estimated dose to victims for physicians to provide treatment in a timely manner.

DCA analysis typically includes the reference method of 500 to 1000 complete metaphases[45], a triage technique of 50 complete metaphases[46, 47], or the "QuickScan method"[48] of 50 metaphases (Table 1). Reference and triage methods also require centromeres to be counted to ensure metaphases are complete. The QuickScan scoring technique was devised by the Biodosimetry Laboratory at Atomic Energy of Canada Ltd. (Chalk River, ON). It is an alternative rapid-scoring approach to the conventional triage technique which rapidly examines metaphases for obvious damage without centromere scoring[48]. However, this technique is only used by experienced scorers for initial screening, rapid scoring for emergency exposures or mass casualty radiation emergency and evaluating the ability of scorers across laboratories. It is also important

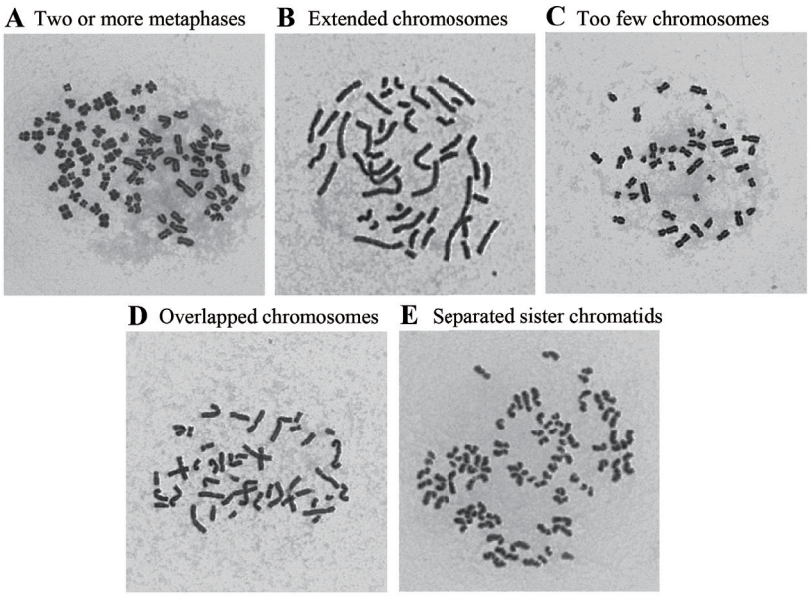

Fig. 5. Metaphases that do not meet selection criteria should be excluded during Dic scoring; (A) Two or more metaphases, (B) unclear or extended chromosomes, (C) too few chromosomes, (D) overlapped chromosomes, and (E) separated centromeres or sister chromatids.

to note that all three methods are found in the IAEA 2011 publication[1], but only the reference and triage method are recognized by the worldwide International Organization for Standardization (ISO)[45].

3.8. Dic scoring criteria and chromosome staining features

Accurate Dic identification in selected metaphases is required to determine the true Dic frequency in exposed subjects[1]. Prior to performing DCA, scoring personnel need to be highly and frequently trained to control inter-laboratory variability and eliminate possibility of scorer error. If Dic frequency is not appropriately quantified, absorbed dose will either be over- or under-estimated. Dic is only scored in metaphases selected based on recommendations from the IAEA 2011 publication. Metaphases must be complete, i.e., to contain 46 (or 45) centromeres, not overlap, and chromosome arms must be distinguishable. Examples of metaphases that should not be scored are provided in Figure 5. Some biodosimetry laboratories will also accept metaphases with 45 centromeres in the metaphase selection criteria because the missing centromere cannot form a Dic (i.e., not influence Dic frequency).

It is often remarked that DCA requires experienced scorers and appropriate slide preparations. This is because incorrect Dic scoring is often the highest cause of uncertainty in dose estimations. Careful observation is needed because a Dic with two adjacent centromeres may be difficult to distinguish from a monocentric chromosome. Understanding chromosome structure and morphology will help in Dic identification. For example, acrocentric chromosomes (i.e., #13, 14, 15, 21, and 22 chromosomes) tend to be arranged with satellite association (Fig. 6), which can help determine if a Dic has been formed with group D or G chromosome (Fig. 7).

In addition to understanding chromosome structure, staining patterns associated with Giemsa-stained chromosomes can also help to identify Dic. Centromeres or satellite regions tend to be more lightly stained (i.e., light gray or white). Both long and short chromosome arms will appear dark gray or black. However, if the chromosome arms are twisted or if chromosomes overlap, the color will appear darker than the rest of the body or other chromosomes (Fig. 8). As previously mentioned, metaphases with overlap should not be counted. However, if the scorer can appropriately distinguish the arms and centromeres using the respective staining features, the cell can be scored. In particular, misinterpretation of staining features should be avoided for Dic when scoring, such that a twisted chromatid arm is not identified as a second centromere (Fig. 9). Scorers should reconfirm that the metaphase is complete and distinguish any fragments before concluding if Dic is present. Images analyzed should be stored, especially those containing suspected Dic, to allow for future validation if needed and provide reproducible results.

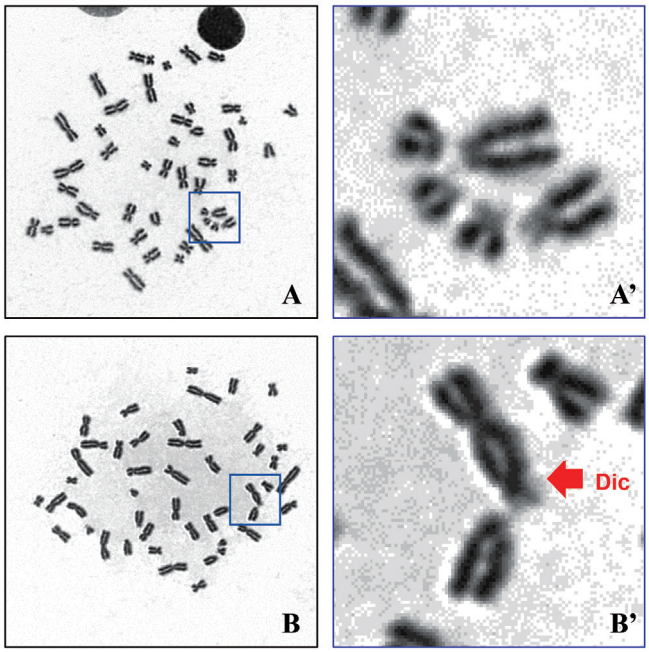

Fig. 6. (A) Acrocentric chromosomes tend to be arranged with satellite association. This helps determine if a Dic has been formed with a group D or G chromosome. (B) An image of a pattern 2 Dic (metacentric chromosome or a submetacentric chromosome mis-repaired with an acrocentric chromosome). (A') and (B') are magnified images of (A) and (B), respectively.

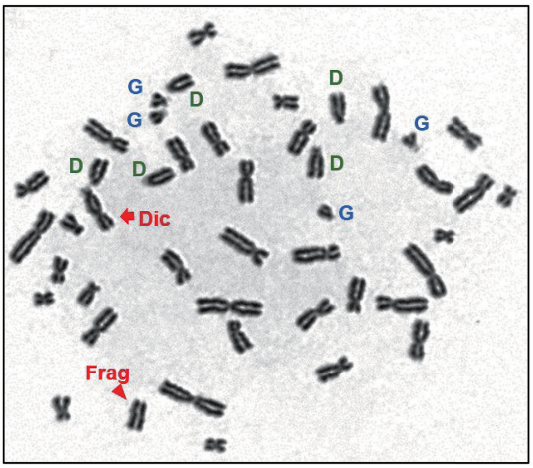

Fig. 7. Example of a female donor's metaphase that includes satellite association with Dic and a D chromosome. Dic is characterized as pattern 2. There are 5 group D chromosomes and 4 group G chromosomes.

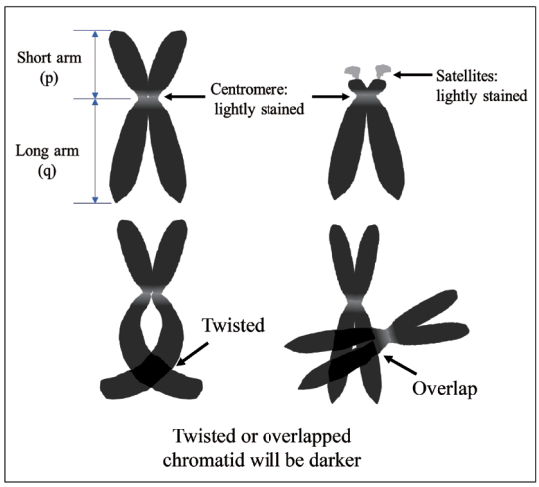

Fig. 8. Chromosome arms are dark gray or black while centromeres are a lighter shade in grayscale images. It is important to clearly identify the staining features of a chromosome during scoring. Chromosomes may twist or overlap causing darker shades and may appear as a Dic. If the staining feature can be identified, then the cell may be scored. If not, we recommend the cell is not used in DCA analysis.

3.9. Dose-response curve and dose estimation

After analyzing an adequate number of lymphocyte metaphases, Dic frequency (i.e., Dic per cell) can be used to estimate a biological absorbed dose by referencing a dose-response calibration curve. This *in vitro* curve is established by radiation exposure of peripheral blood to doses of the appropriate radiation quality following recommended guidelines[1]. More recently, separate curves were constructed for low dose-rates (Table 2), as the molecular mechanisms and the kinetics of Dic induction are influenced by radiation dose, period of exposure, and quality of radiation. Thus, it is recommended that curves used for dose estimation are developed at the laboratory performing the dose assessment for a range of radiation

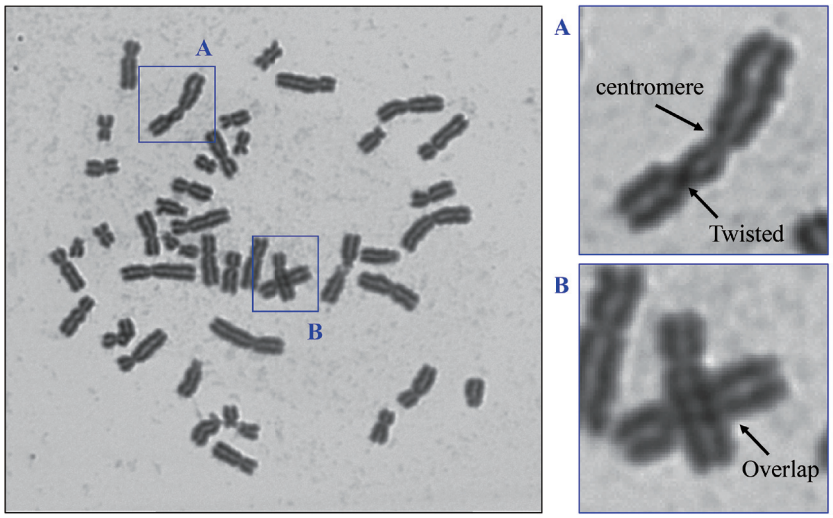

Fig. 9. Example of a Giemsa-stained metaphase spread with a twisted chromosome and overlapping chromosomes. The twisted chromosome may be wrongly identified as a Dic.

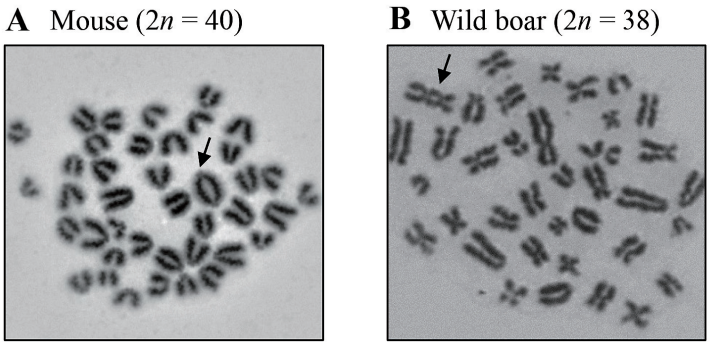

Fig. 10. Dic in non-human biota; (A) mouse and (B) wild boar. $2n$, number of chromosomes in somatic cells.

qualities and dose-rates. The estimated dose obtained from interpolation of Dic frequency to a dose-response curve represents an average absorbed dose to the lymphocytes. This dose is also assumed to approximate a whole-body dose as peripheral blood lymphocytes are mobile and distributed around the body.

The type of radiation contributes to Dic frequency in lymphocytes. For example, high LET radiation, such as fission neutrons and alpha particles, cause significantly higher and complex damage to cells at acute doses and this damage tends to have a linear effect as dose increases. Thus, the dose-response relationship fits a linear regression model $Y = \alpha D$, where Y is the Dic frequency and D is dose. On the other hand, low LET radiation, including X-rays and gamma rays, tends to have a linear-quadratic dose response and there is a good dose-effect relationship in the range of 0.1 to 5.0 Gy for acute exposures. Low LET radiation is best fitted by a linear-quadratic model $Y = C + \alpha D + \beta D^2$.

In addition, Dic frequency follow Poisson statistics for acute whole-body low LET radiation. Statistical verification with dispersion index (i.e., ratio of variance to mean) and suitable Poisson tests (e.g., u-test) will need to be calculated before dose estimation. The objective of curve fitting in a dose-response curve is to determine the values of coefficients C, α and β which best fit the data points. Overall, multiple laboratories have developed dose-response curves showing these tendencies with high and low LET radiation (Table 2).

4. Current issues and applications in biodosimetry

While Dic is preferably used to investigate effects of ionizing radiation exposure and dose estimation, it is important to consider factors that could affect valid and reproducible results in different experimental designs.

Table 2. Previously published constructed *in vitro* dose-response curves for DCA and dose estimation. The curves were based Dic frequency in human lymphocytes exposed to various ionizing radiation sources.

Reference	High or Low LET	Irradiation source	Dose rate (Gy min^{-1})	Dose-response curve equation (High LET, $Y = c + \alpha D$; Low LET, $Y = c + \alpha D + \beta D^2$)		
				c (±SE)	α (±SE)	β (±SE)
Bauchinger *et al.*[49]	Low	^{60}Co	0.017	0.00038 (±0.00013)	0.0090 (±0.004)	0.0417 (±0.0028)
Martins *et al.*[50]	Low	^{60}Co	0.018	0.0011 (±0.0006)	0.0098 (±0.0036)	0.0489 (±0.002)
Schmid *et al.*[51]	Low	^{60}Co	0.033	0.0003 (±0.0002)	0.0139 (±0.0052)	0.0304 (±0.0030)
Abe *et al.*[52]	Low	^{60}Co	0.026	0.0013 (±0.0005)	0.0067 (±0.0071)	0.0313 (±0.0091)
Mendes *et al.*[53]	Low	^{60}Co	0.05	0.0014 (±0.0008)	0.0074 (±0.0069)	0.0449 (±0.0044)
Lindholm *et al.*[54]	Low	^{60}Co	0.24	0.00055 (±0.0024)	0.0135 (±0.0043)	0.0544 (±0.0034)
Wong *et al.*[55]	Low	^{60}Co	0.41	0.000 (±0.000)	0.026	0.0386
Top *et al.*[56]	Low	^{60}Co	0.425	0.0006 (±0.0008)	0.034 (±0.00729)	0.0605 (±0.00282)
Koksal *et al.*[57]	Low	^{60}Co	0.457	0.005 (±0.003)	0.0216 (±0.006)	0.0706 (±0.0025)
Bauchinger *et al.*[49]	Low	^{60}Co	0.5	0.00039 (±0.00013)	0.0107 (±0.0041)	0.0555 (±0.0028)
Beinke *et al.*[58]	Low	^{60}Co	0.64	0.000 (±0.000)	0.0306 (±0.0068)	0.0480 (±0.0036)
Haeri *et al.*[59]	Low	^{60}Co	0.8	0.000 (±0.000)	0.012	0.0461
Voisin *et al.*[60]	Low	^{60}Co	1	0.0004 (±0.0023)	0.0374 (±0.0127)	0.0549 (±0.0034)
Prasanna *et al.*[61]	Low	^{60}Co	1	0.000 (±0.000)	0.098 (±0.021)	0.044 (±0.009)
Stricklin *et al.*[62]	Low	^{137}Cs	0.4	0.0025 (±0.0016)	0.013 (±0.007)	0.065 (±0.003)
Bauchinger *et al.*[49]	Low	220 keV X-rays	0.5	0.000 (±0.000)	0.0404 (±0.03)	0.0598 (±0.017)
Prasanna *et al.*[61]	Low	220-250 keV X-rays	1	0.000 (±0.000)	0.059 (±0.0.0136)	0.029 (±0.0046)
Beinke *et al.*[58]	Low	240 kV X-rays	1	0.0007 (±0.0002)	0.0432 (±0.00459)	0.0630 (±0.0039)
Prasanna *et al.*[61]	High	Fission neutron (0.71 MeV)	0.25	0.000 (±0.000)	0.677 (±0.003)	-
Lloyd *et al.*[63]	High	Fission neutron (0.7 MeV)	0.5	0.000 (±0.000)	0.8349 (±0.003)	-

DCA works well if an exposure is acute and uniform over the whole body with no delay in blood sampling from the initial exposure (i.e., blood sample is typically collected after 24 hours after irradiation). However, if these factors are altered, one should expect an increase of uncertainties for dose estimations.

4.1. Issues

One of the limitations of DCA in nuclear or radiological emergencies is the time and expertise required to perform the scoring of 500 to 1,000 metaphases per sample (i.e., Reference method, Table 1) with an effective dose range between 0.1-5 Gy. This range covers the dose range lower than 0.5 Gy, where peripheral blood lymphocyte depletion is observed and has sufficient sensitivity to evaluate deterministic effects[64]. If the number of metaphases scored is reduced, the range between the upper and lower dose limit will be increased. More than 1,000 metaphases should be scored if assessing exposures below 0.1 Gy or if the exposure was received over a protracted period.

Even though high-throughput automated metaphase image capture and Dic scoring have been developed to quicken DCA analysis[44, 65, 66], the technology can be quite expensive and might require trained scorers to re-evaluate automated Dic frequency. Open-source automated Dic scoring software or alternative staining methods for easier Dic identification must be developed for laboratories who are unable to afford such equipment and software.

The other limitation is the presumably short half-life of Dic and its reduced sensitivity to low dose or low dose-rate ionizing radiation. In general, unstable chromosome aberrations like Dic have a shorter half-life than stable chromosome aberrations, as cells with unstable chromosome aberrations cannot proceed with cell division and are halted during cell-cycle checkpoints or cause cell apoptosis. However, as mentioned in section 2, the estimated Dic half-life ranges from 1.5-3 years in some studies to 4-7 years in others. The authors suspect the discrepancy may be due to the radiation type and quality that was investigated as a longer Dic half-life was estimated in Hiroshima and Nagasaki atomic bomb survivors, while other acute exposure scenarios showed a quicker decline of Dic[67]. Regardless, the half-life has raised concerns on whether Dic can be used to interpret low dose and low dose-rate radiation. DCA may not be feasible if more than ten years have passed since the radiation event. In such cases and in low dose studies, the analysis of stable chromosome aberrations such as translocations is preferred as the endpoint has the sensitivity and longevity to evaluate past irradiated subjects.

4.2. Environmental dosimetry for terrestrial animals

Other than humans, the scientific community has also aimed to protect the environment and wildlife from

harmful effects of ionizing radiation[68]. To advance environmental protection and dosimetry for terrestrial biota, it is necessary to have accurate probabilistic framework for dose assessments of external (and internal) doses for terrestrial animals. Similar to humans, there is extreme diversity of non-human biota, and this creates a major challenge for such assessments. Likewise, Dic can accumulate in animals following ionizing radiation exposure (Fig. 10), and DCA has also been used to estimate the extent of radiation damage after radioactive contamination in areas near the Chernobyl Nuclear Power Plant[69] and Fukushima Dai-ichi Nuclear Power Plant[70, 71]. As mentioned earlier, DCA is more suited for acute radiation exposures from 0.1 to 5 Gy. It is still unclear if the assay is applicable to such cases of chronic radiation exposure to accurately improve wildlife prediction models.

5. Conclusion

In cytogenetic biodosimetry, DCA is the preferred method for evaluating the effects of ionizing radiation and is a valuable dose assessment method. As DCA is highly radiation specific and has an effective dose range between 0.1-5 Gy, it is considered the gold standard in biodosimetry. However, DCA analysis can be labor-intensive, requires expert scorers and takes up a lot of time. Over the years, improvements have been made to reduce the time and expertise needed for Dic scoring. Furthermore, published reports of dose-response curves have shown that the differences exist in measured Dic frequency among laboratories for the same dose. It is currently recommended that each biodosimetry laboratory establish its own Dic dose-response curve using different radiation types, dose-rates, and doses. There is still room for improvement to make DCA more beginner-friendly and applicable for mass-casualty accidents. Such could be addressed by developing open-source AI scoring systems or optimizing staining methodology to reduce scoring time and further improve automated Dic identification.

Point 1. DCA is based on the principle that radiation exposure causes DNA strand breaks.
- Dic can be formed due to a misrepair of DNA double strand breaks, resulting in one chromosome with two centromeres.
- Dic is highly specific and sensitive to ionizing radiation.

Point 2. Many factors influence the Dic frequency.
- Total dose, dose-rate, percentage of body irradiated, and radiation quality will influence Dic frequency.
- Sampling time of heparinized peripheral blood after initial exposure will also influence DCA.
- Scorer's ability and metaphase selection criteria may affect Dic frequency and cause dose under- or overestimation.

Point 3. Absorbed dose to exposed subjects is estimated from Dic frequency and dose-response calibration curves.
- Curves need to be established prior to the analyses and have similar radiation quality to the exposure scenario.
- Scoring 500 to 1000 cells (or 100 Dic) is recommended for the reference method for accurate dose estimation. 50 metaphases (or 30 Dic) can be alternatively scored for triage assessment.

Point 4. DCA issues and limitations
- DCA is the gold standard for cytogenetic biodosimetry. However, limitations such as the shorter half-life compared to stable aberrations, long analysis time and high scorer expertise need to be overcome.
- High-throughput automation including metaphase imaging and Dic scoring do quicken DCA analysis. The technology is expensive and still requires scorer expertise for potential re-evaluation.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Appendix

The dicentric chromosome assay (DCA) follows the general procedures provided by the IAEA manual published in 2011[1] and ISO[2, 3]. The DCA procedures have been adopted by cytogenetic biodosimetry laboratories to assess human blood samples for radiation exposures. Here are detailed protocols of DCA used by the authors' laboratory, which have been adopted and modified from the international protocols.

Please note that once the blood arrives at the laboratory, the researchers should decide if DCA will be performed with isolated peripheral blood mononuclear cells (PBMC) or whole blood. The authors recommend PBMC when possible. Please refer to (**Appendix A**) for PBMC DCA protocol and (**Appendix B**) for whole blood DCA protocol.

Appendix A: DCA protocol for PBMC culture

A.1 Materials and Reagent setup

A. Equipment

ID	Supplier	Cat. #
Biological safety cabinet		
Centrifuge		
Aspiration system		
CO_2 incubator		
Water bath		
Vortex mixer		
Hanabi harvester (optional)	ADSTEC	
Hanabi spreader (manual model)	ADSTEC	
15 mL polypropylene, sterile conical tubes		
50 mL polypropylene, sterile conical tubes		
Portable pipette gun		
Serological pipettes, sterile, 10 mL and 5 mL		
0- 20 µL micropipette + sterile 20 µL tips		
0- 200 µL micropipette + sterile 200 µL tips		
Sterile slugged Pasteur pipette 9"		
Phase contrast microscope pre-cleaned slides, frosted end, 76 x 26 mm		
Premium cover glasses, Size: 50 x 24 mm		
Coplin jar		
Waste container		
Latex gloves		

B. Reagents

ID	Supplier	Cat. #
Histopaque 1077	Sigma-Aldrich	H8889-100ML
RPMI 1640 medium with L-glutamine, phenol red and 25mM HEPES	Gibco	22400089
Fetal Bovine Serum, Prime, Heat-inactivated	Biosource	200P-500HI
Kanamycin sulfate (100X), liquid	Gibco	15160054
KaryoMAX Colcemid Solution in HBSS	Gibco	15210040
PHA Reagent Grade (HA15)	Remel	R30852701
Potassium chloride (KCl)	Sigma-Aldrich	P5405-250G
Methanol		
Acetic acid, glacial		
Giemsa's azur eosin methylene blue solution	Merck	1.09204.0103
Gurr buffer tablets	Gibco	10582013
Malinol 750cps	Muto Pure Chemicals	20093

C. Reagent setup

ID
· Complete medium without PHA (**CM**)*
RPMI 1640 medium supplemented with 20% FBS and Kanamycin sulfate.
· Washing solution (**Wash buffer**)*
RPMI 1640 medium supplemented with 2% FBS and Kanamycin sulfate.
· Hypotonic solution (75 mM KCl)
2.796 g KCl dissolved in 500 mL of distilled water
Sterilize using autoclave (121 ℃ for 20 min), store at room temperature. Warm hypotonic solution at 37 °C before cell fixation.
· Fixative solution
Methanol: acetic acid = 3:1 (prepared on ice for PBMC) **Note**: To freshly prepare fixative prepare 30 min to 2 h before harvest
· Gurr Buffer
Dissolve 1 Gurr buffer tablet in 100 mL of deionized water, sterilize using autoclave (121 ℃ for 20 min), and store at room temperature **
· Giemsa staining solution
4% Giemsa solution in Gurr buffer (pH 6.8)

*FBS will need to be inactivated by heating at 56 ℃ for 30 min prior to making medium and buffer
**The instructions should be modified depending on the type of Gurr tablets purchased.

A.2 Protocol

A. Blood culture and PHA stimulation

Please refer to Series 4[4] for more information regarding culture procedures including the choice of culture medium, serum, and mitogen.

1. Mononuclear cells can be isolated from whole blood by directly layering onto Histopaque 1077 or other ready-to-use tubes for such lymphocyte separation (e.g., BD Vacutainer® CPT™ Cell Preparation tubes, Stemcell Technologies Sepmate™ PBMC isolation tubes). If ready-to-use tubes are used, manufacturer's instructions should be followed.

   The authors recommend the following steps for PBMC isolation if blood is directly layered on Histopaque 1077 in a 15 mL centrifuge tube:
   a. 2.5 mL of Histopaque 1077 into 15 mL centrifuge tube and bring to room temperature.
   b. Mix 1.8-2.4 mL whole blood with 2 mL washing solution (i.e., 1:1 whole blood to washing solution)[5].
   c. Carefully layer the mixed sample (4 mL) onto Histopaque 1077.
   d. Centrifuge at 600 x *g* for 30 min at 20 °C in a horizontal rotor according to the manufacturer's instruction.
   e. After centrifuge, the PBMC layer is then carefully transferred to a sterile conical tube containing 6.5 mL of washing solution.
   f. Centrifuge at 400 x *g* for 5 min and aspirate the supernatant.
   g. Then, proceed to step (2).

2. Resuspend isolated PBMC cell pellet with 1 mL of CM (warmed), pipette well to mix.
3. Transfer 1 mL of PBMC cell suspension into new 15 mL conical tube with 5 mL of CM (warmed).
4. For 6 mL culture, add 30 µl colcemid (final colcemid concentration of 0.03 µg/mL) and 120 µl PHA (final concentration of 180 µg/ml for PHA).
5. Mix gently by vortex.
6. Loosely close the tube cap and start the culture in 5% CO_2 incubator for 48 h.

B. Hypotonic treatment and fixation

1. After blood culture, centrifuge at 400 x *g* for 5 min at 20 °C.
2. Carefully aspirate the supernatant.
3. Resuspend cell pellet with 2 mL of hypotonic solution (37 °C) while vortexing.
4. Incubate at 37 °C for 20 min.
5. Add 200 µL of cold fixative solution, invert the tube 5 to 6 times, and keep on ice for 5 min.
6. Centrifuge at 400 x *g* for 5 min at 4 °C.
7. After centrifugation, carefully aspirate the supernatant.
8. Gently tap the bottom of the conical tube to break up the cell pellet.
9. Resuspend cell pellet with 5 mL of cold fixative solution while vortexing in the same conical tube.
10. Centrifuge at 400 x *g* for 5 min at 4 °C.
11. After centrifugation, carefully aspirate the supernatant.
12. Resuspend in 5 mL of cold fixative solution while vortexing.
13. Centrifuge at 400 x *g* for 5 min at 4 °C.
14. Repeat steps 11-13 twice, discard supernatant and resuspend cell pellet in an optimum volume of cold fixative solution. (Store at -30 °C)

C. Slide preparation

1. Adjust the cell concentration with freshly prepared cold fixative.
2. Drop the 13-17 µl cell suspension onto a clean slide (or use Hanabi spreader to spread cells in a controlled environment).
3. Dry the slide for at least 30 min prior to staining. Slides can be dried quickly in a 50-60 °C oven or dried overnight at room temperature.

Note: Preparation of clean slide

Pre-cleaned slides were kept in 100% Et-OH over 1 week (if necessary).

D. Giemsa staining

1. Prepare the staining solution (4% Giemsa in Gurr buffer, pH 6.8). Note: The concentration of Giemsa may be modified depending on the reagents purchased.
2. Set the slide into an appropriate staining jar.
3. Stain the slide for 10 min at room temperature.
4. After 10 min, gently remove the oxidized Giemsa film formed on the surface of the staining solution.
5. Rinse slides with water.
6. Authors recommend that the processed slides should fully dry before mounting. If dose estimate needs to be reported in a short period (i.e., triage), slides can be dried in 60 °C for 1 h before mounting or immersion oil can be dropped directly on fully dried slides for microscope viewing. In all other cases, slides should be dried at 37 °C for 24 h.

E. Mounting

Mounting media, such as Malinol, are needed for making permanent slides. This ensures that the cells are protected after Giemsa staining and allows for long-term slide storage.

1. Drop the mounting medium (Malinol) on the cover slip.
2. Mount the cover slip gently. Do not make any bubbles.
3. Keep the slide on a flat surface and place it in a clean environment for 2 h to allow the cover slip to firmly adhere to slide.

Appendix B: DCA protocol for whole blood culture

B.1 Materials and Reagent setup

A. Equipment

ID	Supplier	Cat. #
Biological safety cabinet		
Centrifuge		
Aspiration system		
CO_2 incubator		
Water bath		
Vortex mixer		
Hanabi harvester (optional)	ADSTEC	
Hanabi spreader (manual model)	ADSTEC	
15 mL polypropylene, sterile conical tubes		
50 mL polypropylene, sterile conical tubes		
Portable pipette gun		
Serological pipettes, sterile, 10 mL and 5 mL		
0- 20 µL micropipette + sterile 20 µL tips		
0- 200 µL micropipette + sterile 200 µL tips		
Sterile slugged Pasteur pipette 9”		
Phase contrast microscope pre-cleaned slides, frosted end, 76 x 26 mm		
Premium cover glasses, Size: 50 x 24 mm		
Coplin jar		
Waste container		
Latex gloves		

B. Reagents

ID	Supplier	Cat. #
RPMI 1640 medium with L-glutamine, phenol red and 25mM HEPES	Gibco	22400089
Fetal Bovine Serum, Prime, Heat-inactivated	Biosource	200P-500HI
Kanamycin sulfate (100X), liquid	Gibco	15160054
KaryoMAX Colcemid Solution in HBSS	Gibco	15210040
PHA Reagent Grade (HA15)	Remel	R30852701
Potassium chloride (KCl)	Sigma-Aldrich	P5405-250G
Methanol		
Acetic acid, glacial		
Giemsa’s azur eosin methylene blue solution	Merck	1.09204.0103
Gurr buffer tablets	Gibco	10582013
Malinol 750cps	Muto Pure Chemicals	20093

C. Reagent setup

ID
· Complete medium without PHA (**CM**)*
RPMI 1640 medium supplemented with 20% FBS and Kanamycin sulfate.
· Washing solution (**Wash buffer**)*
RPMI 1640 medium supplemented with 2% FBS and Kanamycin sulfate.
· Hypotonic solution (75 mM KCl)
2.796 g KCl dissolved in 500 mL of distilled water
Sterilize using autoclave (121 ℃ for 20 min), store at room temperature. Warm hypotonic solution at 37 °C before cell fixation.
· Fixative solution
Methanol: acetic acid = 3:1 (kept at room temperature for whole blood) **Note:** To freshly prepare fixative prepare 30 min to 2 h before harvest
· Gurr Buffer
Dissolve 1 Gurr buffer tablet in 100 ml of deionized water, sterilize using autoclave (121 ℃ for 20 min), and store at room temperature**
· Giemsa staining solution
4% Giemsa solution in Gurr buffer (pH 6.8)

*FBS will need to be inactivated by heating at 56 ℃ for 30 min prior to making medium and buffer
**The instructions should be modified depending on the type of Gurr tablets purchased.

B.2 Protocol

A. Blood culture and PHA stimulation

Please refer to Series 4[4)] for more information regarding culture procedures including the choice of culture medium, serum, and mitogen.

1. 0.5 mL of whole blood can be directly transferred into a 15 mL sterile conical tube with 5.5 mL of warmed Complete Medium (CM), then proceed to step (1).
2. For 6 mL culture, add 30 µl colcemid (final colcemid concentration of 0.03 µg/mL) and 120 µl PHA (final concentration of 180 µg/ml for PHA).
3. Mix gently by vortex.
4. Loosely close the tube cap and start the culture in 5% CO_2 incubator for 48 h.

B. Hypotonic treatment and fixation

1. After blood culture, centrifuge at 400 x *g* for 5 min at 20 °C.
2. Carefully aspirate the supernatant.
3. Resuspend cell pellet with 5 mL of hypotonic solution (37 °C) and gently invert tube 5-6 times to mix.
4. Incubate at 37 °C for 20 min.
5. Centrifuge at 400 x *g* for 5 min at 20 °C.
6. Carefully aspirate the supernatant.
7. Resuspend cell pellet again with 5 mL of hypotonic solution (37 °C) while vortexing.
8. Add 200 µL of room temperature fixative solution and invert the tube 5 to 6 times.
9. Centrifuge at 400 x *g* for 5 min at 20 °C.
10. After centrifugation, carefully aspirate the supernatant.
11. Gently tap the bottom of the conical tube to break up the cell pellet.
12. Resuspend cell pellet with 5 mL of fixative solution while vortexing in the same conical tube.
13. Centrifuge at 400 x *g* for 5 min at 4 °C.
14. After centrifugation, carefully aspirate the supernatant.
15. Centrifuge at 400 x *g* for 5 min at 4 °C.
16. Repeat Steps 11-13 twice, discard supernatant and resuspend cell pellet in an optimum volume of room temperature fixative solution. (Store at -30 °C)

C. Slide preparation

1. Adjust the cell concentration with freshly prepared cold fixative.
2. Drop the 13-17 µL cell suspension onto a clean slide (or use Hanabi spreader to spread cells in a controlled environment).
3. Dry the slide for at least 30 min prior to staining. Slides can be dried quickly in a 50-60 °C oven or dried overnight at room temperature.

Note: Preparation of clean slide

Pre-cleaned slides were kept in 100% Et-OH over 1 week (if necessary).

D. Giemsa staining

1. Prepare the staining solution (4% Giemsa in Gurr buffer, pH 6.8).
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2. Mount the cover slip gently. Do not make any bubbles.
3. Keep the slide on a flat surface and place it in a clean environment for 2 h to allow the cover slip to firmly adhere to slide.

References

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