Biology Contribution

Detection and Automated Scoring of Dicentric Chromosomes in Nonstimulated Lymphocyte Prematurely Condensed Chromosomes After Telomere and Centromere Staining

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Summary

Premature chromosome condensation (PCC) enables the direct observation of cytogenetic damage in non-stimulated human interphase peripheral blood lymphocytes. Conventional uniform staining of PCC fusions does not allow the identification of dicentrics, the most

Purpose: To combine telomere and centromere (TC) staining of premature chromosome condensation (PCC) fusions to identify dicentrics, centric rings, and acentric chromosomes, making possible the realization of a dose−response curve and automation of the process.

Methods and Materials: Blood samples from healthy donors were exposed to ^{60}Co irradiation at varying doses up to 8 Gy, followed by a repair period of 8 hours. Premature chromosome condensation fusions were carried out, and TC staining using peptide nucleic acid probes was performed. Chromosomal aberration (CA) scoring was carried out manually and automatically using PCC-TCScore software, developed in our laboratory.

Results: We successfully optimized the hybridization conditions and image capture parameters, to increase the sensitivity and effectiveness of CA scoring. Dicentrics,

important biomarkers for recent exposure to radiation. We have combined telomere and centromere staining of PCC fusions to identify dicentrics, making it possible to establish the first dicentrics radiation dose—response curve for a PCC fusion. This improvement permits the automation of the process. This new approach can be used for biological dosimetry in radiation emergency medicine and also for patient follow-up where the rapid and accurate estimation of the dose of genotoxic agents is considered to be a high priority.

centric rings, and acentric chromosomes were rapidly and accurately detected, leading to a linear-quadratic dose—response curve by manual scoring at up to 8 Gy. Using PCC-TCScore software for automatic scoring, we were able to detect 95% of dicentrics and centric rings.

Conclusion: The introduction of TC staining to the PCC fusion technique has made possible the rapid scoring of unstable CAs, including dicentrics, with a level of accuracy and ease not previously possible. This new approach can be used for biological dosimetry in radiation emergency medicine, where the rapid and accurate detection of dicentrics is a high priority using automated scoring. Because there is no culture time, this new approach can also be used for the follow-up of patients treated by genotoxic therapy, creating the possibility to perform the estimation of induced chromosomal aberrations immediately after the blood draw.

Introduction

The current gold standard technique for biological dosimetry for recent exposure to ionizing radiation consists of the scoring of dicentric chromosomes (dicentrics) on metaphases in peripheral blood lymphocytes because of the specificity, precision, and sensitivity (0.1 Gy) of this approach (1). However, this approach presents several major limitations due to the low mitotic index of irradiated cells (especially after high doses), the selection of cells harvested at metaphase, and the long time needed for cell cultures to reach metaphase (2). These limitations hinder the application of this approach in cases where the time after exposure needs to be reduced, such as triage after accidental exposure.

Cell fusion—mediated premature chromosome condensation (PCC), first described by Johnson and Rao in 1970 (3), enables the visualization of chromosome aberrations (CAs) directly in interphase cells. This technique was adapted for the purpose of biological dosimetry with the use of a polyethylene glycol—based methodology for cell fusion of human lymphocytes with mitotic PCC inducer Chinese hamster ovary (CHO) cells (4, 5).

This approach presents a major advantage in that damage can be observed within hours after blood sampling (3 hours vs 49 hours for the gold standard technique) (6). However, the major drawback of this approach is the complexity of the scoring process and the type of damage that can be scored. After Giemsa staining of PCC fusions, it is possible to score the number of human chromosomal pieces and rings, as well as the number of acentric chromosomes in excess of the background frequency. The scoring of acentric chromosomes is limited by the influence of reparation, which decreases the frequency of this

aberration. Dicentrics, which are the most specific to irradiation, cannot be visualized owing to the specific morphology of interphase condensed chromosomes and the inability to detect centromeric regions after uniform staining. Early studies to detect centromeric regions used the C-banding technique (7), permitting the visualization of centromere and heterochromatin regions. This approach, however, gave poor reproducibility and accuracy. Other studies have introduced fluorescence in situ hybridization (FISH) to visualize centromeric regions or specific chromosomes to score CAs of PCC fusions using centromeres or whole-chromosome DNA probes (6). However, the standard FISH technique requires 2 (for DNA centromere or simple chromosome painting) to 5 days (M-FISH) to perform, which is the major limitation of this technique for biological dosimetry, aside from the high cost of the probes (8).

Despite the detection of centromere sequences using DNA probes, PCC fusion has not been widely adopted. The introduction of peptide nucleic acid (PNA) probes to the scoring of radiation-induced CAs, with short hybridization times, high specificity, increased signal intensity, and lower cost, could open new horizons for this application (9-11). However, the long, brightly staining, interstitial telomere sequences of CHO cells impede the detection of the much shorter and more dimly stained telomere sequences in human lymphocytes (12).

In the present study we have shown that telomere and centromere (TC) staining permits the easy detection of dicentrics, centric rings, and acentric chromosomes of lymphocyte PCCs using optimized hybridization conditions and fusion capture of interphase chromosomes. The manual scoring of these CAs proved to be straightforward and reliable. This improvement has made it possible to

establish the first dicentric dose–response curve for a PCC fusion. In addition, a software tool was developed (PCC-TCScore) for the automation of dicentric and centric ring scoring in PCC fusions. Using this approach, it was possible to generate a dose–response curve for dicentric and centric rings indistinguishable from that generated by manual scoring.

Methods and Materials

Lymphocyte isolation and irradiation procedure

Peripheral blood lymphocytes were isolated using Ficoll medium (Ficoll; Biochrom AG, Berlin, Germany), placed in Roswell Park Memorial Institute medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Eurobio, Courtaboeuf, France) and antibiotics (penicillin and streptomycin; Gibco-BRL), irradiated using ^{60}Co (dose rate 0.5 Gy/min) at 5 doses (0, 2, 4, 6, and 8 Gy) at room temperature, and maintained at $37°C$ for 8 hours after irradiation before their fusion to mitotic CHO cells. The choice of this time point is linked to the repair of double-strand breaks (DSBs) during the first hours after irradiation and the formation of dicentrics 8 hours after irradiation.

Premature chromosome condensation

Premature chromosome condensation was performed as previously described [4, 13] using CHO cells. The condensed human chromosomes were easily distinguished from the CHO chromosomes according to their morphologic characteristics. Please see Supplementary Data E1 (available online at www.redjournal.com) for details. This step requires 3 hours.

TC staining

Telomeres and centromeres of PCC fusions were stained using the Q-FISH technique with a Cy-3-labeled PNA probe specific for TTAGGG of telomere and a fluoroscien isothiocyanate-labeled PNA probe specific for centromere sequences (both from Panagene, Daejon, South Korea) [14] (described in Supplementary Data E2; available online at www.redjournal.com).

Slide scanning, interphase acquisition, and CA detection

The PCC fusions were automatically detected using MSearch software (MetaSystems, Altlussheim, Germany). Telomere and centromere–stained PCC fusions were acquired using automated acquisition module Autocapt software (MetaSystems, version 3.9.1) using a Zeiss Plan-Apochromat $63×/1.40$ oil and CoolCube 1 Digital High Resolution CCD Camera (Carl Zeiss, Jena, Germany). Manual scoring of PCC aberrations was performed using Isis software (version 5.5; MetaSystems).

For automatic scoring of CA after TC staining of PCC fusions, the acquired gallery was exported as a series of RGB bitmap images exploitable by any image-processing software. The 3-channel image contained the DAPI (4′,6-diamidino-2-phenylindole) information in the blue channel, and the telomere and centromere information in the red and green channels, respectively. Using the software tool developed in our laboratory (PCC-TCScore), the chromosomes were automatically segmented on the DAPI channel, where those of CHO were detected and filtered. A spatial position map of the telomeres and centromeres was created using information from the red and green channels. The chromosomes were then defined by classifying each object according to its corresponding attributes, and finally each was assigned to a corresponding aberration class (dicentric, centric ring, and acentric chromosome). The results must then be validated by the operator (Supplementary Data E3; available online at www.redjournal.com).

Scoring of CAs and calculated DSBs

Using the same slide, 2 types of manual scoring were performed: the first scoring was performed using uniform staining (in these analyses, reverse DAPI). Only rings and excess acentric chromosomes were scored. The second scoring was performed using TC staining to score dicentrics, centric rings, and acentric chromosomes from interstitial deletions without telomere staining, acentric chromosomes with only 1 telomere, representing terminal deletions, and acentric chromosomes with 2 telomeres derived from the fusion of 2 acentric chromosomes accompanying the formation of dicentrics or acentric rings. Resulting DSBs were calculated. Using uniform staining, rings with the accompanying acentric chromosome were counted as 2 DSBs. Acentric chromosomes were counted as one DSB. All of the information provided by TC staining allowed us to precisely calculate the number of DSBs that generated the CA: either a dicentric or a centric ring with a fragment containing 4 telomeres was considered as 2 DSBs. Excess acentric chromosomes are considered as resulting from 1 DSB for terminal deletions with only 2 telomeres and 2 DSB for interstitial deletion fragments with no telomeres. The formula used to calculate the DSB is provided in Supplementary Data E4 (available online at www.redjournal.com).

Automatic scoring was performed only on TC-stained PCC fusions because there is no existing image analysis system for uniformly stained PCC fusions.

To avoid observer bias, the slides were prepared, coded, hybridized, and analyzed blindly.
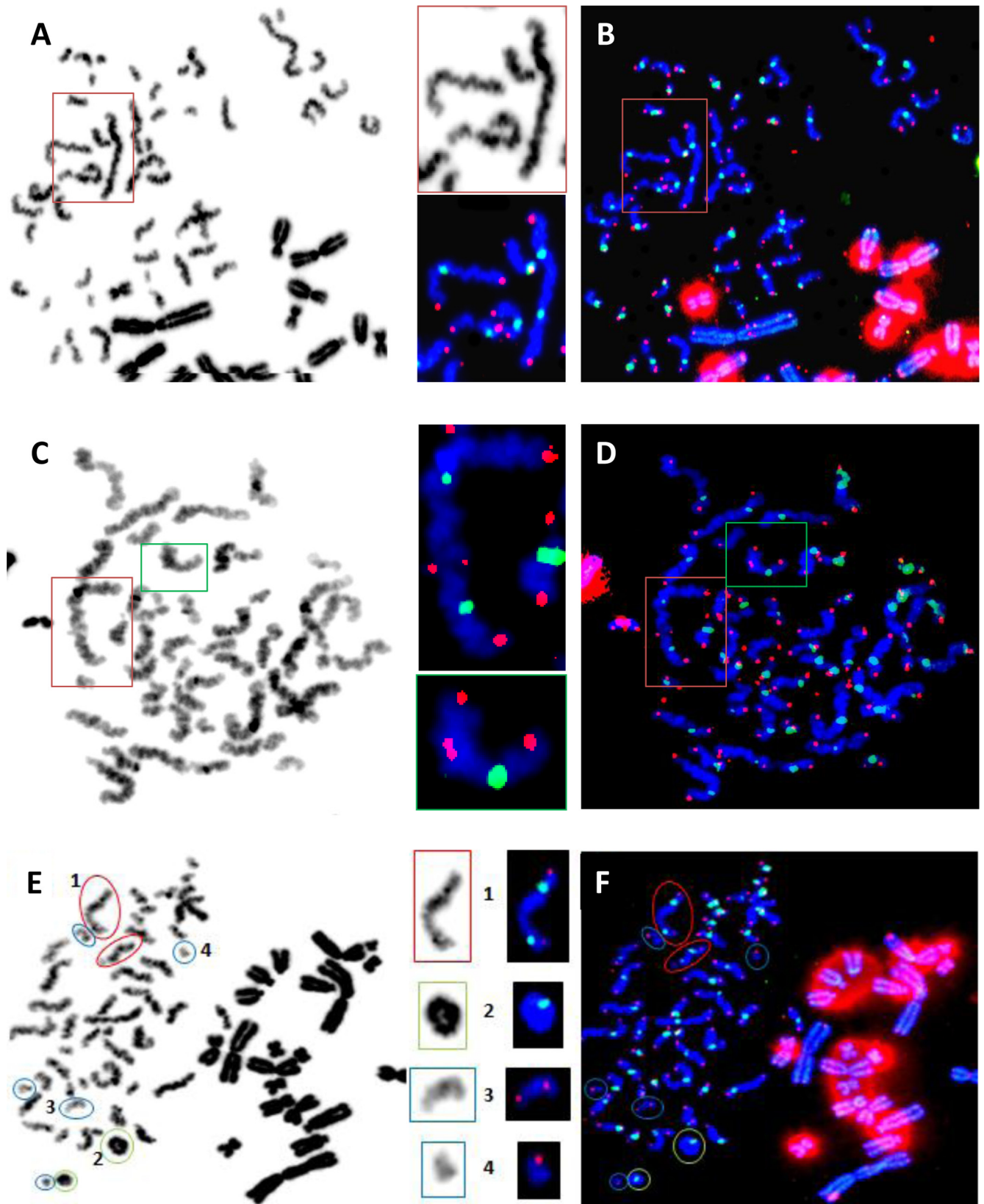
Fig. 1. Application of telomere and centromere (TC) staining to chromosomal aberration (CA) scoring of premature chromosome condensation (PCC) fusions. PCC fusions were subjected to uniform staining (A, C, E) or TC staining (B, D, F). (A) Uniform staining (DAPI [4′,6-diamidino-2-phenylindole] or Giemsa) of a PCC fusion does not allow the visualization of centromeric regions and the identification of dicentrics. (B) Centromere staining permits the scoring of dicentrics, but the

Table 1 Frequencies and distribution of chromosomal aberrations 8 hours after irradiation, detected after manual scoring using telomere and centromere staining

Dose (Gy)	Cells scored	No. dicentrics	No. rings	No. excess acentrics	Dicentrics + ring/cell	Dicentrics/ cell	SE	Dicentrics distribution										σ^2/y	U
								0	1	2	3	4	5	6	7	8	9		
0	101	1	0	1	0.009	0.009	0.009	100	1	0	0	0	0	0	0	0	0	1	0
2	186	62	16	164	0.419	0.333	0.042	134	43	8	1	0	0	0	0	0	0	1.027	0.262
4	164	198	49	266	1.506	1.207	0.085	55	45	43	17	4	0	0	0	0	0	0.99	−0.08
6	139	362	58	331	3.025	2.604	0.136	11	23	38	26	27	9	3	2	0	0	0.91	−0.69
8	124	472	83	373	4.475	3.806	0.175	4	8	15	28	31	16	12	7	2	1	0.86	−1.08

Abbreviations: σ^2/y = index of dispersion; SE = standard bar deviation; U = variance.

Statistical analysis

A script in R was developed taking into account the International Atomic Energy Agency recommendations (1). The curves generated in this study are primarily based on generalized linear models using the *glm* function of the standard *stats* package. Details are provided in Supplementary Data E4 (available online at www.redjournal.com).

Results

Optimization of capture parameters after TC staining of PCC fusions

After developing a specific classifier to automatically search for the PCC fusions, the optimization of PCC fusion capture permitted better visualization of the centromere regions and telomeric sequences, thus helping to resolve overlapping chromosomes, which is difficult in the absence of TC staining (Fig. 1A). However, the capture of telomere sequences has to be adapted to take into account the fact that the interstitial telomere sequences of CHO are much longer than those of human lymphocytes (Fig. 1B). Image capture was carried out using a fixed time by which the signal for the CHO telomeres was saturated, whereas human telomeres were easily visualized. Detection of centromere and telomere signals leads to the more precise scoring not only of dicentrics but also of CA, with better characterization of acentric chromosomes (Fig. 1A-F).

Manual detection of unstable CAs including dicentrics after TC staining of PCC fusions

After uniform staining, it is possible to score DSBs solely according to the presence of excess acentric chromosomes and rings (Fig. 1D) in PCC fusions. After TC staining it was possible to detect dicentrics, centric and acentric rings, and different types of acentric chromosomes (Fig. 1F).

Table 1 shows the number of PCC cells scored manually and the frequency of excess fragments and ring chromosomes detected after uniform staining, as well as the frequency of dicentrics, centric rings, and excess acentric chromosomes after TC staining. In addition, the distribution of dicentrics per cell and their associated σ^2/Y and U values obtained from 0 to 8 Gy are shown. A Poisson distribution was observed at each dose with no dispersion.

A linear dose−response curve was obtained upon the scoring of acentric chromosomes after uniform staining (Fig. 2A), confirming previously published data (Table E1; available online at www.redjournal.com). However, using TC staining, a linear-quadratic dose−response curve was obtained, and a significant difference was observed between the 2 curves ($P < 10^{-4}$). The number of acentric chromosomes detected after TC staining was up to 2- to 3-fold greater at higher exposures than that after uniform staining (Fig. 2A). Figure 2B shows the frequency of different types of acentric chromosomes. We observed a higher frequency of acentric chromosomes with only 1 telomere induced by a terminal deletion. This higher frequency can be related to incomplete DNA repair at 8 hours after higher doses of exposure.

demarcation of the chromosome ends requires telomere staining as demonstrated in this figure (boxed in red). (C) The difficulty in determining the chromosome ends after uniform staining. (D) The importance of telomere staining for the elimination of false dicentrics and for the robust scoring of acentric chromosomes. (E) Dicentrics are difficult to detect (circled in red), leaving only the possibility to detect rings (circled in green) and acentric chromosomes (circled in blue). (F) TC staining of a PCC fusion. After telomere (red) and centromere (green) staining, the following criteria were established: dicentrics, 2 green centromere signals with 2 red telomere signals (1); ring chromosomes, 1 green signal without a red signal (2); and acentric chromosomes, no green signal with either 2 (3) or 1 red signal (4). Numbers in parenthesis refer to the numbers shown in E and F.

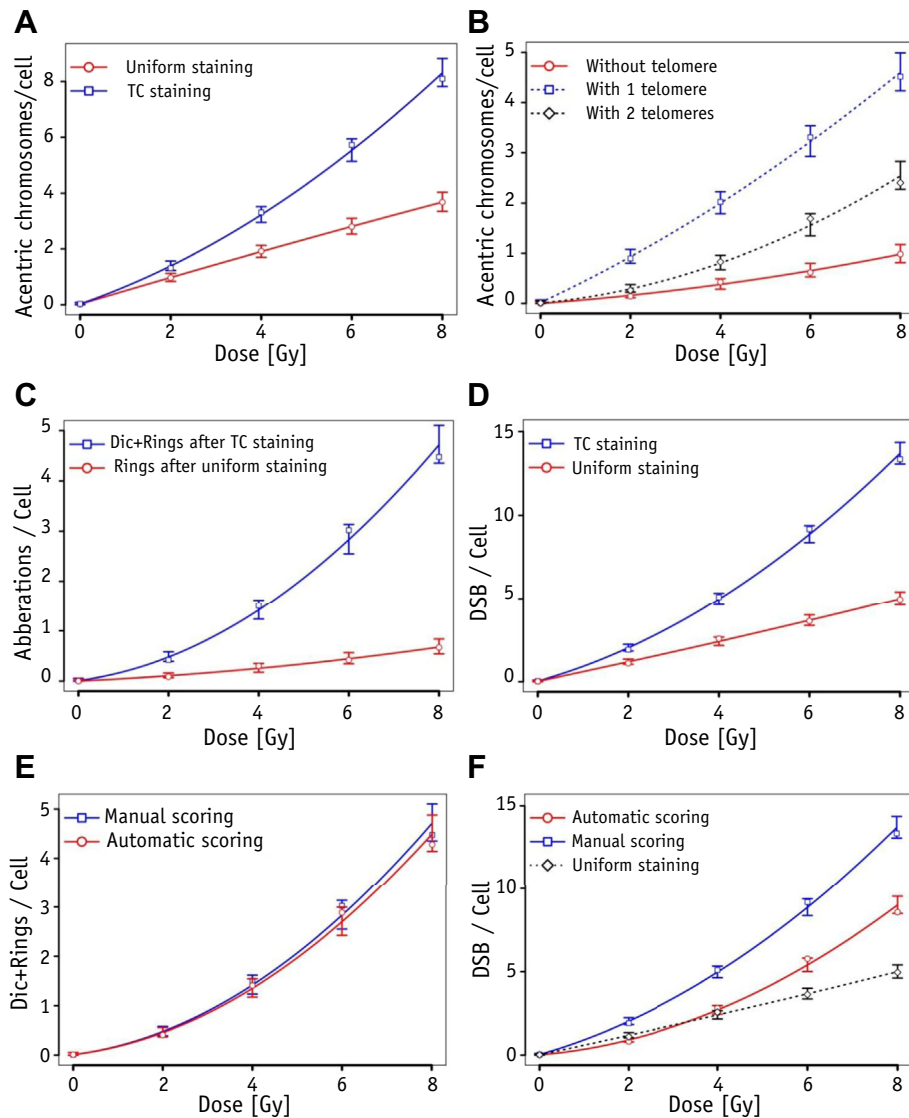**Fig. 2.** Manual and automated chromosomal aberration (CA) scoring in premature chromosome condensation (PCC) fusions after uniform or telomere and centromere (TC) staining. In all cases, error bars represent the 95% confidence limits. (A) Dose−response curves obtained after the scoring of all CAs after uniform or TC staining. (B) Dose−response curves obtained after the scoring of different types of CAs after TC staining. (C) Dose−response curves obtained after the scoring of dicentrics (Dic) and rings after TC or uniform staining. (D) Dose−response curves based on the estimated number of double-strand breaks (DSBs) after the scoring of dicentrics (Dic) and rings after TC or uniform staining. (E) Comparison of dose−response curves for dicentrics and rings per cell after manual or automated scoring after TC staining. No significant difference was observed. (F) Comparison of dose−response curves, including that for uniform staining for calculated DSB per cell.

After uniform staining, a linear dose−response curve was obtained upon the scoring of rings in the PCC fusion (Fig. 2C) with no saturation. After TC staining, a linear-quadratic dose−response curve was obtained with no saturation at up to 8 Gy (Fig. 2C). A significant difference was observed between the dose−response curve obtained for dicentrics plus rings after TC scoring and that obtained for rings only, after uniform staining ($P < 10^{-6}$) (Fig. 2C).

Using the calculated DSBs based on all unstable CAs, a linear dose−response curve was obtained after uniform staining and a linear-quadratic curve after TC staining. The calculated DSBs after TC staining were significantly higher than those observed after uniform staining ($P = .005$) (Fig. 2D). The curve fit coefficients for all dose−response curves are shown in Supplementary Data E5 (available online at www.redjournal.com).

Automated scoring of unstable CAs after TC staining of PCC fusions

The reliable and robust detection of CAs in PCC fusions allows the possibility to automate their scoring, which has not been previously possible. Figure 3A shows a simplified version of the workflow used by PCC-TCScore. The 3-channel image contains the DAPI information in the blue channel and the telomere and centromere information in the red and green channels, respectively. The human chromosomes are filtered to CHO chromosomes using the saturated red channel zone and complementary information about large objects from the DAPI channel (blue block), and the centromeres and telomeres are detected. The chromosomes are then defined by the number of telomeres and centromeres and finally classified according to the aberration class (Supplementary Data E3; available online at www.redjournal.com). This processing scheme allows for batch processing and report generation using a highly intuitive and interactive user interface designed for rapid review and correction by the user (Fig. 3B). The requirements for the use of this software are minimal, allowing its use on any computer, and images can be treated regardless of the type of image capture platform used. An average of 7 seconds per image was required using the software, compared with 2 to 3 minutes for manual scoring.

Linear-quadratic dose−response curves for automated scoring of dicentrics and centric rings, but not taking into account acentric chromosomes, were obtained (Fig. 2E). No significant difference was observed between manual and automated scoring of PCC fusions using PCC-TCScore at up to 8 Gy, whereas those for calculated DSBs based on all CAs (Fig. 2F) did show a significant difference in favor of manual scoring because the current version of the software does not accurately detect acentric chromosomes (Table E2; available online at www.redjournal.com).

Discussion

The dicentric chromosome assay is the "gold standard" biodosimetry method for recent exposure to ionizing radiation (1). Nonetheless, the non-compressible time required to obtain the metaphases remains the major obstacle for obtaining a rapid dose estimate, and the application of this technique for triage of a population after a mass exposure event. The PCC fusion technique induces the premature condensation of chromosomes, allowing rapid scoring of radiation-induced CA, but the complexity of the technique and subsequent analysis of the data restrict its use. Given that we have recently demonstrated the high efficiency of TC staining in the scoring of dicentrics and rings in metaphases (15), we decided to combine this approach with the PCC fusion technique. Thus, in this study we introduce for the first time the scoring of dicentrics, centric rings, and acentric

chromosomes in PCC fusions after TC staining using PNA probes, permitting the reliable detection of all unstable CAs in PCC fusions with a high level of precision and sensitivity. This amelioration allows the automation of the scoring of dicentrics and centric rings. The application of automated software to detect aberrations in PCC fusions would represent a major advance in biological dosimetry after an accident, as well as in the area of radioprotection in general, where conventional cytogenetics is not sufficiently sensitive to detect the effects of exposure to low doses (16).

Many applications of the PCC fusion technique have been used in research and the clinic. In particular, drug-induced PCC assays have been extensively used to investigate the mechanisms underlying the formation of radiation-induced aberrations (17, 18) or bystander effects (19). Premature chromosome condensation has also been used to perform cytogenetic evaluation of quiescent and senescent cells, as well as to increase the number of mitotic cells within tumor samples, allowing for rapid karyotyping after a very short-term culture, permitting the evaluation of karyotype diversity (20). It has also been used to contribute to clinical prediction (21) and the cytogenetic investigation of various diseases (22, 23). Another important application of this technique is the assessment of genotoxic risk immediately after exposure to various reagents (24), and it has also proven useful for investigating chromosomal damage immediately after irradiation, as well as the kinetics of CA formation (25). The PCC technique has been shown to be compatible with a number of chromosome staining techniques, including C-banding, centromere detection by FISH, and chromosome painting (7, 8, 26-29). It has also been recognized as a powerful biodosimetric tool in an internationally standardized technical report (1). Despite the utility of this technique, the scoring of CAs in PCC fusions has, nevertheless, remained technically challenging and time consuming, limiting routine use, even in research, despite the advantages associated with the absence of cell culture and the ability to analyze interphase chromosomes. As such, this technique is used solely by a few specialists. In this work we chose to perform PCC fusions using the CHO cell line as opposed to the drug-induced PCC assay because it does not allow a significant time reduction compared with cell culture (44 hours vs 48 hours for metaphase). In contrast, we performed PCC fusion 8 hours after irradiation, allowing time for DNA repair and the occurrence of dicentrics, centric rings, and acentric chromosomes (3 hours vs 49 hours for metaphase). Indeed, in the case of accidental exposure, the time delay after irradiation, including blood sampling, is variable, and would probably exceed 4 hours without including the additional time for transport and treatment. Because the time of DNA repair may vary between individuals, and owing to the conditions of storage/transport, the ability to score DSBs allows the estimation of the absorbed dose independently of DNA repair processes. It is for this
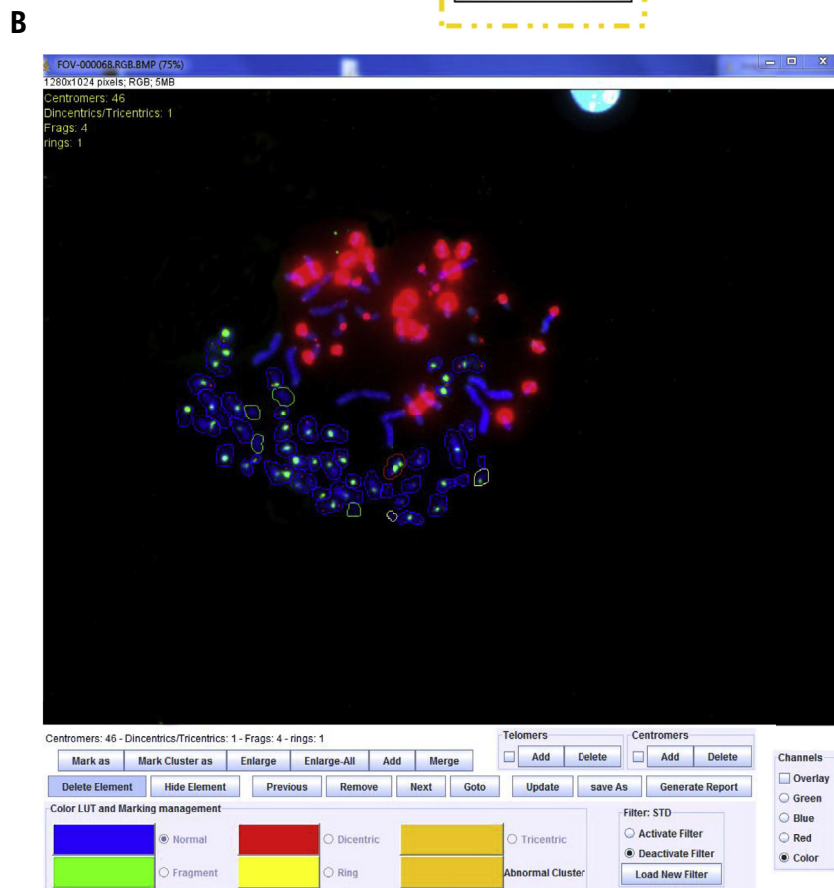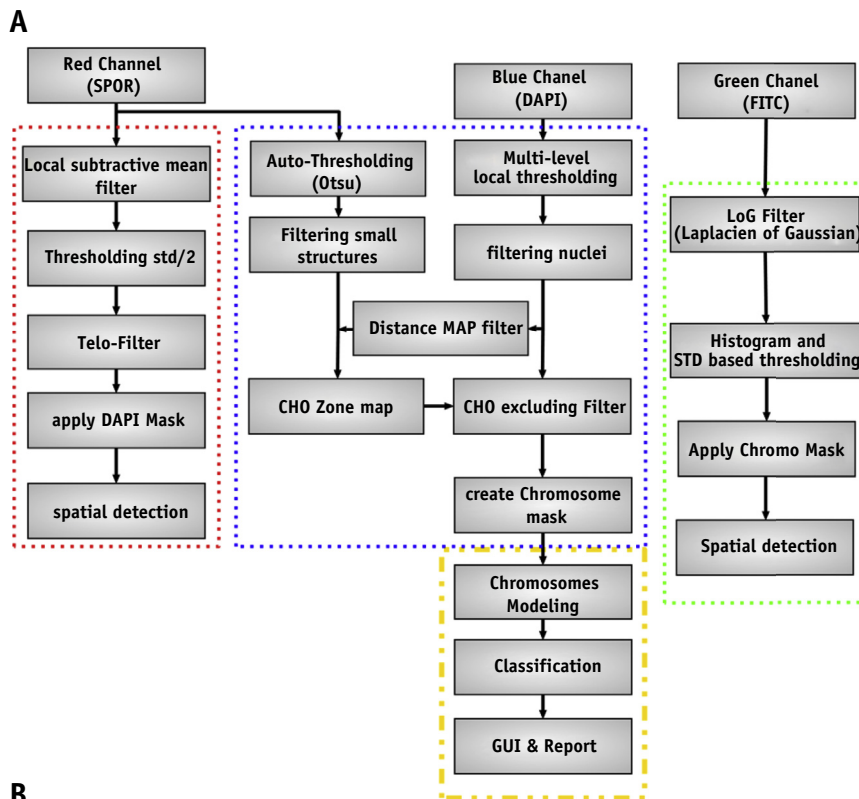
Fig. 3. Summary of the automation process used to develop the softward PCC-TCScore. (A) Chinese hamster ovary (CHO) chromosome is defined as being larger than the human one with an intense telomere signal. To filter out the CHO chromosomes and create a human chromosome map, a CHO zone map is constructed using the saturated red channel zone and complementary information about large objects from the DAPI (4′,6-diamidino-2-phenylindole) channel (blue block).

reason that it is indispensable to take into account all CAs, including all the different types of acentric chromosomes, made possible by TC staining, for estimating the dose.

After uniform staining, we have scored ring chromosomes and acentric fragments. The frequency of chromosome rings is in agreement with previously published data for 8 hours after exposure (30) but higher than that obtained for chemically induced PCC rings (31).

The introduction of TC staining with PNA probes, with higher signal intensity and a short hybridization time, has permitted the identification of centromeric regions and telomere sequences, allowing the precise determination of the number of centromeres in each object and the limits of each chromosome to remove all ambiguity in the scoring of CAs. This improvement has allowed the scoring of all unstable CAs, including dicentrics, and the calculation of the number of resulting DSBs with higher precision, because of the detection of interstitial acentric chromosomes due to telomere staining. Telomere and centromere staining represents a significant improvement compared with other studies that have used centromere staining alone, which was the first attempt to detect dicentrics in PCC fusions, but many uncertainties remained, and there was poor detection of acentric chromosomes.

The combination of telomere and centromere staining with PCC fusion thus represents an important advance in the detection of unstable CAs in PCC fusions. Importantly, because of the higher signal obtained using PNA probes, the manual scoring of dicentrics, centric rings, and acentric chromosomes can be directly carried out at the microscope in the absence of an image analysis system.

After the successful detection of CAs in PCC fusions and the establishment of a dose–response curve, the second step of this work was the semiautomation of the scoring of these aberrations using a new software that we have developed, called PCC-TCScore, which is independent of the image analysis system used. Using PCC-TCScore we have successfully detected on average 95% of dicentrics and centric rings compared with manual scoring, with the level of automated detection at or near 100% below 4 Gy,

falling to 95% at 6 to 8 Gy. This high rate of detection is made possible owing to the robust recognition of CAs after TC staining, the high quality of the PCC fusions, and the validation step by the observer.

It may be equally important to apply these tools to the field of low dose exposure, which is currently a growing public health concern. The PCC fusion technique associated with whole-chromosome painting FISH on 3 chromosomes has been successfully used to measure the biological effects of CT scans and to estimate the absorbed dose (28). Such information is key to advancing our knowledge in the field of low-dose exposure to better define radiation protection standards. Moreover, this assay is currently used in only a limited number of laboratories. Its application for biological dosimetry in an emergency response to radiation exposure would need to be preceded by assimilation of the assay into qualifying laboratories. In both cases the introduction of TC staining will permit more widespread use of PCC fusion techniques and the exploitation of its full potential.

Conclusion

In the present work we demonstrate that the contribution of TC staining to the detection of CAs, including dicentrics, eliminates the time-dependent problem of underestimating the frequency of acentric chromosomes owing to their reparation encountered when using uniform staining. This approach creates new possibilities for the scoring of unstable CAs when conventional techniques are inadequate and when the rapidity of the answer is a prerequisite.

The accurate detection of each type of CA has allowed the development of PCC-TCScore for the scoring of dicentrics and centric rings from captured images of PCC fusions. It will open up new horizons for biological dosimetry, in particular for low, high, and partial-body irradiation, as well as for cytogenetic studies concerning chromosomal instability, the heterogeneity of the karyotype, and when cell culture is not possible. Furthermore, individual responses to genotoxic agents could be

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Telomere location is mapped by filtering red channel information using a local subtractive mean filter and binarizing the result using half of the standard deviation as a threshold. Data are restricted to the human chromosome mask (red block). To detect centromeres, a LoG filter is applied to the green channel, followed by a threshold calculated using histogram information and the data standard deviation value. Results are confined to the human chromosome mask. Finally, the human chromosome mask, and the telomere, and centromere location are merged for chromosome modeling and classification. (B) Screen shot from PCC-TCScore showing a premature chromosome condensation (PCC) fusion from lymphocytes irradiated at 4 Gy. The numbers of centromeres, dicentrics, centric rings, and acentric chromosomes are provided. In this case, the software detected 3 dicentrics (circled in red), 3 acentric chromosomes (circled in green), and 2 abnormal clusters (circled in yellow), permitting the end-user to examine these anomalies in more detail. In the case of overlapping chromosomes, which are difficult to segment, the software does not illuminate the PCC fusion but allows the operator to perform manual corrections. Although the process is fully automated, PCC-TCScore allows the operator to validate the chromosome classification using the attractive and user-friendly interface.

monitored, including the follow-up of patients and their individual response to treatment by radiation or chemotherapy.

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