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Development and application of the PCC biodosimetry method in emergency preparedness

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Abstract

A method for biological assessment of radiation dose for specific application in emergency preparedness was developed. Premature chromosome condensation (PCC) was investigated to provide a potentially faster means of analysis and the ability to assess higher doses than with the dicentric assay which is routinely applied in biodosimetry today.
A review of existing methods was made, followed by experiments determine optimal assay conditions, and evaluations to determination of optimal conditions and the most appropriate endpoints for analyses. Twelve different experimental conditions were examined with four different evaluation approaches. Aspects during optimization such as practicality, speed, and reliability were considered. The conclusion from these studies was a PCC protocol utilizing okadaic acid for induction of PCC cells in stimulated lymphocytes but without the use of colcemid for metaphase arrest with the subsequent evaluation of ring chromosomes. Well-defined criteria were established for evaluation of PCC cells and ring chromosome aberrations. An inter-calibration was made by comparing assessment of ring chromosomes between all three laboratories. Agreement was made to count only rings with observable open spaces or large, obvious rings without open spaces.
The finally a dose response curve for the PCC method was prepared and a comparison of the PCC method to the traditional dicentric assay in triage mode was made. The triage method requires a minimal number of evaluations so that categorization of high, medium and low doses may be made in an emergency situation where large numbers of people should be evaluated. The comparison of the PCC method with the dicentric assay triage method indicated that the PCC assay performed superior to the dicentric assay for evaluation of samples at higher doses, however, the dicentric assay appeared to provide more accurate dose assessment at lower doses.

Key words

Biodosimetry, PCC, dicentric assay, dose reconstruction, emergency preparedness

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Final report of the NKS-B project Biodosimetry Applications in Emergency Preparedness - BioDos

2006-2007

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Abstract

The aim of the 2006-07 NKS-B BioDos project was to establish improved methods for biodosimetry that has specific application in emergency preparedness. In this project, the PCC assay for biological assessment of radiation exposure was established in our laboratories. The range of work covered in our activities included assay optimization, analysis optimization, development of scoring criteria for PCC-rings, comparison of the method to the classical cytogenetic approach, and development of a PCC-ring dose response curve. The results of our work include an optimized approach for preparation and evaluation of the PCC assay for fast biological assessment of radiation dose which could be potentially applied in a triage manner in the event of a significant accident involving many persons.

The BioDos project has further served to build an informal network between the two organisations currently conducting research, STUK and FOI, in this field and a third, NRPA, the Norwegian Radiation Protection Authority, in order to provide capabilities in the event of an emergency and to expand the capacity of the individual labs.

Key words

Biodosimetry, PCC, dicentric assay, dose reconstruction, emergency preparedness

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Executive Summary

The Swedish Defence Research Agency, Finnish Radiation and Nuclear Safety Authority, and Norwegian Radiation Protection Authority have worked together to optimize a method for biological assessment of radiation dose for specific application in emergency preparedness. A variation of the dicentric assay in which chromosome are prematurely condensed (premature chromosome condensation, PCC) was investigated. PCC provides a potentially faster means of analysis as well as the ability to assess higher doses than with the dicentric assay which is routinely applied in biodosimetry.

This work describes the review of existing methods and literature in the field, experiments which were conducted to determine optimal assay conditions, and subsequent evaluations to support determination of optimal conditions as well as the most appropriate endpoints for analyses. A total of 12 different experimental conditions were examined within this work, along with four different evaluation approaches. Aspects of the different approaches such as practicality, speed, and reliability were considered during the review of the methods and experiments conducted within this work.

The conclusion from these studies indicated that a PCC protocol utilizing okadaic acid for induction of PCC cells in stimulated lymphocytes but without the use of colcemid for metaphase arrest with the subsequent evaluation of ring chromosomes. Due to the diminished quality of chromosomes in calyculin A preparations, okadaic acid was chosen and exclusion of colcemid for the same reason.

The next step in this work was to establish well-defined criteria for the evaluation of PCC cells and ring chromosome aberrations. This was completed by conducting an inter-calibration of accessing ring chromosomes between all three laboratories. Agreement was made to count only rings with observable open spaces or large, obvious rings but without observable open spaces due to poorer quality PCC spreads or poor Giemsa staining.

The final step in the BioDos project involved the preparation of a dose response curve for the PCC method and a comparison of the PCC method to the traditional dicentric assay in triage mode. The triage method requires a minimal number of evaluations so that categorization of high, medium and low doses may be made in an emergency situation where large numbers of people should be evaluated. Irradiated blood samples for 10 dose points were prepared and then PCC and dicentric assay cultures were prepared in parallel at STUK. Seven dose points of the PCC samples were evaluated by FOI and STUK to prepare a limited dose response curve for which to be used in the triage comparison. The comparison of the PCC method with the dicentric assay triage method indicated that the PCC assay performed superior to the dicentric assay for evaluation of samples at higher doses, however, the dicentric assay appeared to provide more accurate dose assessment at lower doses.

The results of this project provide a PCC assay method for biological dose estimates for emergency preparedness purposes. The conclusions from our work suggest that an optimal approach in emergency applications would be the parallel culture of blood samples for both PCC and dicentric analysis, followed by triage analysis to indicate high or low dose, followed by more detailed analysis by either for PCC rings or dicentrics depending on the magnitude of dose indicated (above or below 5 Gy) as time allows.

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1. Introduction

1.1 Background

Biological evaluation of radiation exposure, or biodosimetry, is of great importance in radiation emergency situations when individuals are probably not monitored by physical dosimetry or in cases where physical dosimetry is unreliable. In such cases, biodosimetry may be the only available method for assessing an exposure and dose. A reliable dose estimate is needed, particularly in high dose exposures, in order to determine medical treatment for the exposed individual. Furthermore, after an accident or significant radiological event, radiation protection authorities, health services and in some cases, defence agencies will need access to such methods and competence in order to evaluate and reassure potentially exposed individuals. Accident scenarios, such as an explosion occurring in a nuclear facility or the detonation of a nuclear device, would result in highly exposed persons directly involved as well as emergency responders. Therefore, very rapid and reliable dose estimation of numerous individuals would be necessary for life-saving medical decisions.

In the Nordic countries, biological dosimetry is presently performed at two laboratories. It has been established for many years at STUK-Radiation and Nuclear Safety Authority and in the last few years, FOI, the Swedish Defence Research Agency has been developing methods in this area as well. In both laboratories, the classical dicentric assay has been applied as the method of choice for biological dosimetry. This method is internationally acknowledged as the most reliable method for this purpose (see IAEA 2001 technical report series 405). However, limitations which are described below exist with the method. This project proposes to establish improved methods for biodosimetry that has specific application in emergency preparedness. The project serves to build an informal network between the two organisations currently conducting research, STUK and FOI, in this field and a third, NRPA, the Norwegian Radiation Protection Authority, in order to provide competence and capabilities. For preparedness, the network could further function to provide resources, such as additional technical expertise, to speed biodosimetry evaluation in emergency cases where a single lab's capacity could easily be overwhelmed. Likewise, these resources could be extended to the other Nordic countries in the future.

1.2 Current Methods and Limitations

The classical dicentric method is considered as the gold standard in biological dosimetry. In this technique, a small blood sample from an individual is taken, and blood lymphocytes are cultured for 48 hours, followed by evaluation of metaphase chromosomes for specific damage in the form of dicentric chromosomes. While this method has been widely applied for many years and is fully validated for biological dose assessment, some improvements are needed for emergency preparedness purposes. The classical assay requires a two day incubation period followed by at least another day for analysis and dose estimate. In scenarios where high doses are received, timing is critical and an assessment of dose is critical for making life-saving medical decisions. A faster evaluation would be advantageous in the treatment planning process. Furthermore, with high dose exposures, much fewer lymphocytes may be available for evaluation and the response of the assay marker does not follow the linear quadratic curve at very high doses (Lloyd 1983). These problems could result in inaccuracy or even the inability to perform the assay. In recent years, methods describing the use of chemicals to induce premature chromosomal condensation (PCC) in lymphocytes have been reported (Durante 1997, Kanda 1999, Prasanna 2000). The new methods increase the number of cells for evaluation and simplify the technique, thereby decreasing analysis time. In addition, one

variation of the method has been shown to shorten the incubation period for the lymphocytes by one day. Finally, the PCC technique in inducing chromosome condensation and increasing cells for evaluation helps with the problems of cell death and mitotic delay occurring during lymphocyte culture following a high dose of radiation found with the traditional dicentric assay techniques. Therefore, the PCC method can address the shortcomings of the classic dicentric assay by providing a faster assay that is sensitive even at very high doses and potentially easier and hence quicker to analyze. The criticality accident in Tokai-mura can be given as a successful example on the use of the PCC technique (Hayata 2001). Biological dosimetry for three victims was performed by scoring the yields of PCC-rings which resulted in dose estimations of approximately 2, 7 and more than 20 Gy equivalents, respectively (Hayata 2001). With a more rapid and technically less demanding approach for evaluating biological dose, the PCC assay provides a better tool for emergency preparedness application where time is critical and answers are needed fast, both for making medical decisions and for reassuring the public.

2. Purpose

The specific aim of this project is to establish the PCC assay for biological assessment of radiation exposure in the participating laboratories. The first phase of the project has focused on development of optimal assay conditions and optimal analysis methods. Based on the conclusions from this work first phase, the second phase of the project has focused on the development of scoring criteria for PCC rings, development of a PCC - ring dose response curve, and the comparison of the PCC assay to the classical method, the dicentric assay, performed in triage mode.

3. Materials and Methods

3.1 Technical Exchange between Partners

The partners from FOI, NRPA, and STUK met at FOI in Umeå, 1-2 February 2006, to initiate the project and plan in detail the activities for the year. This meeting consisted of a literature review and discussion, and the results from the meeting were documented in a protocol from the meeting. The partners met in July 2006 in conjunction with the BiodosEPR conference in Bethesda, MD, USA. A follow-up discussion on the progress of the project took place and partners reviewed pictures of cells obtained by each participant. A working meeting for the conclusion of the 2006 project work and for preparing the report and activities for continuation was held at STUK in Helsinki 28-29 November 2006.

The second phase of the project was continued according to schedule by a visit from FOI to STUK in the spring of 2007 to conduct a large experiment for which to generate all of the samples required for the PCC dose response curve and dicentric assay comparison. A working meeting intended to refine scoring criteria was held at the NRPA in August 2007 in conjunction with the NKS-B status seminar in which the progress of BioDos was presented.

Samples have also been periodically exchanged. In the first phase of the project, slides of Giemsa stained PCC preparations from FOI were made sent to the NRPA for evaluation and comparison of recorded frequencies. Unstained slides from FOI were sent to STUK for FISH painting experiments. STUK also made available Giemsa stained replicates from PCC experiments conducted there. Samples prepared in the second phase of the project were distributed by STUK to FOI and NRPA as needed.

3.2 Experiments

3.2.1 Preparation of Samples for Culture and Analysis Comparisons

The following laboratory experiments were conducted by FOI to generate PCC cells for evaluation. FOI generated PHA stimulated samples with chemical induction of PCCs by the phosphatase inhibitors, okadaic acid (OA) and calyculin A (Cal A) for Giemsa and FISH evaluations and unstimulated PCC chemically induced cells with kinase for FISH evaluation. Each experiment was conducted on whole blood exposed to 3 doses: 0, 4, and 8 Gy (^{137}Cs). Conditions 1 and 2 were replicated at least once during the course of this work.

1. Chemical induction without colcemid in stimulated cells (Kanda 1999)
    a. OA, 500nM last 1 hr 48hr incubation
    b. Cal A, 50nM last 1 hr 48hr incubation
2. Chemical induction with colcemid in stimulated cells (Durante 1998)
    a. OA, 500nM 0.04ng/ml colcemid after 24hr last 1 hr 47hr inc.
    b. Cal A, 50nM 0.04ng/ml colcemid after 24hr last 1 hr 47hr inc.
3. Chemical induction with colcemid in stimulated cells (Lamadrid 2006)
    a. OA, 500nM 0.04ng/ml colcemid after 24hr last 1 hr 48hr inc.
    b. Cal A, 50nM 0.04ng/ml colcemid after 24hr last 1 hr 48hr inc.
4. Chemical induction facilitated by cyclin B kinase and ATP in unstimulated cells (Prasanna 2000)
    a. OA, 500nM 50u/ml kinase 3 hr inc. (after 24hr repair time)
    b. Cal A, 50nM 50u/ml kinase 3 hr inc. (after 24hr repair time)

In brief, for the stimulated cultures (1, 2, and 3 above), 20% FCS, 2 % PHA, 1 % Na Heparin, and media containing glutamine and gentamycin were used. The unsitmulated cultures (4) had the same media minus the PHA but including kinase and ATP for activation.

3.2.2 Preparation of Samples for FISH Painting Experiments

STUK conducted the following experiment to generate samples for FISH experiments. Blood samples were collected and irradiated using a Co-60 source (0.47 Gy/min) for preparation of the following doses: 0, 2.5, 5, 7.5 and 10 Gy. Samples were then incubated in 37 ºC water bath for about 2 hours for "repair" time. Lymphocyte separation was conducted using Histopaque and Accuspin tubes. Cultures were prepared (RPMI1640, 20%FCS, 1%PS, 1%L-glutamine, 1% PHA) with 0.5 to 0.7 x 10^6 cells/ml in 5 ml culture tubes. Cells were cultured for 48 hours at 37 ºC. Okadaic acid added at 47 hours to a final concentration of 500 nM. Cells were routinely harvested and slides prepared.

3.2.3 FISH Painting Experiments

A variety of FISH painting experiments were tested. One incorporated whole chromosome probes for chromosomes 2 (FITC), 4 (Cy3) and 12 (FITC) from Cambio. Single chromosome probes as well as different combinations of probes were used during several painting experiments. FISH painting was conducted according to manufacturer's instructions with slight modifications: denaturation for 1min 30sec or less (PCC cells tend to be more sensitive

for denaturation than metaphases). DAPI was used as the counterstain with triple painting and propidium iodide (PI) for FITC single probes.

3.3 Scoring Criteria

Strict scoring criteria (only rings with easily observed open spaces) as well as less stringent scoring criteria (all reasonably certain rings, even when open spaces are not observed) were tested by each of the 3 evaluators for evaluation of 2 samples (OA/ no colcemid/ STUK/ 2.5 and 10 Gy samples; 500 cells each). Scoring down the microscope rather from pictures was determined necessary in order to reliably identify both categories of rings. An effort was made to only evaluate complete metaphases; however counting of 46 chromosomes was not considered mandatory.

3.4 PCC Curve and Dicentric Assay Comparison

3.4.1 Preparation of Samples

Whole blood was drawn from a single volunteer into 20 vacutainer tubes and irradiated by a ^{60}Co gamma source in STUK's Radiation Metrology Laboratory at 0.33 Gy/ min to provide the following doses: 0, 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 Gy. The samples were kept in a 37°C water bath and inverted for mixing as necessary under the irradiation period. The blood samples were then incubated for 2 hours at 37°C to allow for repair of DNA damage.

Lymphocytes were separated using Ficoll and Acuspin tubes and placed at a density of 0.4 10^2/ ml and placed into replicate cultures. Both the PCC protocol using okadaic acid without colcemid in a 48 hour cell culture (RPMI with 20% FCS and 1% PHA) as developed in this work and standard procedures for metaphase preparation for dicentric analysis were performed. Sample tubes were blinded by an independent party by placing coded labels on samples. Cells were harvested and slides of selected samples were prepared.

3.4.2 PCC Dose Response Curve

The doses selected for the analysis for the preparation of the dose response curve were: 0, 1, 2.5, 5, 10, 15, and 20 Gy. The limited PCC dose response curve was developed by blinded analysis of at least 100 PCC rings or 500 PCC cells by each evaluator for each dose point selected and data for each point by each evaluator was combined.

3.4.3 PCC-Ring Assay and Dicentric Assay Triage Comparison

Coded slides for each evaluator were prepared from three samples from matching PCC and dicentric assay preparations. Blinded analyses of the samples were conducted by the evaluators in triage mode, i.e. limited number of evaluations: 300 PCC cells or 30 PCC rings; 50 metaphase cells or 30 dicentrics.

Dose estimates were prepared according to each laboratory's own dicentric dose response curve for gamma radiation (NRPA's estimate was prepared using STUK's curve). The PCC data was prepared using the linear curve described in this work.

4. Results and Discussion

4.1 Evaluation of Culture Conditions

Several different culture methods were tested and compared to determine optimal assay conditions. The variables included the type of phospatase inhibitor used, the use of kinase in unstimulated cultures, the length of the culture, and the use of colcemid to prevent cell division. The preparations from each set of experiments were compared for the quality and quantity of cells rendered. These aspects are listed below.

Induction Approaches	Test Conditions	Evaluation
Okadaic acid	+/- colcemid	Quality of cells
Calyculin A	Variable incubation times	Yield of scorable cells
Okadaic acid + kinase		
Calyculin A + kinase		

The percent of PCC cells in different phases, scoreable and non-scoreable PCCs, and total PCC induction for different experiments were prepared for comparison (data not shown). While the percent of OA induced PCCs are lower than Cal A, the quality of the resulting PCC cells are more variable in those preparations.

4.1.1 Kinase Method

The method of PCC induction utilizing cyclin B kinase together with phosphatase inhibitors (OA or Cal a) in unstimulated lymphocytes did not produce adequate quality of cells; i.e. not enough condensation nor enough cells in number for a good evaluation. Furthermore, the kinase is a very labile enzyme which must be stored at extremely cold temperatures and may only be reliably used for a few months after receipt. The enzyme is also quite expensive and not readily available. It is made in batches and may not be uniform or consistent between batches. These PCC cells which show only the slightest amount of condensation require FISH for any evaluation or analysis. For these reasons, this approach does not appear to be accessible to many labs.

4.1.2 Choice of Phosphatase Inhibitor

Experiments with both phosphatase inhibitors, Cal A and OA, have been conducted. In general, with the “optimal” concentration of either phosphatase inhibitor, Cal A results in a higher yield of PCC cells in comparison to OA. However, the Cal A induced cells are not of the same quality as OA induced cells. Cal A induced PCC cells tend to be somewhat shorter making rings harder to identify. In experiments without colcemid, OA indicated higher PCC ring yields in comparison to Cal A induced PCC cells which might be an indication that rings can more easily be identified in these preparations due to improved quality of the cells.

For practical concerns, OA may be reconstituted in media and stored in practical aliquots. Cal A is recommended to be reconstituted in a tiny volume of ethanol of which 1.25 μL of

chemical is placed in cultures. There is concern over reproducible pipetting of small volumes as well as evaporation of EtOH resulting in altered concentration of reagent.

4.1.3 Colcemid verus No Colcemid

Colcemid is used in the classic dicentric assay to arrest cells in metaphase and prevent cells from continuing on to divide. The use of colcemid after 24 hours of culture in Durante's method (1998) and later in Lamadrid's method (2006) is presumably to help ensure that cells are in first division. Some cells containing unstable aberrations either may not go on to divide or may not retain the unstable aberrations in later divisions. A percent of the ring chromosomes, the aberration type prioritized in this work, are likely to be lost during subsequent divisions. Hence the ring frequency may be underestimated with inclusion of second division cells.

The primary concern with the use of colcemid is the resulting quality of PCC spreads. Prolonged exposure to colcemid tends to degrade the chromosome quality and result in the shortening of chromosome arms which would then make the identification of rings more difficult. To determine whether colcemid had an effect on chromosome morphology, preparations from cultures with and without colcemid were evaluated for the quality of chromosomes to determine if colcemid did result in chromosomes that are more difficult to evaluate. In general, the conclusion was that preparations from cultures with prolonged colcemid treatment contained chromosomes that were more condensed and shorter. This did appear to affect the ability to evaluate ring chromosomes. The samples exposed at 8 Gy prepared without colcemid showed higher frequencies of rings than those from colcemid experiments (see Figure 1). This observation is an indication that rings are more easily identified in preparations that have not used colcemid. This factor must compensate for any underestimations due to inclusion of second division cells. However, whether this will impact reproducibility must still be addressed.

4.1.4 Other Considerations

One special consideration which became apparent during the experiments was the differential sensitivity to the cells during the bloating step of the cell harvest. In several experiments, cells suffered from over-bloating, resulting in loss of many cells. A limited bloating time appears to be particularly important. However, while Cal A treated cells seemed particularly sensitive to hypotonic treatment, OA treated cells actually appears to be rather resistant.

The difference in culture time between Durante (1998) and Lamadrid (2006) which was 47 or 48 hours did not seem to make a difference in the yield or quality of PCCs.

4.2 Evaluation of Analysis Approaches

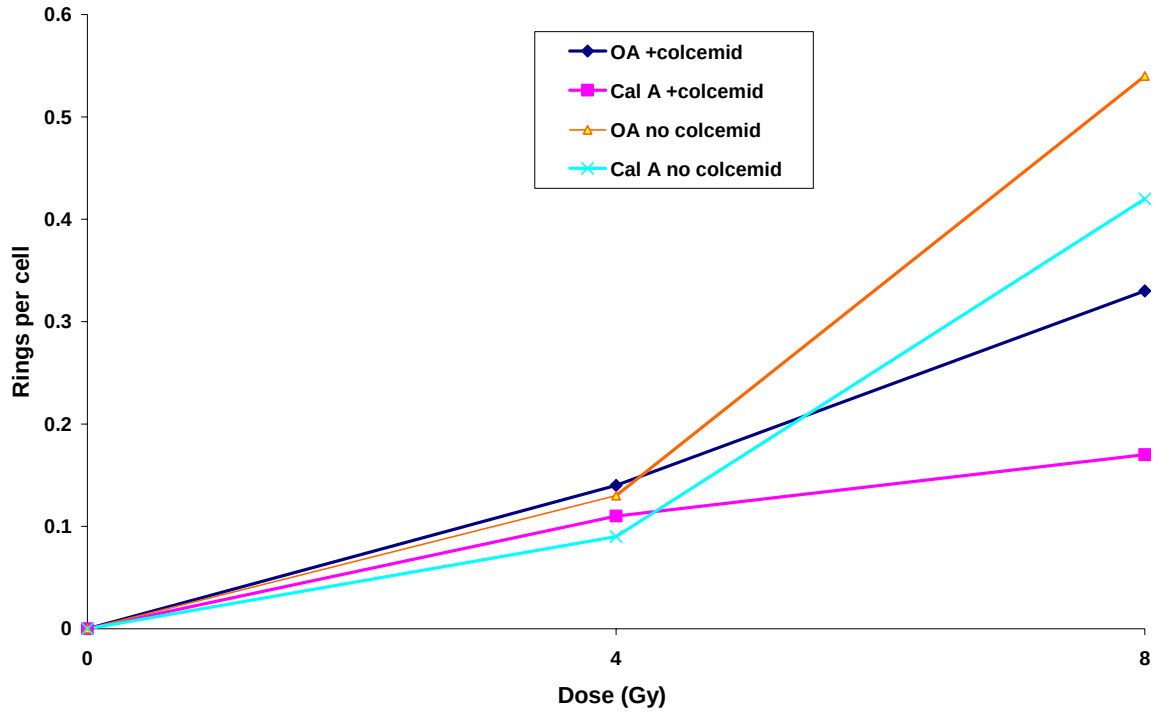
Analysis of fragments, rings, other aberrations, spots and aberrations identified by FISH, were all reviewed for use in an optimized method of PCC application in emergency preparedness.

Analysis Approaches	Consideration
Excess fragments	Reliability (comparability to standard method)
Ring chromosomes	Time required for analysis
Aberrations detected by FISH	Technical difficulty
"Spot" technique (FISH)	

4.2.1 Analysis of Giemsa Stained Slides

Giemsa stained PCC cells were evaluated from each of the cultures conditions except for the kinase method (condition 4) which rendered very slightly condensed cells that could only be analyzed with FISH painting. For the two main conditions (1 and 2), data on the frequency of rings and excess fragments were collected and are shown in Figure 1. Too few metaphases were distinct enough to reliably evaluate other aberrations such as dicentric chromosomes in the Giemsa stained PCCs.

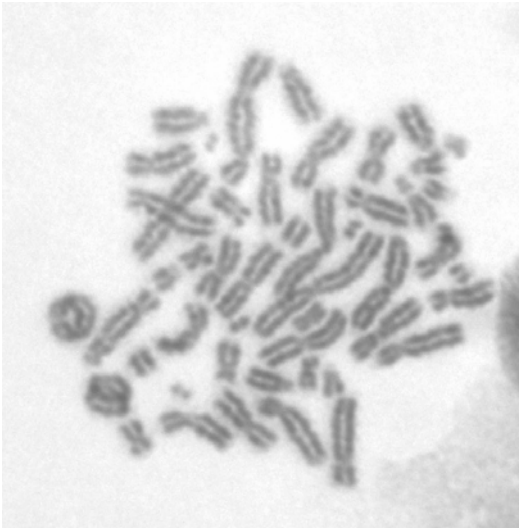
Figure 1. Illustration of the dose response of rings, from 0, 4, and 8 Gy γ radiation in PCC induced cells from different culture conditions.

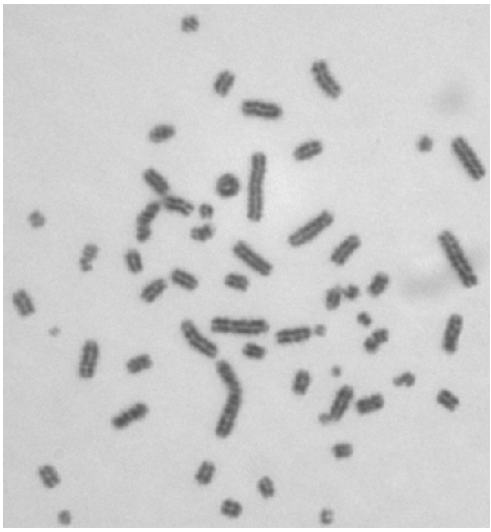


Evaluation of excess fragments requires counting of many chromosomes and pieces for every cell. Uncertainty was common in the evaluation of fragments since many chromosome pieces could be observed even in control samples, perhaps arising from treatment with the chemicals. Since, for emergency preparedness purposes, the method for analysis of PCCs should be fast, the evaluation of fragments was excluded.

Rings evaluations went fairly quickly and to date afford the most practical method for fast PCC evaluations. Some uncertainty in the identification of rings was found but could be addressed by better defining the criteria for identification of rings.

Figure 2. An examples of a.) M phase PCC cell induced by OA with colcemid after 8 Gy γ radiation and b.) a PCC cell induced by Cal A with colcemid after 8 Gy γ radiation.





4.2.2 Analysis of FISH Painted Slides

From the collective FISH painting experiments, only the triple painting, i.e. FITC-Cy3-DAPI was successful enough to enable scoring of the OA induced PCC cells. At control level and lower doses, the scoring of aberrations was relatively easy but at higher doses (7.5 and 10) the portion of complex aberrations became too large for reliable scoring and could not easily be defined by routine FISH terminology. An alternative evaluation approach could be evaluating the number of chromosome breaks in the painted chromosomes.

The data obtained from the FISH experiments on OA induced PCCs at different doses based on a modest number of cells (Table 1) are shown in Figure 4. Unstable aberrations include dicentrics, acentrics, and ring chromosomes. Stable aberrations include translocations and insertions. Due to the fact that centromere probe was not used, the distinction between dicentrics and translocations was not possible at a very accurate level and thus the total aberration scores may be the most relevant variable.

Figure 3. Examples of FISH painted PCC cells a.) induced by Cal A using 2 probes and b.) OA induced cell (10 Gy exposed) exhibiting several rearrangements. Note that the FISH painted PCC in (a) can be reliable evaluated while the Giemsa stained cell would be too difficult to discern.

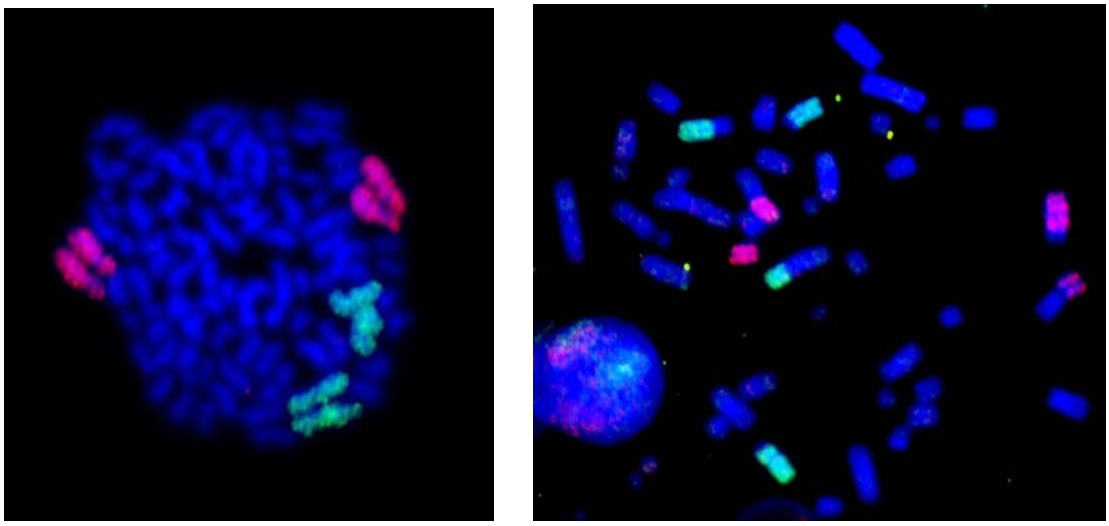
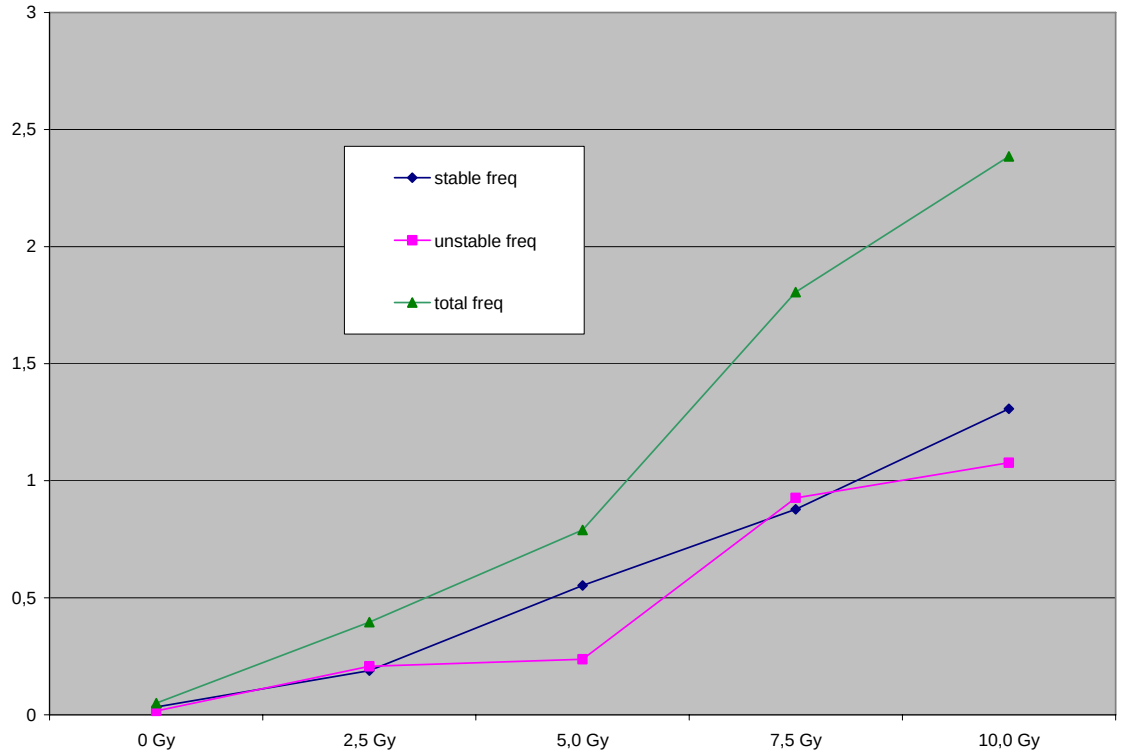


Table 1. FISH painting results for OA induced PCC cells.

Dose	cells	stable aberra	unstable aberr	total	stable freq	unstable freq	total freq
0 Gy	60	2	1	3	0.033	0.017	0.05
2.5 Gy	53	10	11	21	0.189	0.208	0.396
5.0 Gy	38	21	9	30	0.553	0.237	0.789
7.5 Gy	41	36	38	74	0.878	0.927	1.804
10.0 Gy	39	51	42	93	1.308	1.077	2.385

Figure 4. Aberration frequencies from Table 1 plotted. Note that the results are not converted according to genomic correction factors for different types of aberrations.



FISH painting was conducted on slides with kinase PCC preparations for the "spot" counting analysis. A few slides were tested but the results were not promising. The main problem with this approach is the difficulty of defining the spots in an accurate manner and thus to distinguish one spot from another. While the method in principle affords a faster overall PCC method, reliability in evaluations are limited and the method has some degree of technical difficulty. Further considerations for use of kinase in PCC experiments include the considerable cost of the kinase, lability of the enzyme, and variation of the enzyme performance from batch to batch.

The kinase PCC method does not seem to afford a practical approach for emergency preparedness. For the other OA and Cal A induced PCCs, FISH painting takes some additional laboratory time also limiting its usefulness for a fast method for emergency preparedness. In general, FISH based analysis methods do show promise for addressing more mechanistic questions regarding chromosome damage, but may not be practical for the needs presented here.

4.3 Establishment of Scoring Criteria for Rings

Two scoring criteria for evaluation have been discussed; one following Kanda (1999) which includes all spherical shaped PCC rings, and another (Lamadrid 2006) which strictly limits ring evaluation to those which clearly defined open space can be observed. The less restrictive criteria provide a higher ring frequency and steeper dose response but at the same time introduce greater uncertainty into the analyses and more subjective bias.

The outcome of analyzing rings from 2.5 and 10 Gy samples indicated that larger variation in ring frequencies resulted between individual evaluations. Larger variation was observed between observers as well as among repeated evaluations from the same sample by the same

observer. These results indicated the need for very strict criteria for evaluation of rings, i.e. evaluation of only rings with discernable open spaces.

Table 2. Results from calibration of ring scoring criteria.

	Frequencies (rings per cell ± S.E.)		
	FOI	STUK	NRPA
10 Gy	0.41 ± 0.028	0.41 ± 0.029	0.34 ± 0.026
2.5 Gy	0.046 ± 0.009	0.047 ± 0.008	0.042 ± 0.009

During this phase of our work, we found that the quality of the PCC slides and the clarity of the Giemsa stain could affect the observed frequency of rings in PCC cells. This consideration could affect the overall uncertainty associated with ring evaluations; however, we believe that the results provide an adequate assessment for our purposes.

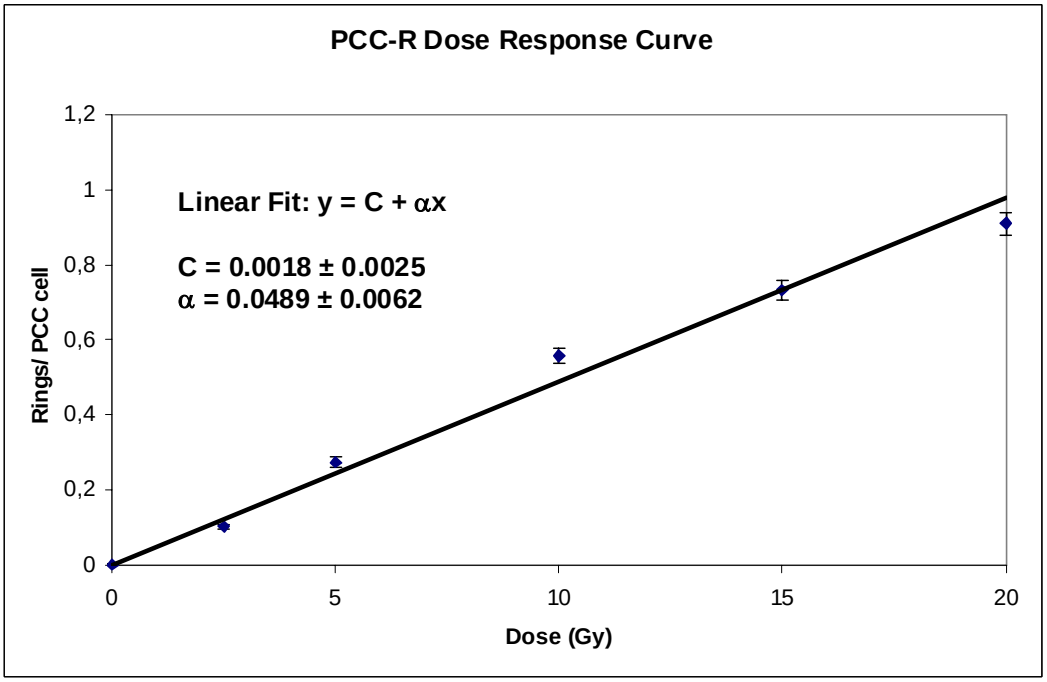
4.4 PCC – Ring Dose Response Curve

A limited dose response curve for the PCC- ring assay was prepared by evaluating a critical number of cells for 7 dose points (0, 1, 2.5, 5, 10, 15, and 20 Gy). The data from the primary evaluators at STUK and FOI were statistically coherent and thus allowed us to combine and model the data for the best fit for the curve. Fitting was performed according to standardized methods (IAEA 2001). The best fit for the curve was determined to be a linear model with exclusion of the 1 Gy dose point. The data included in the curve are shown in Table 3 below and the curve is illustrated in Figure 5 below.

Table 3. Combined data used in preparation of the PCC-ring dose response curve.

Dose (Gy)	PCC cells	PCC Rings	PCC rings/ cell
0	2026	4	0.002 ± 0.001
2.5	2243	230	0.103 ± 0.007
5	1372	377	0.275 ± 0.014
10	1340	748	0.558 ± 0.020
15	987	723	0.733 ± 0.027
20	988	899	0.910 ± 0.030

Figure 5. PCC-ring dose response curve using a linear fit.



4.5 Comparison of PCC – Ring Analysis and Dicentric Assay

The dose estimates were calculated according to the corresponding dose curve. For the dicentric assay, the curves established in each laboratory for gamma-irradiation were used (NRPA's data were obtained from STUK's curve). The dicentric assay curves from STUK and FOI only covers doses up to 5 Gy and therefore the dose estimates for R (7.5 Gy) and G (10Gy) samples should not be considered reliable. For the PCC data, the new linear dose response curve was applied.

Table 4. Dose estimates and confidence intervals for dicentric assay and PCC-ring analysis in triage evaluations.

	Dose Estimate and Confidence Intervals			
Sample	STUK1	STUK2	FOI	NRPA
Dicentric Assay				
T (2.5 Gy)	2.6 (2.0-3.0)	2.2 (1.5-2.8)	3.3 (2.2-4.6)	
R (7.5 Gy)	6.2 (4.8-7.1)	6.0 (4.7-7.1)	10.3 (8.2-12.7)	5.0 (3.9-5.8)
G (10 Gy)	7.3 (5.7-8.6)	8.8 (6.9-10.3)	15.2 (12.2-18.5)	
PCC Assay				
T (2.5 Gy)	1.0 (0.4-1.6)	1.6 (0.8-2.7)	1.2 (0.5-1.8)	
R (7.5 Gy)	5.9 (3.6-8.1)	5.0 (3.1-6.9)	7.8 (4.4-11.3)	4.8 (3.0-6.6)
G (10 Gy)	12.8 (8.1-17.6)	16.2 (10.2-22.2)	12.2 (6.9-17.6)	

5. Conclusions

In order to assess the best technique for dose estimation based on PCC induction, a variety of culture parameters were investigated during this work. The quality and quantity of PCC cells produced and the reliability during evaluation of different aberration frequencies were also assessed. With these aspects in mind, the most promising PCC assay for the purpose of fast dose estimation of a large number of casualties encompassing a wide range of doses is the method using okadaic acid (without colcemid) during a 48 hour cell culture and subsequent evaluation of ring chromosomes. Since both calyculin A and colcemid seem to have deleterious effects on chromosome quality, reproducible evaluations may be more difficult for samples prepared using them. Although not using colcemid may affect the number of second divisions in the resulting preparations, this may not affect analysis if further work indicated highly reproducible evaluations.

FISH chromosome painting applications do not seem well suited for an emergency preparedness method where fast analysis is critical. The complex nature of aberrations at higher doses induces difficulties in evaluation. Furthermore, although in principle a faster method, the kinase method for obtaining PCCs did not produce high numbers or high quality PCCs, and evaluations with FISH techniques did not result in reliable evaluations to any degree. The method is not highly accessible and further work is not advocated.

The second phase of this project continued with the establishment of scoring criteria for ring chromosomes in PCC preparations. Based on our work, the evaluation of rings that have clearly defined open spaces and those large spherical rings without open spaces seems to provide the most reproducible results.

The PCC dose response data seems to indicate that a linear fit best describes the data. Other models containing a quadratic and even third degree term were also feasible, but the more complicated models did not show a better fit. However, the linear fit of the curve may be most applicable and relevant at doses 5 Gy and above. The triage comparison, while very limited, does provide some indication that the PCC assay performs better for the higher doses (above 5 Gy) and the dicentric assay performs better at the lower doses (below 5 Gy).

The dicentric assay is known to be reliable only up to 5 Gy, since saturation of aberration yield occurs at higher doses. On the other hand we have shown here that the PCC ring assay shows a linear increment of aberrations up to very high doses. Thus, for emergency preparedness applications, the dicentric assay and PCC assay cultures could be run in parallel, evaluated in triage mode by either assay to indicate the degree of the doses, followed by more detailed evaluation by the appropriate assay as time allows. Both assays maybe analyzed very quickly in triage mode and provides for a greater capacity in the event of a large number of samples to be analyzed. The approach of running samples in parallel would allow for optimal assessment of a wide range of doses in as efficient manner as possible.

6. Future

As a continuation of this work, an exercise which simulates a case where triage would be needed, i.e. a mass casualty/ catastrophe scenario has been planned. An appropriate scenario will be developed independently and a large number samples irradiated accordingly. The workload will be distributed among the partners and analyzed using the PCC-R assay methods developed in this work in triage mode to test the ability to place the sample assessments into clinically relevant dose categories.

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Abstract

A method for biological assessment of radiation dose for specific application in emergency preparedness was developed. Premature chromosome condensation (PCC) was investigated to provide a potentially faster means of analysis and the ability to assess higher doses than with the dicentric assay which is routinely applied in biodosimetry today.

A review of existing methods was made, followed by experiments determine optimal assay conditions, and evaluations to determination of optimal conditions and the most appropriate endpoints for analyses. Twelve different experimental conditions were examined with four different evaluation approaches. Aspects during optimization such as practicality, speed, and reliability were considered.

The conclusion from these studies was a PCC protocol utilizing okadaic acid for induction of PCC cells in stimulated lymphocytes but without the use of colcemid for metaphase arrest with the subsequent evaluation of ring chromosomes.

Well-defined criteria were established for evaluation of PCC cells and ring chromosome aberrations. An inter-calibration was made by comparing assessment of ring chromosomes between all three laboratories. Agreement was made to count only rings with observable open spaces or large, obvious rings without open spaces.

The finally a dose response curve for the PCC method was prepared and a comparison of the PCC method to the traditional dicentric assay in triage mode was made. The triage method requires a minimal number of evaluations so that categorization of high, medium and low doses may be made in an emergency situation where large numbers of people should be evaluated. The comparison of the PCC method with the dicentric assay triage method indicated that the PCC assay performed superior to the dicentric assay for evaluation of samples at higher doses, however, the dicentric assay appeared to provide more accurate dose assessment at lower doses.

This project suggests a PCC assay method for biological dose estimates for emergency preparedness purposes. The conclusions indicate that an optimal approach in emergency applications would be the parallel cultures for both PCC and dicentric analysis, followed by triage analysis to indicate high or low dose, followed by more detailed analysis by either for PCC rings or dicentrics depending on the magnitude of dose.

Key words Biodosimetry, PCC, dicentric assay, dose reconstruction, emergency preparedness