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«Φυσική και Τεχνολογικές Εφαρμογές»

Ανάπτυξη αυτοματοποιήσιμης micro-PCC μεθόδου βιοδοσιμετρίας για ραδιολογικά συμβάντα μεγάλης κλίμακας

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

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Development of an automatable micro-PCC biodosimetry assay for large-scale radiological emergencies

MSc Thesis

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Πρόλογος

Η ευρεία χρήση των ιοντιζουσών ακτινοβολιών υπογραμμίζει την αναγκαιότητα αξιολόγησης και εκτίμησης των επιπτώσεων στην υγεία που εγκυμονεί μια υπερέκθεση ή ένα ατύχημα με ιοντίζουσες ακτινοβολίες. Ιδιαίτερα μετά από ραδιολογικά συμβάντα / ατυχήματα μεγάλης κλίμακας, εκατοντάδες ή ακόμη και χιλιάδες άνθρωποι θα μπορούσαν να εκτεθούν σε διαφορετικές δόσεις ακτινοβολίας. Επομένως, η ανάπτυξη ευαίσθητων και αξιόπιστων βιοδεικτών και τεχνολογίας, με δυνατότητα αυτοματοποίησης των απαραίτητων διαδικασιών για την ταχεία εκτίμηση δόσεων, θα συμβάλλει στην εξατομίκευση του κινδύνου και την κατηγοριοποίηση των εκτιθέμενων ατόμων ώστε να τους παρασχεθεί η βέλτιστη ιατρική βοήθεια. Προς επίτευξη του στόχου αυτού, στην παρούσα διπλωματική εργασία διερευνάται η δυνατότητα εφαρμογής του φαινομένου της πρόωρης χρωμοσωματικής συμπύκνωσης (PCC) σε λεμφοκύτταρα περιφερικού αίματος μέσω της σύντηξής τους με μιτωτικά κύτταρα Χάμστερ - Chinese Hamster Ovary (CHO) cells, για την ανάπτυξη μιας ταχείας, ελάχιστα επεμβατικής και αυτοματοποιήσιμης micro-PCC μεθόδου βιολογικής δοσιμετρίας, χρησιμοποιώντας όγκους αίματος 30-100μl και πλάκες 96 φρεατίων.

Στο πλαίσιο αυτό και σε συνεργασία με τον Δρ. Α. Γεωργακίλα, Αν. Καθηγητή του τομέα Φυσικής της Σχολής Εφαρμοσμένων Μαθηματικών και Φυσικών Επιστημών του Εθνικού Μετσόβιου Πολυτεχνείου, η διπλωματική εργασία εκπονήθηκε στο εργαστήριο Υγειοφυσικής, Ραδιοβιολογίας & Κυτταρογενετικής υπό την επίβλεψη της Δρ. Γ. Τερζούδη, Ερευνήτριας Α' του Ινστιτούτου Πυρηνικών & Ραδιολογικών Επιστημών & Τεχνολογίας, Ενέργειας & Ασφάλειας του ΕΚΕΦΕ «ΔΗΜΟΚΡΙΤΟΣ».

Με εκτίμηση, εκφράζω θερμές ευχαριστίες στον Δρ. Α. Γεωργακίλα για την υποστήριξη, το ενδιαφέρον του και τις πολύτιμες συμβουλές του. Θα ήθελα επίσης να ευχαριστήσω θερμά τη Δρ. Γ. Τερζούδη για την πολύτιμη και καθοριστική καθοδήγηση καθ' όλη τη διάρκεια εκπόνησης της διπλωματικής αυτής εργασίας σχετικά με την κατανόηση των μεθόδων βιοδοσιμετρίας και των φυσικών και βιολογικών παραμέτρων που ήταν θεμελιώδη για την υλοποίηση του πειραματικού μέρους. Η επιστημονική της εμπειρία με βοήθησαν καταλυτικά στο σχεδιασμό, ερμηνεία και συγγραφή των πειραματικών αποτελεσμάτων για τη δημοσίευσή τους σε διεθνή επιστημονικά περιοδικά. Ευχαριστώ επίσης θερμά τη Δρ. Τερζούδη διότι μου έδωσε την ευκαιρία να συμμετάσχω σε ερευνητικά προγράμματα και να παρουσιάσω τα αποτελέσματα της εργασίας αυτής σε διεθνή επιστημονικά συνέδρια.

Τέλος, τις θερμότερες ευχαριστίες μου επιθυμώ να απευθύνω σε όλο το επιστημονικό και τεχνικό προσωπικό του Εργαστηρίου Υγειοφυσικής, Ραδιοβιολογίας & Κυτταρογενετικής, καθώς και στους γονείς και τον αδελφό μου για την κατανόηση και τη θερμή τους συμπαράσταση καθ' όλη τη διάρκεια του κύκλου αυτού των σπουδών μου.

Abstract

In radiation accidents and large-scale radiological emergencies, a fast and reliable triage of individuals according to their degree of exposure is important for accident management and identification of those who need medical assistance. In this work, the applicability of cell-fusion-mediated premature chromosome condensation (PCC) in G_0-lymphocytes is examined for the development of a rapid, minimally invasive and automatable micro-PCC assay, which requires blood volumes of only 100µl and can be performed in 96-well plates, towards risk assessments and categorization of individuals based on dose estimates. Chromosomal aberrations are visualized for dose-estimation analysis within two hours, without the need of blood culturing for two days, as required by conventional cytogenetics. The various steps of the standard-PCC procedure were adapted and, for the first time, lymphocytes in blood volumes of 100µl were successfully fused with CHO-mitotics in 96-well plates of 2ml/well. The plates are advantageous for high-throughput analysis since the various steps required are applied to all 96-wells simultaneously. Interestingly, the morphology of lymphocyte PCCs is identical to that obtained using the standard PCC-assay, which requires much larger blood volumes and 15ml tubes. The use of only 1.5ml hypotonic and Carnoy's fixative per well offers high-quality PCC-images. The micro-PCC assay can be also combined with fluorescence in situ hybridization (FISH), using simultaneously centromeric/telomeric (C/T) peptide nucleic acid (PNA) probes. This allows dose assessments on the basis of accurate scoring of dicentric and centric ring chromosomes in G_0-lymphocyte PCCs, which is particularly helpful when further evaluation into treatment-level categories is needed. Dose assessments using Giemsa-stained or C/T-FISH-stained PCCs were facilitated by the use of specialized software (MetaSystems) and appropriate calibration curves. The micro-PCC assay has significant advantages for early triage biodosimetry when compared to other cytogenetic biodosimetry assays. It is rapid, cost-effective, automatable, and could thus pave the way to its subsequent automation.

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1 Περίληψη

1.1 Σκοπός

Μετά από ατυχήματα και ιδιαίτερα μετά από ραδιολογικά συμβάντα μεγάλης κλίμακας, εκατοντάδες ή ακόμη και χιλιάδες άνθρωποι θα μπορούσαν να εκτεθούν σε άγνωστες και ενδεχομένως διαφορετικές δόσεις ακτινοβολίας και συνεπώς στους κινδύνους που εγκυμονεί η έκθεση σε ιοντίζουσες ακτινοβολίες. Αποτελεί επομένως υψηλή προτεραιότητα η τεχνολογική ανάπτυξη βιοϊατρικών εργαλείων και ευαίσθητων βιοδεικτών με δυνατότητα αυτοματοποίησης των απαραίτητων διαδικασιών για την ταχεία εκτίμηση δόσεων και κατηγοριοποίηση των εκτιθέμενων ατόμων βάσει εξατομίκευσης του κινδύνου ώστε να τους παρασχεθεί η βέλτιστη ιατρική βοήθεια. Προς επίτευξη του στόχου αυτού, στην παρούσα διπλωματική εργασία διερευνάται η δυνατότητα εφαρμογής του φαινομένου της πρόωρης χρωμοσωματικής συμπύκνωσης (PCC) σε λεμφοκύτταρα περιφερικού αίματος μέσω της σύντηξής τους με μιτωτικά κύτταρα Χάμστερ, για την ανάπτυξη μιας ταχείας, ελάχιστα επεμβατικής και αυτοματοποιήσιμης micro-PCC μεθόδου βιοδοσιμετρίας.

Όταν ένας μεγάλος αριθμός ατόμων εκτίθεται σε ακτινοβολία, είναι επιτακτική ανάγκη στο πλαίσιο αντιμετώπισης και διαχείρισης του συμβάντος να οριστεί ένα κατώφλι απορροφουμένης δόσης, π.χ. τα 2Gy ως όριο αποκοπής, το οποίο θα επιτρέψει την κατηγοριοποίηση των εκτιθέμενων ατόμων του πληθυσμού. Για δόσεις κάτω από το όριο αποκοπής, δεν θα χρειασθούν άμεσα μέτρα ιατρικής περίθαλψης των εκτιθέμενων αλλά παρακολούθηση της υγείας τους. Εντούτοις, για τα άτομα με δόσεις πάνω από την τιμή αποκοπής, θα ήταν απαραίτητη η άμεση ιατρική περίθαλψή τους για τη βελτίωση των ποσοστών επιβίωσης. Το όριο αυτό αποκοπής ορίζεται κατά κανόνα στα 2Gy, ωστόσο θα μπορούσε να καθοριστεί και υψηλότερα εάν ο αριθμός των υπερεκτεθειμένων ατόμων υπερβαίνει τις δυνατότητες των διαθέσιμων ιατρικών υποδομών και εγκαταστάσεων.

Η ανάπτυξη μιας ταχείας και αξιόπιστης μεθόδου βιοδοσιμετρίας για την αξιολόγηση ατυχημάτων και ραδιολογικών συμβάντων μεγάλης κλίμακας θα επιτρέψει επομένως την ταξινόμηση των εκτιθέμενων ατόμων σε τρεις κατηγορίες: α) σε αυτούς που αναμένεται με βεβαιότητα να εμφανίσουν καθορισμένα αποτελέσματα από την έκθεσή τους στην ακτινοβολία, για τους οποίους η άμεση ιατρική παρέμβαση είναι ζωτικής σημασίας, β) σε εκείνους με ενδιάμεση έκθεση κοντά στην οριακή τιμή δόσης, για τους οποίους θα χρειασθεί επίσης ιατρική παρακολούθηση για την άμβλυνση των βραχυπρόθεσμων, μεσοπρόθεσμων και μακροπρόθεσμων επιπτώσεων της έκθεσης και τέλος γ) σε αυτούς που έχουν εκτεθεί σε χαμηλές δόσεις, για τους οποίους δεν αναμένονται καθορισμένα αποτελέσματα αλλά μπορεί να απαιτηθεί μακροπρόθεσμος έλεγχος της υγείας τους.

Η ανάπτυξη μιας ταχείας και ελάχιστα επεμβατικής μεθόδου βιοδοσιμετρίας θα συμβάλλει επομένως ουσιαστικά στην εξατομίκευση του κινδύνου και κατηγοριοποίηση των εκτιθέμενων ατόμων μετά από ραδιολογικά συμβάντα μεγάλης κλίμακας, όπου η έκθεση σε ακτινοβολία ενδεχομένως να εγκυμονεί σοβαρούς κινδύνους σε ένα μεγάλο τμήμα του πληθυσμού. Στο πλαίσιο αυτό, ο κύριος στόχος της διπλωματικής εργασίας επικεντρώνεται στην τεχνολογική ανάπτυξη της micro-PCC μεθόδου, με την έννοια ότι θα μπορεί να εφαρμοστεί σε πολύ μικρούς όγκους αίματος των 50-150μl, για την επίτευξη πρόωρης χρωμοσωματικής συμπύκνωσης στα λεμφοκύτταρα χρησιμοποιώντας πλάκες 96 φρεατίων των 2ml έκαστο. Η χρήση των 96 φρεατίων προσδίδει στη micro-PCC μέθοδο δυνατότητα επεξεργασίας και ανάλυσης ενός μεγάλου αριθμού δειγμάτων και ουσιαστικά αποτελεί τη βάση για την αυτοματοποίηση των απαιτούμενων διαδικασιών και κατηγοριοποίηση των εκτιθέμενων ατόμων για την αποτελεσματική αντιμετώπιση ραδιολογικών συμβάντων. Η micro-PCC μέθοδος που προτείνουμε για την ταχεία εκτίμηση δόσεων επιτρέπει κατά ένα μοναδικό τρόπο την απεικόνιση και ανάλυση χρωμοσωματικών αλλοιώσεων εντός δύο ωρών απευθείας στα πρόωρα συμπυκνωμένα χρωματοσώματα (PCCs) των G_o-λεμφοκυττάρων, χωρίς δηλαδή την ανάγκη καλλιέργειας των δειγμάτων αίματος για δύο ημέρες. Επισημαίνεται ότι οι συμβατικές κυτταρογενετικές μέθοδοι βιοδοσιμετρίας απαιτούν τουλάχιστον 48-52 ώρες καλλιέργειας των

δειγμάτων αίματος των εκτιθέμενων στην ακτινοβολία για την ανάλυση των χρωμοσωματικών αλλοιώσεων στη μετάφαση.

1.2 Η αναγκαιότητα εφαρμογής της πρόωρης χρωμοσωματικής συμπύκνωσης (PCC)

Παρά το γεγονός ότι έχουν προταθεί αρκετοί κυτταρογενετικοί βιοδείκτες για την αξιολόγηση έκθεσης σε ακτινοβολία, κανένας από αυτούς δεν ικανοποιεί όλα τα κριτήρια ενός ιδανικού βιοδείκτη που θα μπορούσε να χρησιμοποιηθεί ως ένα αξιόπιστο βιολογικό δοσίμετρο για την εκτίμηση δόσεων. Ένας ιδανικός βιοδείκτης θα πρέπει να είναι ειδικός για την ακτινοβολία, ευαίσθητος ακόμη και σε χαμηλές/σχετικά χαμηλές δόσεις (<0.5 Gy), αναπαραγώγιμος και ικανός να διακρίνει μεταξύ μερικής ή ολόσωμης έκθεσης. Επιπλέον, θα πρέπει να είναι σε θέση να παρέχει αξιόπιστες εκτιμήσεις απορροφούμενης δόσης ανεξάρτητα από την χρονική στιγμή της έκθεσης και η ανάλυση του βιοδείκτη για τον προσδιορισμό της δόσης θα πρέπει να είναι ταχεία, ιδίως όταν απαιτείται επείγουσα κατηγοριοποίηση των εκτιθέμενων μελών του πληθυσμού. Για ένα ιδανικό βιοδείκτη, η συλλογή των απαιτούμενων βιολογικών δειγμάτων από τους εκτιθέμενους θα πρέπει να είναι εύκολη και μη επεμβατική, ενώ οι διαδικασίες προετοιμασίας παρασκευασμάτων και ανάλυσης θα πρέπει να παρέχουν τη δυνατότητα αυτοματοποίησής τους. Αυτό θα συμβάλλει ουσιαστικά στην χρήση τους για την αντιμετώπιση έκτακτων περιστατικών και ραδιολογικών συμβάντων μεγάλης κλίμακας. Τέλος, οι μεταβλητές που θα μπορούσαν να επηρεάσουν τη μεθοδολογία ανάλυσης και εκτίμησης της δόσης θα πρέπει να είναι καλά τεκμηριωμένες και οι διαδικασίες βαθμονόμησης και διακρίβωσης θα πρέπει να καθορίζονται με σαφήνεια μέσω διεργαστηριακών μελετών πριν υιοθετηθεί η μεθοδολογία από ένα εργαστήριο βιοδοσιμετρίας.

Μεταξύ των διαφορετικών κυτταρογενετικών μεθόδων που χρησιμοποιούνται για την εκτίμηση απορροφούμενων δόσεων σε εκτιθέμενα άτομα, ο ποσοτικός προσδιορισμός της δόσης βάσει ανάλυσης των δικεντρικών χρωματοσωμάτων (DC) παραμένει ο ευρύτερα χρησιμοποιούμενος.

Η ανάλυση αυτή βασίζεται ουσιαστικά στον προσδιορισμό της συχνότητας επαγωγής από την ακτινοβολία δικεντρικών χρωματοσωμάτων και κεντρικών δακτυλίων στα λεμφοκύτταρα του περιφερικού αίματος των εκτεθειμένων ατόμων. Εντούτοις, η κυτταρογενετική αυτή μέθοδος έχει ένα σημαντικό μειονέκτημα σε σχέση με το συνολικό χρόνο που απαιτείται για την εκτίμηση των δόσεων. Για την ανάλυση των χρωμοσωματικών αλλοιώσεων στην μετάφαση απαιτείται καλλιέργεια των Τ-λεμφοκυττάρων του περιφερικού αίματος τουλάχιστον για δύο ημέρες. Ως εκ τούτου, η ανάλυση δικεντρικών χρωματοσωμάτων στη μετάφαση δεν ανταποκρίνεται στην απαίτηση για ταχεία εκτίμηση της δόσης, η οποία αποτελεί υψηλή προτεραιότητα για την εκτίμηση του κινδύνου και γενικά για την ιατρική αντιμετώπιση έκτακτων ραδιολογικών συμβάντων.

Από την άλλη πλευρά, είναι ενδιαφέρον να σημειωθεί ότι μετά την επαγωγή των μοριακών αλλοιώσεων στο DNA των λεμφοκυττάρων από την ακτινοβολία και την ενζυματική επεξεργασία των αλλοιώσεων αυτών, οι χρωμοσωματικές αλλοιώσεις, που προκύπτουν από τη λανθασμένη επιδιόρθωση του DNA, σχηματίζονται μέσα σε 8 ώρες μετά την έκθεση στην ακτινοβολία και δεν απαιτούν για τον σχηματισμό τους καλλιέργεια 48-52 ωρών και διέγερση των Τ-λεμφοκυττάρων ή αναδιπλασιασμό του DNA τους. Συνεπώς, οι βιοδείκτες έκθεσης όπως τα δικεντρικά, οι δακτύλιοι και τα χρωμοσωματικά θραύσματα θα υπάρχουν ήδη στα λεμφοκύτταρα τη στιγμή που τα δείγματα αίματος φθάνουν στο εργαστήριο για ανάλυση. Η ταχεία ποσοτικοποίηση των χρωμοσωματικών αυτών αλλοιώσεων μπορεί, επομένως, να επιτευχθεί κατά ένα μοναδικό τρόπο, χωρίς την καλλιέργεια των δειγμάτων αίματος, εκμεταλλευόμενοι το φαινόμενο της πρόωρης συμπύκνωσης χρωμοσωμάτων (PCC). Συγκεκριμένα, η micro-PCC μέθοδος που προτείνουμε επιτρέπει εντός 2 ωρών και για ένα μεγάλο αριθμό δειγμάτων να απεικονισθούν για ανάλυση οι χρωμοσωματικές αλλοιώσεις που επάγονται από την ακτινοβολία όχι μόνο σε μη διεγερμένα (G0) Τ- λεμφοκύτταρα, αλλά επίσης και στα Β-λεμφοκύτταρα του περιφερικού αίματος.

1.3 Στόχοι και Επιτεύγματα

Για την επίτευξη του κύριου στόχου της διπλωματικής εργασίας και την ανάπτυξη της micro-PCC μεθόδου ταχείας εκτίμησης δόσεων χρησιμοποιώντας πολύ μικρούς όγκους αίματος για την επαγωγής PCC σε λεμφοκύτταρα μέσω κυτταρικών συντήξεων σε πλάκες 96 φρεατίων, τα διάφορα στάδια του συμβατικού πρωτοκόλλου PCC διαφοροποιήθηκαν ουσιαστικά. Συγκεκριμένα, για πρώτη φορά στη διεθνή βιβλιογραφία λεμφοκύτταρα σε όγκους αίματος 50-150μl, η λήψη των οποίων είναι δυνατόν να γίνεται από το δάχτυλο ενός ατόμου, συντήχθηκαν επιτυχώς με CHO μιτωτικά κύτταρα σε δοκιμαστικούς σωλήνες των 2ml, καθώς επίσης και σε πλάκες 96 φρεατίων των 2ml έκαστο. Οι πλάκες των 96 φρεατίων είναι πιο κατάλληλες για την ταχεία εκτίμηση δόσεων καθώς τα διάφορα στάδια που απαιτούνται για την προετοιμασία PCC λεμφοκυττάρων γίνονται ταυτόχρονα και στα 96 φρεάτια, χωρίς την ανάγκη διαχωρισμού των λεμφοκυττάρων από τα ερυθρά αιμοσφαίρια με χρήση διαλύματος φικόλης. Αυτό επιτρέπει την ταχεία και ταυτόχρονη ετοιμασία χρωμοσωματικών παρασκευασμάτων για εκατοντάδες δότες αίματος και την έγκαιρη εκτίμηση των δόσεων τους στην περίπτωση ενός ραδιολογικού συμβάντος μεγάλης κλίμακας.

Τα αποτελέσματα έχουν ενδιαφέρον καθότι η μορφολογία των PCC λεμφοκυττάρων με την micro-PCC μέθοδο είναι ουσιαστικά ίδια με εκείνη του συμβατικού πρωτοκόλλου το οποίο όμως απαιτεί πολύ μεγαλύτερους όγκους 1-2 ml φλεβικού αίματος ανά άτομο και δοκιμαστικούς σωλήνες των 15ml. Επίσης, η χρήση μόνο 1,5ml υποτονικού διαλύματος και η μονιμοποίηση των κυττάρων δύο φορές με 1,5ml μονιμοποιητή (Carnoy's fixative) στους μικρούς σωλήνες ή τις πλάκες των 96 φρεατίων αποδεικνύεται να προσφέρει εικόνες PCC υψηλής ποιότητας μετά την χρώση τους με Giemsa. Η ανάλυση των χρωμοσωματικών θραυσμάτων στα PCC λεμφοκυττάρων για την εκτίμηση των δόσεων διευκολύνεται πολύ με τη χρήση του ειδικού λογισμικού Ikaros της εταιρείας MetaSystems και βασίζονται σε κατάλληλες καμπύλες βαθμονόμησης οι οποίες και κατασκευάσθηκαν στο πλαίσιο της παρούσας διπλωματικής εργασίας.

Για τις περιπτώσεις υπερέκθεσης ατόμων των οποίων τα δείγματα αίματος φθάνουν στο εργαστήριο 8 ώρες μετά την έκθεση ή και αργότερα, η μέθοδος micro-PCC συνδυάστηκε επιτυχώς με την τεχνική Fluorescence In Situ Hybridization (FISH), χρησιμοποιώντας ταυτόχρονα centromeric/telomeric (C/T) ανιχνευτές PNA (Peptide Nucleic Acid). Η προσέγγιση αυτή επιτρέπει την εκτίμηση δόσεων βάσει της συχνότητας των δικεντρικών και κεντρικών δακτυλίων των οποίων η ανάλυση είναι δυνατόν να γίνει με μεγάλη ακρίβεια στα πρόωρα συμπυκνωμένα χρωματοσώματα των λεμφοκυττάρων, χρησιμοποιώντας τη micro-PCC μέθοδο. Μετά την ταχεία κατηγοριοποίηση των εκτιθέμενων ατόμων χρησιμοποιώντας τη micro-PCC μέθοδο και τη χρωστική Giemsa, οι εκτιμήσεις δόσεων με το συνδυασμό της τεχνικής FISH μπορούν να είναι ιδιαίτερα χρήσιμες για την επιβεβαίωση περιστατικών υπερέκθεσης και ιδιαίτερα όταν απαιτείται περαιτέρω κατηγοριοποίηση των εκτιθέμενων σε υποκατηγορίες με σκοπό τη βέλτιστη ιατρική τους αντιμετώπιση και παρακολούθηση. Οι εκτιμήσεις δόσεων με το συνδυασμό micro-PCC και FISH διευκολύνονται πολύ με τη χρήση του ειδικού λογισμικού ISIS της εταιρείας MetaSystems και βασίζονται σε κατάλληλες καμπύλες βαθμονόμησης οι οποίες και κατασκευάσθηκαν επίσης στο πλαίσιο της παρούσας διπλωματικής εργασίας.

1.4 Συμπεράσματα και Προοπτικές

Τα αποτελέσματα αποδεικνύουν ότι η micro-PCC μέθοδος που αναπτύχθηκε έχει σημαντικά πλεονεκτήματα σε σύγκριση με άλλες κυτταρογενετικές μεθόδους που εφαρμόζονται σήμερα για σκοπούς βιοδοσιμετρίας και εξατομίκευσης κινδύνου από έκθεση σε ιοντίζουσες ακτινοβολίες. Ιδιαίτερα, σε σύγκριση με τη μέθοδο ανάλυσης δικεντρικών χρωματοσωμάτων και κεντρικών δακτυλίων στη μετάφαση, που επί του παρόντος θεωρείται ως η πρότυπη μέθοδος βιοδοσιμετρίας, η μέθοδος micro-PCC είναι πολύ ταχύτερη, ευαίσθητη, αξιόπιστη και εύχρηστη. Επειδή, σε αντίθεση με τις συμβατικές κυτταρογενετικές μεθόδους, δεν απαιτεί καλλιέργεια λεμφοκυττάρων, τα δείγματα αίματος δεν κινδυνεύουν από τυχόν μολύνσεις που ενέχει η καλλιέργειά τους και τα χρωμοσωματικά παρασκευάσματα ετοιμάζονται για ανάλυση μέσα σε 2 ώρες αντί των 48-52 ωρών που απαιτεί η συμβατική μέθοδος. Επιπλέον, επειδή κατά

τη διάρκεια της καλλιέργειας των δειγμάτων αίματος πολλά από τα αλλοιωμένα από την ακτινοβολία λεμφοκύτταρα καθυστερούν στην G2-φάση του κυτταρικού κύκλου (G2-block), ή και πεθαίνουν λόγω του μηχανισμού απόπτωσης, η μέθοδος micro-PCC μπορεί να ανιχνεύσει περισσότερες χρωμοσωματικές αλλοιώσεις και είναι επομένως πιο ευαίσθητη. Ιδιαίτερα, επειδή απαιτεί όγκους αίματος μόνο 50-150μl, η λήψη των οποίων είναι εύκολο να γίνει από το δάχτυλο ενός ατόμου, η micro-PCC μέθοδος είναι πιο εύχρηστη και η ανάλυση μόνο 10-20 λεμφοκυττάρων μπορεί να δώσει αξιόπιστη εκτίμηση της δόσης. Αυτό οφείλεται στο γεγονός ότι η ανάλυση βασίζεται στα επαγόμενα από την ακτινοβολία χρωμοσωματικά θραύσματα επιπλέον των 46 χρωματοσωμάτων τα οποία αποτελούν το γονιδίωμα του ανθρώπου και ο αριθμός 46 είναι πολύ σταθερός στα υγιή άτομα. Σε αντίθεση τα δικεντρικά χρωματοσώματα που επάγονται από την ακτινοβολία είναι σπάνια, ιδιαίτερα στις χαμηλές δόσεις, και ως εκ τούτου για την ανίχνευσή τους με τη συμβατική μέθοδο απαιτείται η ανάλυση εκατοντάδων ή και χιλιάδων λεμφοκυττάρων στη μετάφαση. Επιπρόσθετα της εφαρμογής της μεθόδου micro-PCC με τη χρωστική Giemsa, ο συνδυασμός της μεθόδου αυτής με την τεχνική FISH για τον ακριβή προσδιορισμό δικεντρικών χρωμοσωμάτων μπορεί να είναι χρήσιμος για την επιβεβαίωση τυχόν υπερεκθέσεων και την κατηγοριοποίηση των εκτιθέμενων σε υποκατηγορίες με σκοπό τη βέλτιστη ιατρική τους αντιμετώπιση και παρακολούθηση. Τέλος, η δυνατότητα που έχει η micro-PCC μέθοδος να χρησιμοποιεί πλάκες 96 φρεατίων της προσδίδει το πλεονέκτημα ανάλυσης ενός μεγάλου αριθμού δειγμάτων και αποτελεί επομένως τη βάση για την αυτοματοποίησή της.

Περαιτέρω ανάπτυξη της μεθόδου micro-PCC βρίσκεται σε εξέλιξη κυρίως όσον αφορά την αυτοματοποίηση των σταδίων που απαιτούνται για την επεξεργασία των δειγμάτων αίματος, την ετοιμασία των χρωμοσωματικών παρασκευασμάτων και την ανάλυση των χρωμοσωματικών αλλοιώσεων. Η αυτοματοποίηση της μεθόδου για την εκτίμηση δόσεων αποτελεί μελλοντική πρόκληση και η υλοποίησή της θα συμβάλλει ουσιαστικά στην εξατομίκευση του κινδύνου από έκθεση σε ιοντίζουσες ακτινοβολίες και στην αποτελεσματική αντιμετώπιση ραδιολογικών συμβάντων μεγάλης κλίμακας. Για την επίτευξη του στόχου αυτού συνεργαζόμαστε με την εταιρεία MetaSystems (Γερμανία) και το Κέντρο Ραδιολογικών Ερευνών του Πανεπιστημίου

Columbia, ένα από τα κέντρα CMCR (Centers for Medical Countermeasures Against Radiation) των ΗΠΑ, το οποίο μετά από διεθνή διαγωνισμό επέλεξε για χρηματοδότηση την παρούσα έρευνα για την ανάπτυξη της micro-PCC μεθόδου (Opportunity Funds Management Core of the Centers for Medical Countermeasures against Radiation Consortium, USA: grant number U19AI067773). Η εταιρεία MetaSystems έχει παγκόσμια πρωτοπορία και εξειδίκευση σε λογισμικά αυτοματοποίησης απεικονιστικών συστημάτων για εφαρμογές στην Κυτταρογενετική, ενώ το Κέντρο Ραδιολογικών Ερευνών του Πανεπιστημίου Columbia έχει πολύτιμη εμπειρία σε ρομποτικές πλατφόρμες για το χειρισμό δειγμάτων αίματος και ανάλυσης χρωμοσωματικών αλλοιώσεων και άλλων κυτταρογενετικών βιοδεικτών. Επιπλέον, η συνεργασία μας με τον Dr. Adayabalam S. Balajee, Διευθυντή του Εργαστηρίου Κυτταρογενετικής Βιοδοσιμετρίας στο Radiation Emergency Assistance Center, Oak Ridge Institute for Science and Education των ΗΠΑ, θα συμβάλλει στη διεύρυνση των εφαρμογών της μεθόδου micro-PCC και ενδεχομένως στην ανάπτυξη νέων βιοδεικτών έκθεσης σε ακτινοβολία που να βασίζονται σε χρωμοσωματικές αναδιατάξεις όπως αυτές που δυνατόν να ανιχνευθούν κατά μοναδικό τρόπο στα πρόωρα συμπυκνωμένα χρωμοσώματα λεμφοκυττάρων με τη τεχνική mFISH (multicolor Fluorescence In Situ Hybridization).

2 Introduction

2.1 Rationale for the Thesis

Following a large-scale radiological event, hundreds or thousands of people could be potentially exposed to unknown and variable doses of radiation. Therefore, it is of high priority to use biomedical tools, sensitive biomarkers and automatable methods in order to reflect promptly the biological importance of the radiation exposure [1-3]. At present, it is estimated that the throughput of a biodosimetry laboratory is a few tens of samples per day and even large cytogenetic biodosimetry laboratory networks can only analyse a few hundred of samples per day [4-6]. For large-scale incidents, rapidity and ease of screening are essential in order to obtain quick radiological dose and risk assessments [7]. This will enable categorization of individuals according to the degree of their exposure and, subsequently, identification of those who need medical assistance, which is essential for optimal post-exposure management [8-10]. Towards this goal, two main approaches of biodosimetry, biologically-based and physically-based, have been developed and essentially three criteria are minimally necessary for an effective biodosimetric technique: the dose can be assessed promptly after-the-fact; the technique can assay at the level of an individual; and the technique can provide information sufficient to determine what actions should be taken for that individual [11].

Biologically-based biodosimetry approaches are based on biological processes or biomarkers that can be affected by ionizing radiation (IR) allowing thus a dose of radiation to be estimated. The status and suitability of current biomarkers for radiation exposure have been reviewed recently [12-16]. Particularly, cytogenetic biomarkers are the most widely used and, at present, several well-established biological dosimeters exist, some of which have been proposed for high-throughput analysis [17-21]. Cytogenetic biodosimeters can offer accurate dose estimates but, are both time-and labour-consuming and therefore not ideal for use in radiological mass-casualty scenarios where short turnaround times and high throughput are of prime importance [9]. When an ideal biomarker is used for early triage biodosimetry in radiological events, the collection of

the required biological samples from the potentially exposed individuals should be easy and non-invasive, while the procedures involved for dose and risk assessments should be rapid and automatable. The latter will pave the way to the subsequent automation of the assay's workflow so that it could be used in the event of a large-scale radiological emergency. The state-of-the-art advances in radiation biodosimetry for mass casualty events involving radiation exposure have been also reviewed recently [22, 23].

Among the different cytogenetic assays used for triage biodosimetry and the estimation of absorbed doses in exposed individuals, the dicentric chromosome (DC) assay remains the most widely used. The DC assay is essentially based on the analysis of dicentrics and centric ring chromosomes present in the peripheral blood metaphase lymphocytes of the exposed individuals. Nevertheless, this assay has a significant drawback with respect to the time needed to obtain dose estimates for rapid decision on the right line of medical treatment. It requires culturing of peripheral blood T-lymphocytes for two days before allowing the analysis of chromosomes at metaphase. Hence, it fails the requirement of rapid dose estimation, which is a high priority in radiation emergency medicine.

On the other hand, it is noteworthy that following radiation-induced DNA damage and the enzymatic repair processing of the lesions, which depend on the quality of radiation and the complexity of DNA damage [24], chromosomal rearrangements in the nuclei of blood lymphocytes are well known to be formed within 8h post-irradiation, without requiring blood culture, T-lymphocyte stimulation and DNA replication for their formation. Following accidental exposure, the biomarkers of exposure i.e. dicentric and centric ring chromosomes as well as residual chromosomal fragments, will be present, therefore, as single chromatid chromosomes in the blood lymphocytes by the time the blood samples reach a reference Biodosimetry Laboratory for dose estimation. Consequently, rapid dose and risk estimates can be only achieved by taking advantage of the unique features of the premature chromosome condensation (PCC) phenomenon in blood lymphocytes [25-27]. This phenomenon enables visualization, analysis and quantification of chromosomal aberrations directly in unstimulated G_0-peripheral blood T- as well

as B-lymphocyte prematurely condensed chromosomes (PCCs), without requiring a two-day blood culture [28].

2.2 Objectives and Achievements

In the present thesis, the main objective is to explore the applicability of the PCC phenomenon in order to devise an automatable micro-PCC assay using very small blood sample volumes below 100µl and 96-well plates enabling rapid dose and risk assessments and the categorization of individuals according to the degree of their exposure in the event of a large-scale radiological emergency. Towards this goal, the various steps of the conventional PCC procedure were adapted in order to make use of fingerstick-derived blood samples from each individual. This simple and rapid method for blood sample collection allows screening of large numbers of people while the 96-well plates enable the simultaneous preparation of chromosomes for multiples of 96 individuals. The yield of Giemsa stained lymphocyte prematurely condensed chromosomes (PCCs) in excess of 46 PCCs was used as a rapid, inexpensive and minimally invasive biomarker of radiation exposure. The number of 46 PCCs is remarkably stable in healthy individuals since it constitutes the human genome. Consequently, this allows to link every single excess fragment above 46 to radiation exposure.

For the standardization of the micro-PCC assay, appropriate calibration curves were constructed, while its applicability and reliability for dose assessment were compared to the conventional dicentric chromosome assay, through the evaluation of speed of analysis and minimum number of cells required for each method. When further evaluation into treatment-level categories is needed in the event of an accident, the micro-PCC assay has the potential to be combined with fluorescence in situ hybridization (FISH), using simultaneously centromeric/telomeric (C/T) peptide nucleic acid (PNA) probes. This would enable early dose assessments and categorization of exposed individuals on the basis of accurate scoring of dicentric and centric ring chromosomes in G_o-lymphocyte PCCs. The micro-PCC assay for high-throughput analysis developed in the present thesis is automatable and could thus pave the way to its subsequent automation, which is critically needed for timely triage biodosimetry in mass-casualty radiological emergencies.

3 General Background

3.1 Needs for effective biodosimetry measurements in large-scale radiation exposure

Currently, awareness is growing regarding the possibility that large-scale radiological accidents or terrorism acts could result in potential radiation exposure of hundreds or thousands of people and that the present guidelines for evaluation after such an event are seriously deficient. There is a great and urgent need, therefore, for after-the-fact biodosimetric methods enabling the assessment of radiation doses, which must be at the individual level, timely, accurate, and plausibly obtained in large-scale disasters[7, 11].

By definition biodosimetry means, the quantification of absorbed doses with the help of biological material obtained from exposed individuals. Ideally, the biodosimetric methods for estimating human radiation exposure must have first the ability to determine which individuals did not receive a significant exposure so they can be removed from the acute response system. Second, must have the capacity to classify those initially assessed as needing further evaluation into treatment-level categories, and third, must have the ability to guide both short- and long-term treatments. Towards this goal, significant attempts have been made for the development of biodosimetric methods using appropriate biomarkers based on biological or physical parameters and considering their features such as speed, accuracy, capacity and ease of getting information for dose assessment. Specifically, there are two main methods of biodosimetry, biologically-based and physically-based, and in practice their use indicates that combining physical and biological techniques may sometimes be most effective.

Biologically-based biodosimetry approaches are essentially based on biological processes or parameters that are affected by IR. Examples include cytogenetic approaches enabling the visualization and evaluation of chromosome integrity following the initial radiation-induced damage at the DNA level. The subsequent activation of repair/misrepair mechanisms may cause production of unusual chromosomal aberrations in case the enzymatic repair processing of DNA

damage is not error free. Dose estimation using cytogenetic analysis is based on the relationship between chromosome aberration frequency and the amount of dose absorbed. The preferred choice of sample to analyze aberration frequency is the blood lymphocytes as they are easy to collect, culture and processing for biodosimetric studies. Exposed lymphocytes show different types of chromosome aberrations like dicentric chromosome (DC), centric ring, acentrics and translocation, all of which can be related to dose. For reliable dose estimation, there are several important biological parameters such as low background frequency, specificity to IR, a clear dose-effect relationship for high and low linear energy transfer (LET) radiation with different dose and dose rates, and most importantly reproducibility and comparability of in vitro to in vivo results [29].

Physically-based biodosimetry approaches are mainly based on physical parameters measured in the tissues of exposed individuals. Examples include, levels of long-lived radiation-induced free radicals detected by Electron Paramagnetic Resonance (EPR)[30-32] and Optically Stimulated Luminescence (OSL)[33]. EPR dosimetry is especially well-developed and established as one of the principal methods for estimating doses many years after an exposure. Originally, this technology was based on exfoliated teeth but more recently, EPR has been used for acute dosimetry using measurements of teeth in situ [34, 35] and fingernail clippings [36, 37].

The use of biodosimetry to measure radiation dose after-the-fact has become a very important and high-priority field due to the need for governments to be prepared for the heightened potential for exposures of large numbers of individuals from radiological accidents or terrorism acts [7]. Estimating radiation doses would greatly help to medically evaluate the injured in four different ways. Biodosimetry would help estimate how many people received doses that did not require acute care, classify those patients who need further evaluation into treatment-level categories, guide actual treatment, and help providers and patients with the long-term consequences of exposures to IR, including planning for treatment and patient compensation.

3.2 Triage patients

The next level of use of biodosimetry, which could follow the initial screening, would be to assist in assigning individuals as rapidly and effectively as possible into major action classes. The number of categories would depend on the volume of people triaged for care and the capabilities of the medical care system for addressing their treatment. Under some circumstances, such as the limited availability of stem-cell transplantation, it would be desirable for the biodosimetry technique to provide reliable estimates for subclasses of risk so that the limited capabilities for high-intensity treatments could be used most effectively. In general, the exposed individuals can be assigned into three categories [11].

Category 1. Identify false positives and those near 2 Gy—These individuals would not need urgent medical care. They might possibly need to be evaluated for risks of long- term effects but would have little need for prompt actions. Individuals assigned to this category could leave the emergency medical care system, at least during the period of time when there is greatest stress and potential for overwhelming the system.

Category 2. Admit patients into the medical care system for observation and, as needed, active medical care—This would be done to reduce the probability of a near-term deleterious clinical course due to ARS. This group is likely to require active symptomatic medical care and may also receive complex (and potentially risky) more aggressive treatments, such as bone marrow transplantation and/or high doses of radiation- mitigating drugs. The assignment of individuals into this action class would typically occur when the dose is in the range of 3–8 Gy.

Category 3. Provide palliative or expectant care—This level would identify individuals whose radiation exposure is too high for effective active or mitigating therapy. The actual threshold level may vary under the conditions of the event and the ability of the system to provide advanced care; however, a likely threshold would be 8 Gy. If fewer individuals are involved and the treatment capability is not overwhelmed, the threshold for entry into this category would probably be increased. On the other hand, if the healthcare system was potentially

overwhelmed, the dose range for active treatment (i.e., placement into Category 2) might be narrowed on both ends. That is, more people could be placed into Category 1 (by raising the minimum dose to qualify for active treatment) and more placed into Category 3 (by lowering the maximum dose to qualify for active treatment).

Many of the useful characteristics of biodosimetry techniques for this more refined sorting into action categories would differ from those required for the initial triage. The information would not need to be available as rapidly. While it would be desirable to avoid the need to transport the samples, it would sometimes be feasible to transport samples, especially to nearby facilities such as an emergency center set up near the event site. The throughput could be less. Techniques for measuring dose could include bringing expert operators to the site. It would be important for the technique to have a low false-assignment rate, i.e., neither assigning too high nor too low a category or subcategory. For this purpose, an estimate of dose within ±0.5 to 1.0 Gy of the actual dose is probably sufficient, because the known variation in response among individuals receiving the same exposure dose is likely to render more precise estimates of dose clinically irrelevant.

3.3 Biomarkers of radiation exposure

Exposure to radiation induces certain changes on the proteins, carbohydrates, lipids, nucleic acids and gene expression in the exposed cell, which are collectively known as biomarkers. In particular, traversal of IR in a cellular system can bring about a variety of changes such as base damages, alkylation, intercalation adduct formation, nucleotide modifications, single strand and double strand breaks in the deoxyribonucleic acid (DNA). Those changes can result either due to direct deposition of energy on the nucleic acids (direct action) or can be mediated by the release of electrons and generation of free radicals like $OH^{\cdot}$, released at some point in the interaction with water (indirect action) and membrane (lipid peroxidation) which surrounds the cells. The biomarkers are classified based on the changes being looked into like chromosomal aberrations, alterations in cell number, change in an enzyme level and or activity, proteins, or expression of

genes, etc [38, 39]. Of late based on the temporal parameters, it has been classified into markers of exposure, marker of susceptibility, markers of late effects and markers of persistent effects[16]. Thus, the manifestations of any of those changes are resulted due to the traversal of ionization track and deposition of energy in exposed cells/tissues. A summary of biomarkers of radiation exposure reported in the literature is given in Figure 1.

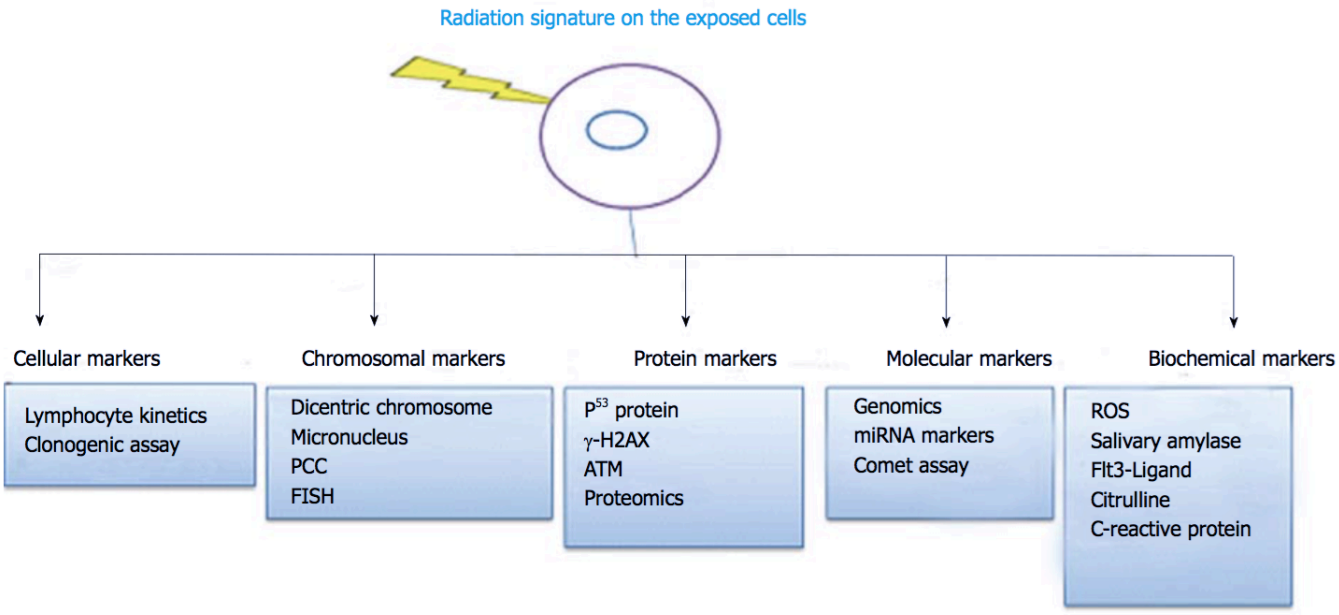

Figure 1. Biomarkers of exposure to ionizing radiation. FISH: Fluorescence in situ hybridization; PCC: Premature chromosome condensation; ROS: Reactive oxygen species; ATM: Ataxia telangiectasia mutated [23].

Upon the energy deposition of radiation in the exposed cells, many changes can be induced and in turn the cells respond to those changes explicitly activation player molecules involved in check points activation, DNA repair and apoptosis [40]. The end result and fate of the cells depends on the many physical parameters of the incident photon as well as the cellular biological machinery. The chromosome aberrations are formed predominantly due to the repair activation that results in a not perfect rejoining or mis-rejoin to form chromosome aberrations. Thus, the aberration produced depends on the number of breaks, chromatids and chromosomes as well as its

proximity of induced breaks involved [41]. The type, complexity and frequency of aberrations induced by radiations are diverse, which are traditionally being used to quantify and relate to the absorbed dose (Figure 2). Chromosomal changes are named based on the methodology employed, or stain used (Giemsa or fluorescence) to observe those changes or the end product (micronucleus, translocations) [42].

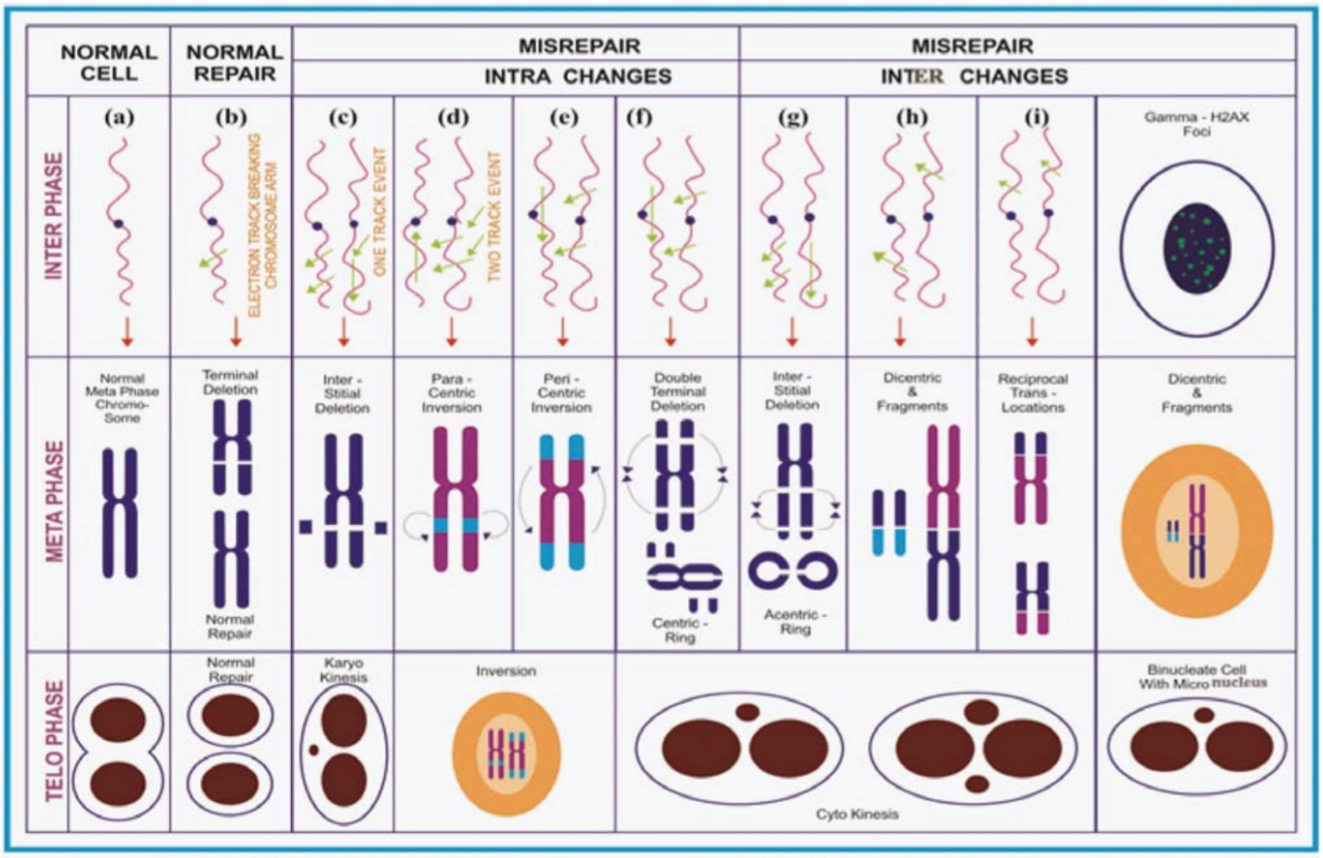

Figure 2. Diagrammatic illustration on the formation of ionizing radiation induced chromosome aberrations [23].

3.4 Cytogenetic biomarkers of radiation exposure

Cytogenetics focuses on the study of chromosomes, in particular chromosomal anomalies. Several cytogenetic endpoints are routinely used as biomarkers of exposure as they show a high degree of specificity and sensitivity. Other cytogenetic measurements might be useful as

biomarkers of late effects but need to be validated against well-defined outcomes/endpoints. Cytogenetic biomarkers are summarized in Table 1.

Table 1. Cytogenetic biomarkers [16].

Biomarkers	Assays/methodology	Sensitivity	Specificity to IR and confounders	Time window after exposure during which assays might be performed	Biological material needed to perform the assays[a]
Dicentrics	Dicentric chromosome assay	0.1–5 Gy	Almost exclusively induced by IR	Before renewal of PBL	WB: fresh; PBMC: fresh and frozen[b]
Translocations	Single colour FISH G-banding	0.25–4 Gy	Confounding factors: smoking; strong age effect	Years	WB: fresh; PBMC: fresh and frozen[b]
CCR	Multiple colour FISH	Unknown	High LET and heavy ion exposure	Before renewal of PBL	WB: fresh; PBMC: fresh and frozen
PCC	PCC assay combined or not to FISH chromosome painting or c-banding	PCC fragments: 0.2–20 Gy PCC rings: 1–20 Gy	IR specific to a large extent	PCC fragments: ideally immediately after exposure PCC rings: before renewal of PBL	PBMC: fresh and frozen
Telomere length	Flow cytometry Quantitative-FISH Southern blot qPCR	Not yet established	Not specific: modulated by viral infection Potential confounders: age, oxidative stress	Not yet established	WB, PBMC, cell lines: fresh and frozen
Micronuclei	Cytokinesis block micronucleus assay Micronucleus centromere FISH assay for low doses Flow cytometric detection of DNA in reticulocytes	0.2–4 Gy[c] but limited sensitivity at doses <1 Gy. Selective scoring after centromere FISH: ~100 mGy	Not specific: modulated by genotoxins Confounding factors: age, gender	In lymphocytes: before renewal of PBL In reticulocytes: not yet established	WB, PBMC: fresh and frozen[b]; Reticulocytes: fresh

CCR: complex chromosomal rearrangement; FISH: Fluorescence in situ hybridization; PBMC: Peripheral Blood Mononuclear Cell (lymphocytes, monocytes, etc.); PBL: Peripheral blood lymphocytes (T lymphocytes for assays requiring cycling cells); PCC: premature chromosome condensation; qPCR: quantitative polymerase chain reaction; WB: whole blood.

[a] Sample storage conditions listed when known.

[b] Frozen samples give lower yields of scorable cells.

[c] Dose range for photon equivalent acute whole-body exposure 24 h ago.

3.4.1 Dicentric chromosomes

The dicentric chromosome, which is a chromosome with two centromeres instead of its normal structure with one centromere (Figure 3), is the most widely used cytogenetic biomarker for dose assessment following exposure to IR. Its formation is a complex event because it needs double strand breaks (DSBs) in at least two different chromosomes. The DSBs should be in close proximity to each other so that they will have a high probability for misrepair and formation of abnormal structures [43].

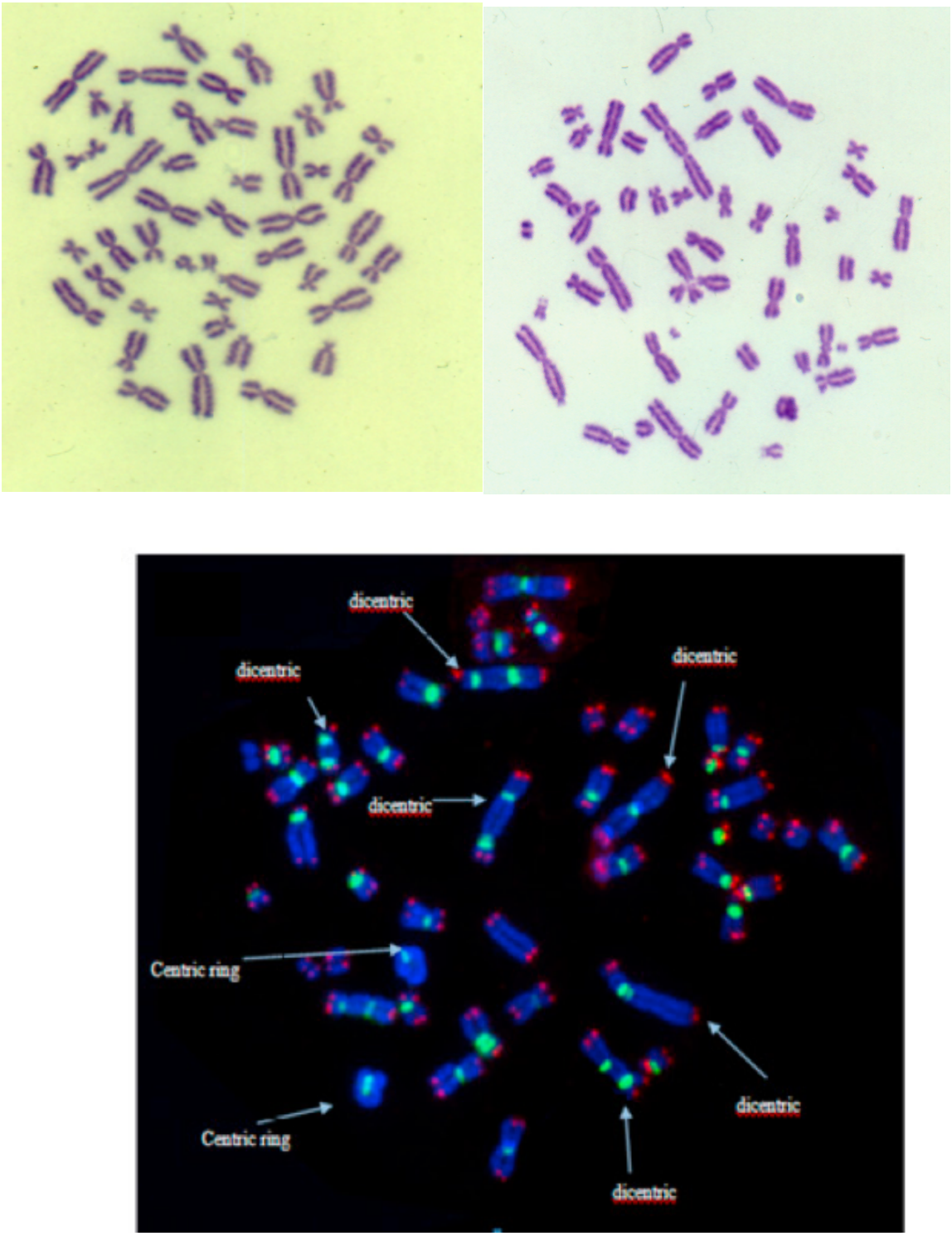

Figure 3. Examples of normal chromosomes (top left), dicentric and centric ring chromosomes (top right) as visualized by Giemsa staining; or by combining Centromeric/Telomeric (C/T) Peptide Nucleic Acid (PNA) probes with the FISH technique (bottom).

In order to estimate the dose from accidental exposures, appropriate dose-response curves should be constructed in every biodosimetry laboratory for different radiation qualities by irradiating in vitro heparinized peripheral blood samples using sufficient dose points. The irradiated blood samples are then cultured for 48-50h under aseptic conditions in the presence of phytohaemagglutinin (PHA) and microscope slides are prepared with good quality of metaphase chromosomes and high mitotic index.

Stained slides with Giemsa are used to measure the number of dicentric and centric ring chromosomes at each dose point and their frequency per lymphocyte metaphase is used to construct a reference dose-response curve. Representative images of normal metaphase and a metaphase with DC obtained from human blood lymphocytes exposed to 60Co-γ-irradiation are given in Figure 3 top. DC scoring can be supplemented with the application of C/T-FISH, which simultaneously stains centromeres and telomeres (Figure 3 bottom). This technique enables the detection of true dicentrics in ambiguous cases with complex configurations, which is technically challenging with classical Giemsa staining only [1, 44].

The constructed dose-response curve under in vitro conditions follows the equation $Y=C+\alpha D+\beta D^2$ or $Y=C+\alpha D$, depending upon the quality of radiation, and can be applied for estimating doses of the exposed individuals, where: Y is the yield of dicentrics plus centric rings, D is the dose, C is the background frequency, α is the liner coefficient, and β is the dose squared coefficient (Figure 4). The ratio of α/β can be referred to as the cross-over dose. It is equal to the dose at which the linear and the quadratic components contribute equally to the formation of dicentrics. It has been shown that the number of DC obtained with a given amount of dose is the same when irradiated either in vitro or in vivo condition [45].

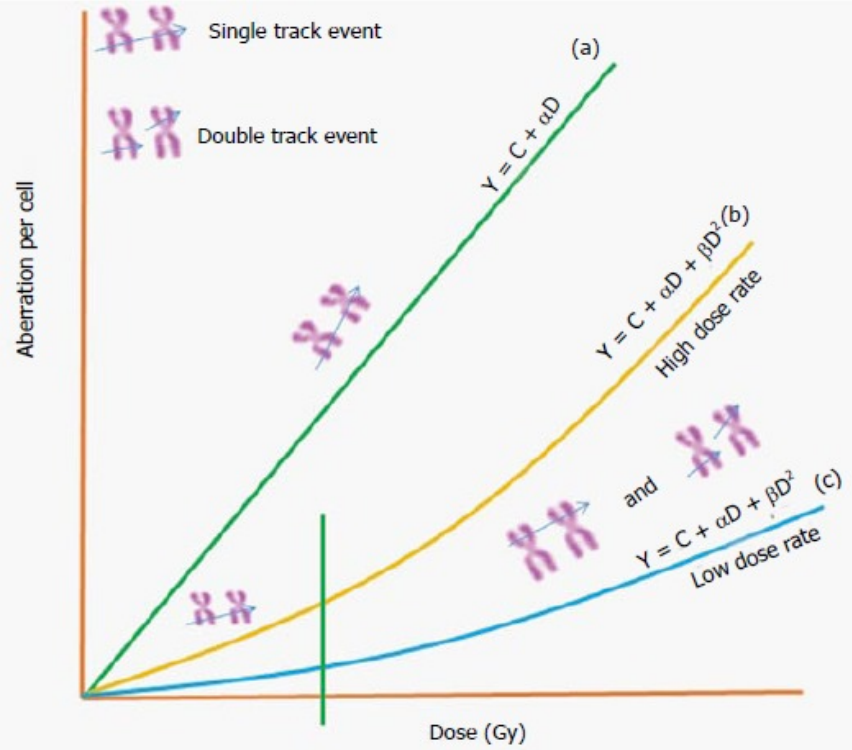

Figure 4. Dose response relationship for chromosome aberrations induced by different types of ionizing radiations[23]

The linear component (αD) often interpreted as the number of aberrations formed due to the traversal of a single particle track and is expected to be independent of dose-rate. The dose squared (βD^2) term is formed due to the interaction between two independent particle tracks and its degree determined by the time interval between the two tracks.

With low LET radiation such as X rays and γ-radiation, the ionization at any particular dose will be randomly distributed between cells, particularly since there will be a very large number of tracks. The DNA damage as well as the chromosomal aberrations will be also randomly distributed between cells. With high LET, or densely-ionizing radiation, the ionization tracks will be non-randomly distributed between cells, with the energy being deposited in more “discrete packets”, and consequently the induced aberrations will be non-randomly distributed between cells. Therefore, for low LET radiation, there is greater probability that two lesions within the target will be induced by two ionization events along the same track, resulting in two consequences. In

the case of high LET radiation, there is a greater probability that two lesions within the target to be produced by one track, and the dose-response curve mostly follows the equation $Y = C + \alpha D$ (Figure 4).

A useful comparative term to describe the deposition of energy by different types of radiation is the linear energy transfer expressed in keV/µm (LET), and the track average appears to be the better quantity to describe the relative biological effectiveness (RBE) variations for chromosomal damage[1, 46]. The track average for LET of 250 kVp (kilovolts peak) X rays is about 2 keV/µm, as compared with heavy charged particles that have track average LET values of 100-2000 keV/µm. The quantity of energy deposited per micrometer of track will determined the biological effectiveness of different types of radiation. By definition, the relative biological effectiveness (RBE) is defined as the ratio of the dose of the reference radiation (200-250 kvp of X rays) to the dose of the particular radiation being studied that produces the same biological effect.

The dicentric chromosomes are stable within non-dividing cells such as G0-lymphocytes but as the half-life of blood lymphocytes is in the order of months/years depending on the sub-population, the dicentric is the biomarker of choice for investigating recent exposure to IR. In general, as there are no major confounders influencing the yield of dicentrics, its natural occurrence is very low (generally in the order of 0.5–1/1000 cells scored) [1]. Individual dose assessment can be achieved for homogeneous whole-body exposures to doses as low as 100 mGy for low-linear energy transfer (LET) IR if up to 1000 cells are analyzed.

The scoring of dicentrics based on chromosomal morphology requires expertise, and time to analyze a large number of cells required particularly for low dose exposures. However, after exposure to low doses, the calculated estimates often carry large uncertainties, mainly due to the insufficient number of cells scored. Since the dicentric assay is very laborious, counting sufficiently large numbers of cells will be a limiting factor and will limit the possibilities for adequate dose estimation in the low dose range on an individual level. Automated systems are under development and provide very reproducible results but their major limitation is the dicentric's detection efficiency that remains around 50–70% [1]. Nevertheless, automated dicentric assays are currently being investigated in the framework of the European Multi- biodose

project (http://www.multibiodose.eu) that is aimed at analysing and adapting biodosimetric tools to manage high scale radiological casualties.

In addition to acute whole-body exposures, dose estimation for protracted and partial-body exposure can also be achieved by scoring dicentrics in lymphocytes. It should be noted that in order to estimate dose, calibration curves are necessary. Although the scoring of dicentrics is most suitable as a biomarker for external exposures [47], it can also be informative after internal exposures to radionuclides that disperse fairly uniformly around the body. Isotopes of caesium and tritiated water are two such examples [1]. Dicentric aberrations are unstable because their frequency decreases with the turnover of peripheral blood lymphocytes. Thus, for reliable dose assessment, dicentric aberration assays should be performed within a few weeks of exposure. If performed later, the precision of the assay is diminished as the dose calculation requires the use of half-time estimates for the disappearance of dicentrics [1].

3.4.2 Translocations

In contrast to dicentrics, reciprocal translocations are chromosomal aberrations that can persist in peripheral blood lymphocytes for years and can thus be used as biomarkers of past exposures [1]. This persistence reflects the presence of translocations in the lymphatic stem cells and is affected by many factors. These include exposure conditions, such as dose rate and whole- body vs. partial-body exposure. As translocations may be parts of complex chromosomal rearrangements (CCRs) [48] that are unstable (see Section 4.1.3), it is essential to distinguish their origin within the cell. The cells that are scored also influence measurements and dose calibration for translocations, as it has been shown that the presence of unstable chromosomal aberrations in the same cell reduces the frequency of translocations with time [49]. Therefore, only cells free of any unstable chromosome damage should be scored.

The FISH technique or ‘‘chromosome painting’’ is commonly used for the detection of inter-exchanges, such as translocations and dicentrics (Figure 5). Currently the assay could be semi-

automated, for instance through the use of a metaphase finder system, but there is still a need for a fully automated image analyzer that would reliably differentiate normal cells from cells with chromosomal aberrations [50, 51]. Rapid developments in the probe labelling methodology, optics and imaging modalities, the assay has evolved in different directions like m-FISH, SKY-FISH, and m-band where exchanges involved in any chromosomes or regions within chromosomes can be identified easily similar to that, GTG-banding technique have been in use for the identification of aberrations in individual chromosomes as well as in entire genomes. It was an attractive option for many years back; however, RT measurements with latest FISH technology, and G-banding, in dosimetry is limited because of either time factor and/or cost factor. However, it can provide a true estimation of translocation frequency by analyzing the individual chromosomes for chronic dose estimation.

A common feature of translocations among non-exposed subjects is the large inter-individual variation in their number, age being the most important confounding factor [52]. Therefore, natural occurrence and accumulation with lifespan can confound very low dose exposure estimations. Despite the strong age-effect for translocations, subjects of the same age may show large variation in translocation frequencies. The reasons for the age-dependent baseline frequency is not fully established, but some of them may be linked with cellular mechanisms changing with age such as DNA repair. From a number of confounders tested, smoking has been demonstrated to increase translocation frequencies in some studies, but not in others, possibly due to variations in cigarette types or numbers smoked [50]. Other sources of variation may include clastogenic agents in the diet or environment, gender, ethnicity and genetic polymorphisms in genes involved in cellular defense mechanisms. Individual dose assessment using translocations is strongly dependent on the personal baseline frequency of this aberration, and from a practical point of view, the time required for the analysis would severely limit the use of such an approach for triage biodosimetry in large studies.

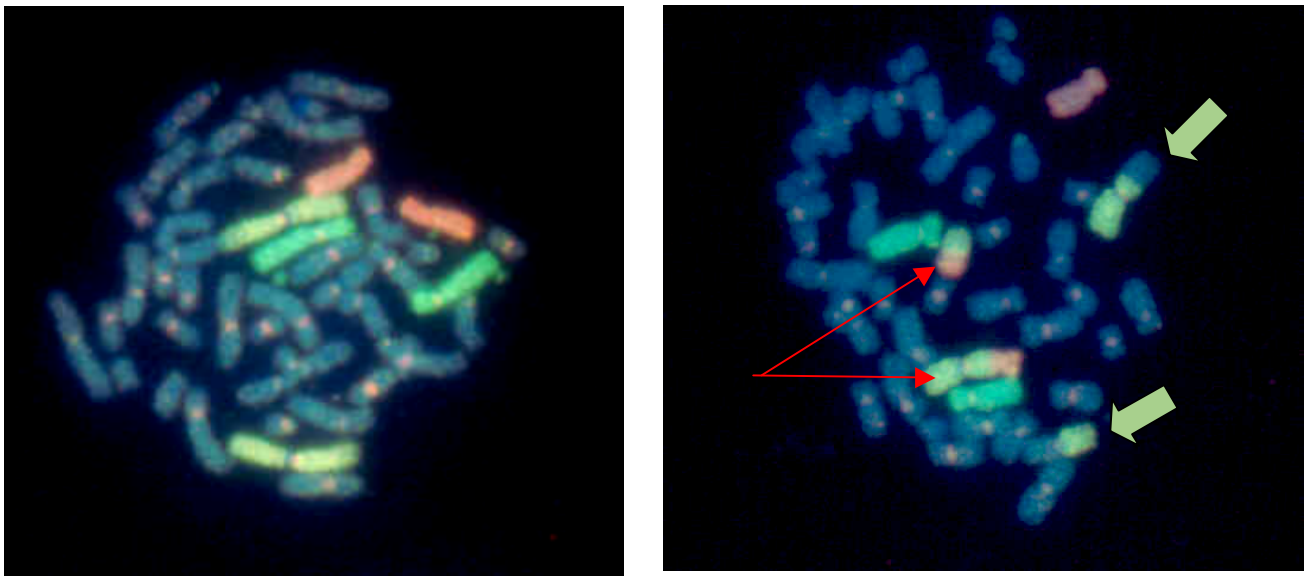

Figure 5. Human lymphocyte metaphase illustrating FISH-based chromosome 'painting' to detect chromosomes 1 (yellow/green), 4 (green) and 8 (orange) using whole chromosome and pan-centromeric probes (left). A dicentric chromosome and its associated acentric fragment (red arrows) as well as a reciprocal translocation of chromosome 1 illustrated by the two bicolored chromosomes (green arrows) (right)

3.4.3 Micronuclei

Micronuclei (MN) form when mainly fragments or intact chromosomes are not properly segregated into daughter cell nuclei at anaphase but instead remain in the cytoplasm after cell division. Generally, they are regular in shape with a similar staining intensity to that of daughter nuclei and they can be visualized as small spherical objects using any conventional DNA dye (Figure 6). In comparison to most other cytogenetic techniques, MN are far easier to score both manually and using automated microscopy slide scanning and image analysis systems [1, 53, 54]. As MN form only during cell division and, like dicentrics, are lost when cells continue to divide. Fenech et al [55] developed a simple, most effective and reliable methodology to select cells between first and second mitosis division using cytochalasin-B to inhibit cell division at cytokinesis in a cycling cell and named as cytokinesis blocked micronucleus (CBMN) assay. Indeed, reliable quantitative results are achieved by blocking the cell cycle progression of PHA-

stimulated lymphocytes at the stage of cytokinesis after the first mitosis, and by performing microscopic scoring of MN only in binucleated cells. An important caution is that many factors like age, genetic makeup and storage of blood samples could influence the dose estimation using the MN assay [56]. Similar to DC many laboratories have established dose response curves to estimate the dose; it follows linear-quadratic pattern despite the fact that there are differences in the obtained co-efficients among the established laboratories.

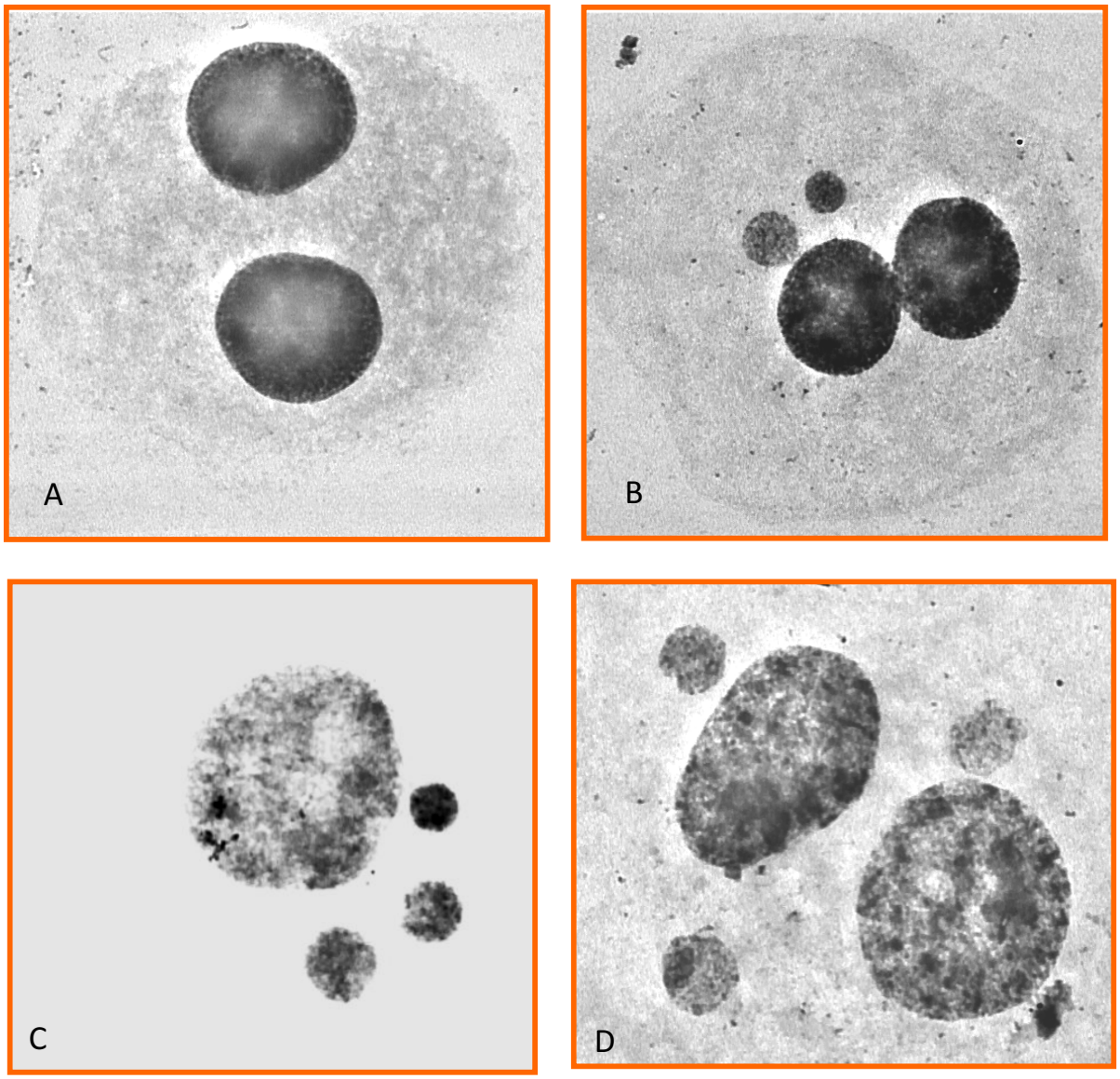

Figure 6. Normal cell division (A), aberrant divisions with 2 (B), 3 (C) and 4 (D) micronuclei.

MN reflects chromosomal damage and thus it is a useful index for monitoring environmental effects on genetic material in human cells [57]. Due to the simplicity and the rapidity of scoring, this assay has shown promising potential in the triage medical management. However, due to background frequency of spontaneous MN frequency (0.002 to 0.036/cells) the sensitivity is 0.25 Gy [1]. The CBMN assay in addition to measuring the MN, it can also be used to measure nuclear-plasmic bridges, nuclear buds, necrotic cells, apoptotic cell and nuclear division rate collectively known as cytome assay [58]. Several studies have been carried out using the MN analysis in vitro and in vivo, for the purposes of biological dosimetry. A good correlation between the doses estimated from the MN frequency was observed in radiation workers [59] and in thyroid cancer patients undergoing radioiodine treatment [60]. A large volume of published reports for in vitro dose response curves is available. MN measured by cytokinesis-blocked micronucleus (CBMN) assay show promise as a biomarker for individual radiosensitivity and susceptibility to environmental carcinogens [61]. Consistent with this notion, a recent twin study provided evidence for the high heritability of baseline and induced MN frequencies [62].

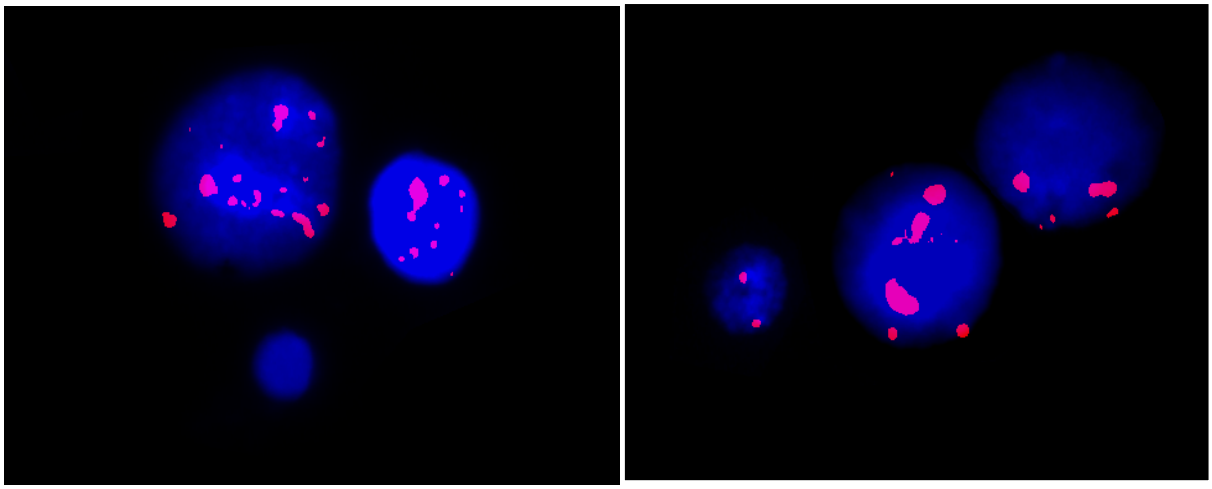

Figure 7. Binucleanted cells showing a centromenre negative MN (left) and a centromere positive MN (right). Centromeres are stained with a pan-centromeric probe (spectrum orange) and nuclei and MN are counterstained with DAPI.

Due to variable base levels in different individuals, the standard CBMN assay cannot detect acute whole-body doses below 200 mGy for low-LET IR. In addition, a wide range of clastogenic and aneugenic agents (i.e. agents causing chromosome breakages and abnormal number of chromosomes, respectively) can induce MN, and confounding factors include age and gender. Most of this background 'noise' of MN in non-exposed individuals can be attributed to the loss of one intact copy of the X-chromosome.

Selective scoring of MN that are negative for centromere-specific FISH signals (Figure 7) can significantly improve the sensitivity to a minimum detectable acute whole-body gamma-ray dose of 100mGy for individuals [63]. First steps have been made towards the development of an automated analysis system for the micronucleus centromere assay [64] which would enable large-scale studies of cohorts exposed to low-to-moderate radiation doses. Other limitations of the CBMN assay include the minimum delay of 3 days between sampling and first results becoming available, loss of the signal with lymphocyte turnover (as discussed above for dicentrics) and its inability to detect non- uniform exposures [63].

3.4.4 Premature chromosome condensation (PCC)

When cycling cells enter mitosis, their nuclear membrane is dissolved and chromatin condenses into the familiar shaped of metaphase chromosomes by the histone phosphorylation processes of phosphokinases, which are generated during G2 to M-phase transition. Therefore, measurement of chromosomal aberrations by means of the conventional metaphase method requires that the cells of interest are in mitotic phase where chromosomes are highly condensed and visible.

Alternatively, techniques have been developed to cause chromatin that is not at mitosis to condense prematurely in cycling, quiescent or even in non-cycling cells. This phenomenon is termed premature chromosome condensation (PCC), and it can be induced by fusing interphase cells to mitotic Chinese hamster ovary (CHO) or HeLa cells using Sendai virus or, particularly for

lymphocytes, using the fusing agent polyethylene glycol (PEG). PEG is used for lymphocytes since cell fusion by means of Sendai virus requires cells with membranes especially receptive to the virus particles and it has been reported that G_0 lymphocytes cannot be satisfactorily fused using the Sendai virus. This difficulty was overcome for the purpose of biological dosimetry with the use of PEG for PCC induction in peripheral blood G_0 lymphocytes, as shown in Figure 8 [1, 2].

The major advantage of the PCC method is that radiation-induced chromosomal damage can be observed shortly after blood sampling, and it is also possible to score centric ring chromosomes, dicentrics and translocations if the PCC method is combined with FISH chromosome painting or C-banding [1] [25, 65]. The PCC technique is a very useful research tool to probe the immediate post-irradiation processes and kinetics of chromosomal break restitution and/or misrepair to form aberrations (i.e. dicentrics and translocations). These studies demonstrate that the dicentrics, complete and incomplete translocations and acentric fragments, that one sees eventually at metaphase, are formed essentially in G_0 -lymphocytes and, therefore, their formation do not require lymphocyte stimulation, culture and DNA replication.

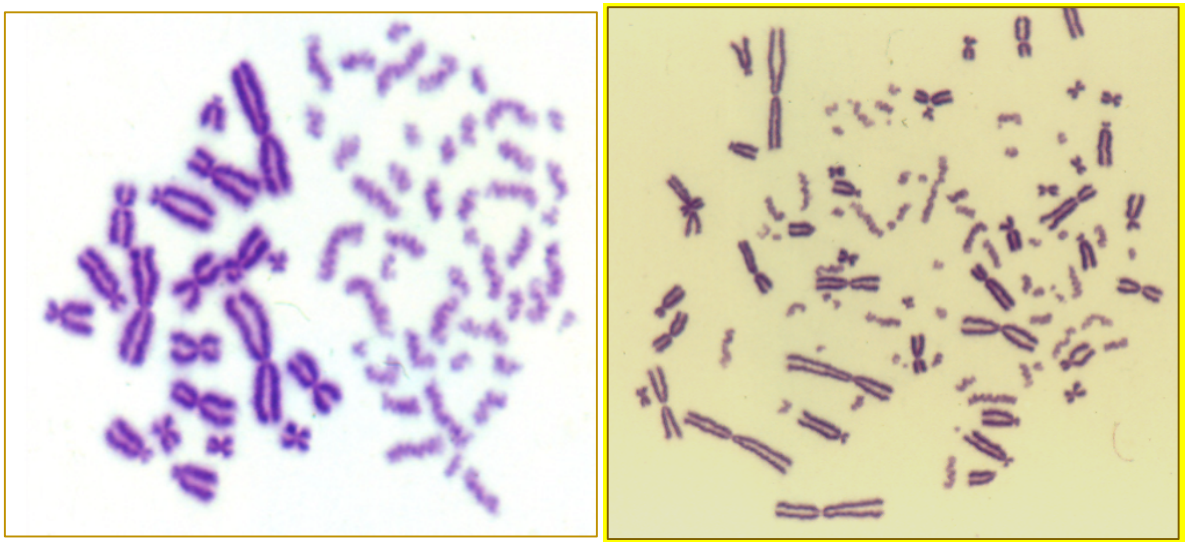

Figure 8. Premature chromosome condensation induced by PEG-mediated fusion in an unirradiated human lymphocyte fused with a mitotic CHO cell. Forty-six distinct single chromatid PCCs can be seen (left). In irradiated quiescent cells a number of excess PCC fragments (>46 chromosomes for human) can be scored (right).

3.5 DNA and nucleotide pool damage Biomarkers

IR induces directly or indirectly, via ionization events, a variety of DNA lesions that could be exploited as potential biomarkers of exposure [66]. It is estimated that exposure of mammalian cells to 1 Gy of gamma or photon radiation can lead to 1000 single-strand breaks (SSB), 500 damaged bases, 40 double strand breaks (DSBs) and 150 DNA-protein cross-links [67]. However, the formation of these DNA lesions is not unique to IR and thus they cannot be used per se as biomarkers of exposure unless a number of confounding factors are taken into account, including age, syndromes associated with oxidative stress and exposure to other genotoxins (including smoking, certain occupational settings and chemotherapy). DNA strand breaks can be measured directly or by using surrogate endpoints such as the presence of γH2AX foci or assays such as the comet assay. The nucleotide pool is also target of IR and indirectly of oxidative stress, and in recent years the use of 8-oxo-dG has been suggested as a potential biomarker.

3.5.1 DNA single/double strand breaks (DSBs)

Since SSB and especially DSBs are highly characteristic of the DNA lesions formed after exposure to IR, assays detecting their formation and persistence or the individual's ability to repair this type of damage can be used as biomarkers for exposure as well as for individual radiation sensitivity. To measure DNA SSB and/or DSB generated by IR several techniques with different sensitivities in terms of the lesions detected and the level of detectable DNA damage exist. These include techniques such as alkaline or neutral filter elution [68], alkaline unwinding [69], sucrose gradient centrifugation [70] or pulsed field gel electrophoresis [71]. However, the utility of such techniques for molecular epidemiological studies investigating low doses effects is limited as none can be used to investigate DNA damage after exposures to doses under 2 Gy and at the single cell level.

The comet assay is a relatively easy, quick and automatable test to detect direct DNA-damage at

the single cell level and with a higher sensitivity compared to methods described above. Its main advantage is the requirement of only minimal numbers of cells (~10.000) or volumes of whole blood (10 µl) [72]. It has been widely used to measure both in vitro and in vivo DNA damage and repair following exposure of mammalian cells to various genotoxic agents including some chemicals, IR and non-IR [73]. The assay can be performed in neutral or in alkaline conditions. Both methods detect SSB and DSB however the alkaline assay is often used to detect SSB and alkali-labile sites whereas the neutral comet assay is often used to detect DSBs, although it has to be noted that this variant of the comet assay lacks sensitivity and specificity. With the alkaline comet assay an irradiation dose range from 100 mGy to 8 Gy can be investigated as well as DNA repair capacity. Because it can be automated, the assay is highly suitable for a screening assay in human populations although some important criteria need to be taken into consideration: (a) the specificity for radiation exposure is low, since oxidative stress in general induces single strand breaks detected by the assay, and (b) inter-experimental variability is very high in the test system, so the test has to be performed under highly standardized conditions with an automated analyses and the inclusion of reference samples so that comparisons can be made over-time [74]. In addition to being used as an endpoint to assess DNA damage levels, the comet assay can be used as a bioassay for instance to evaluate DNA repair capacity. Using such an approach, biological samples are irradiated in vitro and the level of persisting DNA damage with time used as a biomarker of susceptibility. The results from such assays need to be interpreted with caution as a number of confounding factors including age of the subject, smoking and diet have been reported.

In general, the yield of formation of radiation-induced DNA lesions per dose unit is very low and many of them are similar to those generated by endogenous stress. Even in the absence of radiation, oxidative DNA lesions are detected in cells at levels around one modification per million DNA bases, and after exposure of low doses may be difficult to interpret in terms of radiation exposure biomarkers. Emphasis should be, therefore, on DNA lesions produced predominantly by ionizing radiation such as complex DNA lesions and lesions generated in clusters [24, 75]. High LET radiation produces greater lesion clustering due to the confined energy deposition since direct ionizations along the DNA backbone will generate DSBs that can be only 10-20 bp apart

leading to strand cross linking. However, the detection and quantification of clustered lesions because of their very nature remains technically challenging. Yet, detection of clustered lesions is particularly interesting because it has the potential to be radiation specific. This could be technically feasible using surrogate endpoints signaled by the accumulation of phosphorylated proteins at the damage sites such as γH2AX, i.e. the H2AX histone phosphorylated on Ser-139, which form the typical DNA repair foci detected by immunological based techniques.

3.5.2 γH2AX and DNA repair

Following exposure to IR, molecules of histone H2AX at the broken site are rapidly phosphorylated on serine 139 in the C-terminus. In continuation, multiple factors involved in DNA repair and chromatin remodelling are assembled at the broken site and form the γH2AX foci [76]. Such γH2AX foci are simply visualized with antibodies to γH2AX with each DSB yielding one focus.

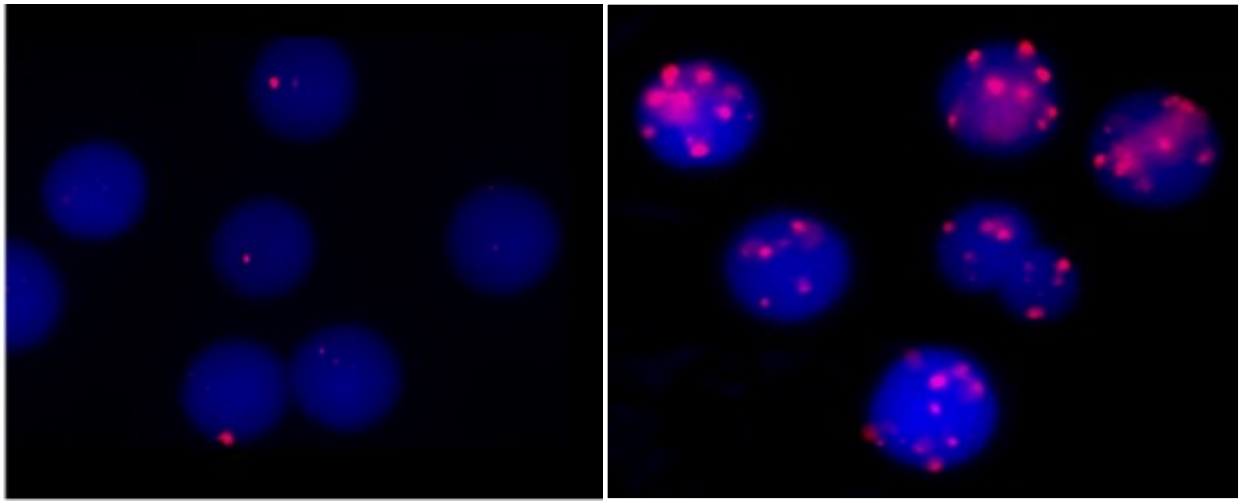

Figure 9. γH2AX foci in control (left) and irradiated with 1Gy (right) human lymphocytes.

The accumulation of γH2AX foci in the cell nucleus at the site of DNA DSBs occurs within minutes after the induction of DNA damage (Figure 9). The maximum yield of foci is detected within 30–

60 min after irradiation, depending on the dose, and after this, the number of foci usually decreases to baseline level within days [77]. The measurement of γH2AX foci by means of immunofluorescence microscopy could be used as a direct endpoint assessing the formation of damage and therefore as a biomarker of exposure, whereas its persistence with time in irradiated samples can be used to evaluate DNA repair capacity and thus as a biomarker of susceptibility [77].

Several studies on patients medically exposed to low dose radiation have shown that the γH2AX assay is very sensitive, and that foci after doses below 20–10 mGy can be detected. A study on patients with differentiated thyroid carcinoma who underwent 131-Iodine therapy showed that exposure to radionuclide incorporation can be detected by γH2AX assay in mononuclear peripheral blood leukocytes after absorbed doses to the blood below 20 mGy [78]. Studies investigating γH2AX foci in blood samples of CT scanned patients before and after the CT scan, have shown that γH2AX focus induction are sensitive enough to be used as biological dosimeters [79]. However, extensive use of the assay as a biomarker of exposure directly in biological samples obtained from a study subject is regarded as limited due to the fast decline of the signal as well as variation of foci frequencies between individuals. Indeed, variables like age, smoking, oxidative stress, inflammation, heat, genetic factors, etc., have been reported to influence the γH2AX foci levels. Moreover, a difference in γH2AX foci yields obtained from the same samples by different laboratories and methodical underestimation of doses has been reported as a major concern when using flow cytometry; variations in foci loss during shipment of blood samples, or variations in immunofluorescence staining quality were listed and should be minimized to reduce the uncertainties [80]. It was also emphasized that one should not look upon any calibration curves for this assay as in the DC and MN assays. Alternatively, the γH2AX foci assay should be frequently recalibrated to take into account any drift in foci yields, and protocols should be optimized to reduce variability and ensure consistency.

Despite these limitations, if samples can be obtained in appropriate time windows (1-12 hrs), the γH2AX assay has potential to reveal low dose hypersensitivity [81]. Löbrich et al. have investigated loss of γH2AX foci in lymphocytes of CT scanned patients and have demonstrated

that the loss of γH2AX foci correlated with DSB repair [79]. Interestingly, one radiosensitive patient presented elevated residual foci after CT, suggesting an impaired DNA repair. The kinetics of γH2AX foci loss might thus be used as a biomarker of susceptibility in in vivo or in vitro studies. However, as discussed above, due to the fast decline of the signal after exposure, in vitro irradiation of subject's cells to detect a loss in γH2AX foci might be more feasible in a large scale epidemiological study.

When considering γH2AX foci frequency or foci loss as potential biomarkers of exposure or susceptibility, it should be kept in mind that previous studies have provided evidence that at very low doses (1.2–10 mGy), the loss of foci in irradiated mice or nondividing primary human fibroblasts is impaired over several days compared to higher dose exposure [82]. Therefore, the use of γH2AX foci loss as a biomarker of susceptibility in studies investigating the effects of less than 10 mGy might not be suitable because the kinetics of foci loss will be difficult to establish. Regarding the use of γH2AX foci frequency as a biomarker of very low dose exposure, the persistence of the foci might be an opportunity to increase the time frame of sampling and testing after irradiation and also to discriminate between irradiated and non-irradiated cells.

Optimization of γH2AX foci analysis according to cell cycle phases and its limitations has been reviewed recently elsewhere [83]. Automation may improve the possibilities to apply the technique as a biomarker in large scale accidents and molecular epidemiological studies to investigate radiation sensitivity. There are two ways to automate the assay: (a) flow cytometric analysis, and (b) automated microscopic analysis. Flow cytometric analysis has the disadvantage that only total fluorescence instead of single foci are analyzed. As mentioned above for the dicentric assay and the MN assay, the automation of γH2AX assay for rapid triage in case of mass casualty scenario is one of the topics of interest in the European Multibiodose project.

3.5.3 Extracellular 8-oxo-dG

Recently, attention has been drawn to the nucleotide pool as a target of IR and it has been

suggested that extracellular 8- oxo-dG primarily originating from the sanitization process of 8-oxo- dGTP, may serve as a biomarker of the intracellular oxidative stress [84]. The mechanism(s) of radiation induced nucleotide pool damage are not well understood, dose–response relations suggest that in the low dose region (1–100mGy) radiation will trigger a stress response reaction leading to an endogenous formation of ROS that is the dominating cause of 8- oxo-dGTP production [85]. The dose–response relations are not linear and saturates for doses in the 0.1–1 Gy range. Regarding radiation-induced endogenous formation of ROS, mechanistic studies in cellular model systems have shown that it is transient with a time span over a few hours.

In parallel with the formation of 8-oxo-dGTP, the levels of the nucleotide pool sanitizing enzyme with 8-oxo-dGTPase activity (hMTH1) were increased following exposure to low dose radiation suggesting that subsequent ROS production will trigger the activation of cellular defense systems against oxidative stress [85]. Increased serum levels of 8-oxo-dG have also been suggested to correlate with inflammatory responses – that are known to generate ROS – in a group of haemodialysis patients [86]. Thus, although extracellular 8-oxo-dG may be used as a biomarker of oxidative stress it lacks the specificity for a biomarker of exposure to IR.

4 Materials and methods

4.1 Cell cultures and preparation of PCC-inducer mitotic CHO cells

Chinese hamster Ovary (CHO) cells were grown in McCoy's 5A (Biochrom), culture medium supplemented with 10% FBS, 1% l-glutamine and 1% antibiotics (Penicillin, Streptomycin), incubated at 37 °C in a humidified atmosphere with 5% CO_2. CHO cultures were maintained as exponentially growing monolayer cultures in 75 cm^2 plastic flasks at an initial density of 4 x 10^5 cells/flask. Colcemid (Gibco) at a final concentration of 0.1 µg/ml was added to CHO cultures for 4 hours and the accumulated mitotic cells were harvested by selective detachment. Once a sufficient number of mitotic cells had been obtained, they were used as supplier of mitosis-promoting factors (MPF) to induce PCC in human lymphocytes. Generally, the mitotic CHO cells harvested from one 75 cm^2 flask were used for 2-3 fusions.

4.2 Lymphocyte isolation, irradiation conditions and conventional cell fusion mediated premature chromosome condensation

Human lymphocytes were separated from heparinized blood samples using Biocoll separating solution (Biochrom). The blood sample was diluted 1:2 in RPMI-1640 without FBS, and was carefully layered on top of an equal amount of Biocoll in a test tube before centrifugation at 400g for 20 min. Collected lymphocytes were washed with 10 ml culture medium, centrifuged at 300g for 10 min and kept in culture medium (RPMI-1640 supplemented with 10% FBS, 1% glutamine and antibiotics). Lymphocytes isolated from 1-2ml of blood were used for each experimental point, mixed with CHO mitotics and fused by means of Polyethylene Glycol (PEG) (Figure 10).

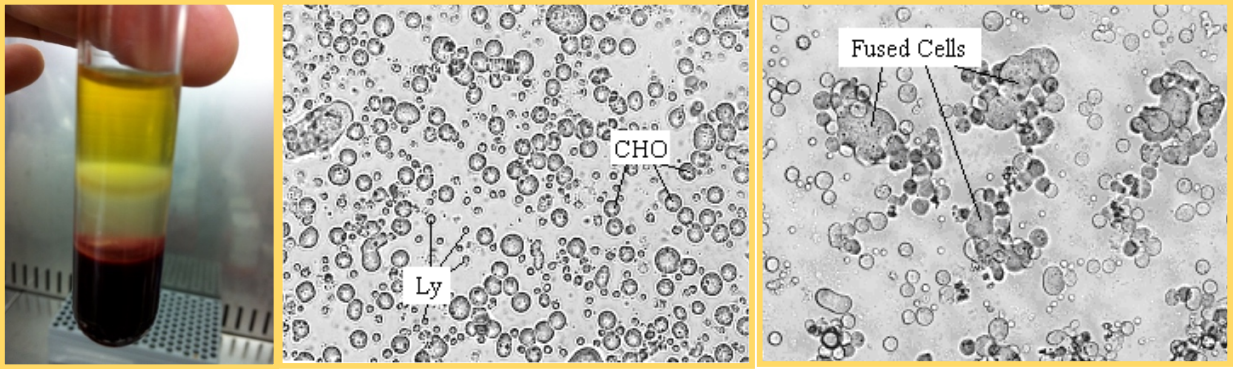

Figure 10. Human lymphocytes (Ly) are isolated using Biocoll separating solution (left), mixed with mitotic CHO cells (centre) and fused by means of PEG (right).

Irradiation was carried out in vitro using a Gamma Cell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) at room temperature and at a dose rate of 20cGy/min. Different irradiation times were applied in order to deliver to the blood samples doses ranging from 0.5 to 6Gy.

Mitotic CHO cells harvested from a 75 cm^2 flask were used for 2-3 fusions. Briefly, lymphocytes and mitotic CHO cells were mixed in serum-free RPMI-1640 medium containing HEPES (25mM) in a 15ml round-bottom culture tube in the presence of colcemid. After centrifugation at 200g for 6 min, the supernatant was discarded without disturbing the cell pellet, keeping the tubes always inverted in a test tube rack on a paper towel in order to drain the pellet from excess liquid. While holding the tubes in an inverted position, 0.15 ml of 45% (w/v) PEG (mol wt 1,450, Sigma-Aldrich/serum-free RPMI 1640 with Hepes) was injected forcefully against the cell pellet using a micropipette and immediately after the tube was turned in an upright position and held for about 1 min. Subsequently, 1.5 ml of phosphate buffered saline (PBS) was slowly added, the tube was shaken gently and the cell suspension was centrifuged at 200g for 6 min. The supernatant was discarded and the cell pellet was resuspended gently in 0.7 ml RPMI-1640 complete growth medium with colcemid. To optimize cell fusion when a low number of lymphocytes is available, complete lymphocyte growth medium, containing 1% phytohemagglutinin (PHA) and 10% FBS, was used [28, 87]. After 60–75 min at 37^{o}C, cell fusion and PCC induction was completed (Figures 11 and 12). Afterwards, cells were treated with hypotonic solution KCl (0.075 M), and fixed with methanol:glacial acetic acid (3:1, v/v), following standard cytogenetic procedures with

centrifugation at 250g. Chromosome spreads were prepared, slides were air-dried and stained with 3% Giemsa in Sorensen buffer solution. The PCC fragments per cell in excess of 46 PCCs were scored for each experimental point using light microscopy and the analysis was greatly facilitated by an image analysis system (Ikaros, MetaSystems).

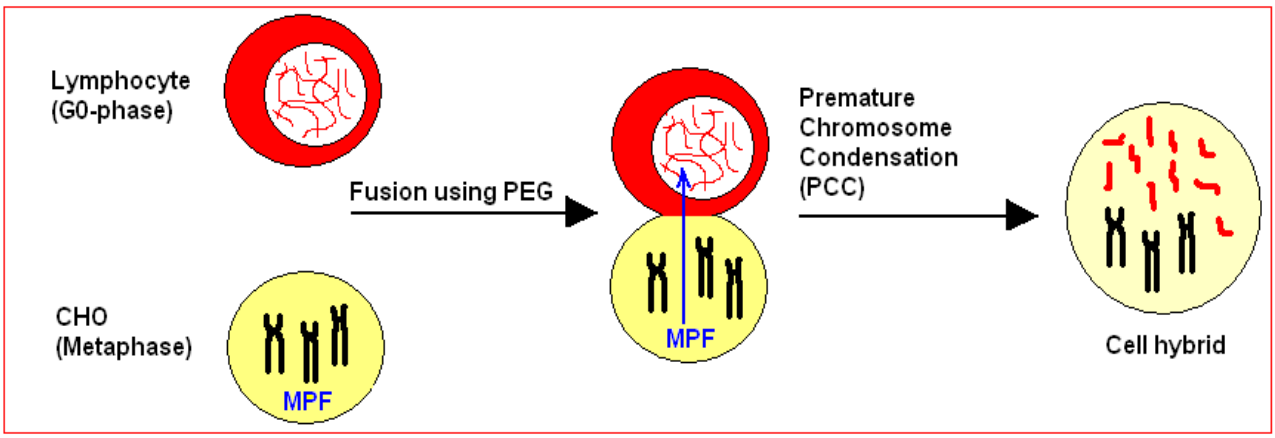

Figure 11. The presence of Mitosis-Promoting Factors (MPF) in the hybrid cells disolves the nuclear membrane of interphase lymphocytes and condenses their diffuse chromatin into distinct chromosomes, enabling thus their visualization.

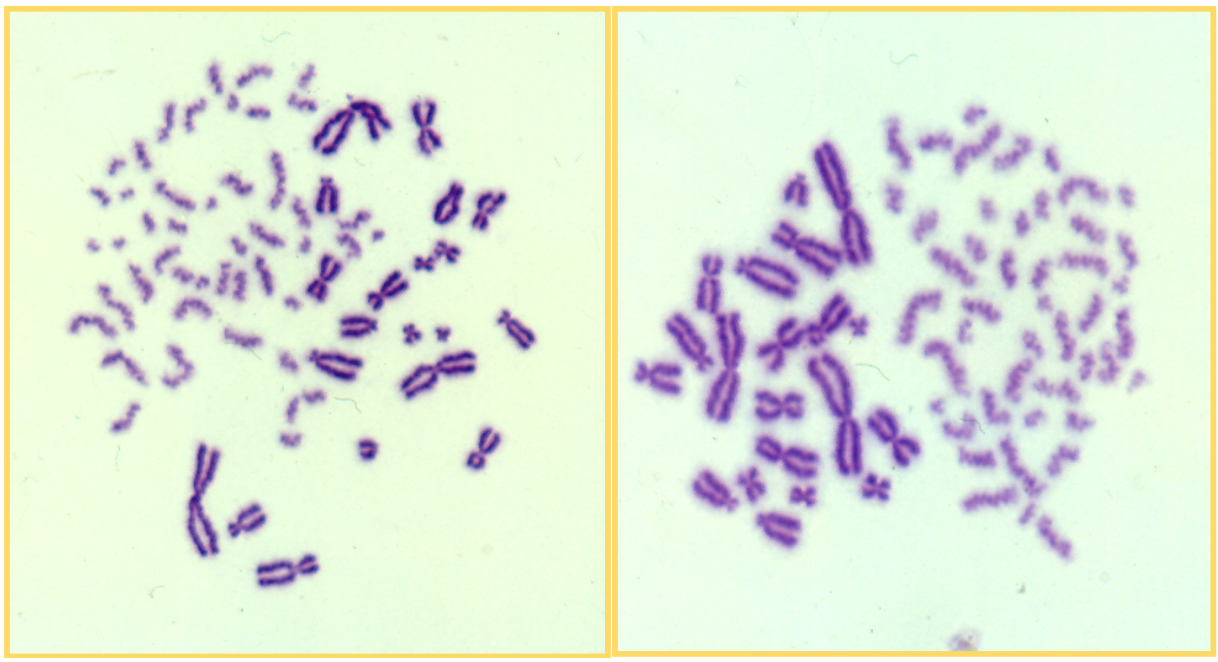

Figure 12. Darkly stained double-chromatid chromosomes represent hamster CHO mitotics while the lighter-stained single-chromatid chromosomes represent the lymphocyte PCCs.

4.3 Development of a micro-PCC assay as a rapid and minimally invasive automatable tool for early triage biodosimetry

A detailed protocol for the conventional PEG-mediated cell fusion and PCC induction in lymphocytes using mitotic CHO cells was established and standardized in our laboratory to facilitate the adoption of this technically demanding but not yet widely used PCC assay by other biodosimetry laboratories. This protocol was shared with members of the European Biodosimetry Network (RENEB) in order to harmonize and standardize the scoring criteria for the construction of reference calibration curves, and use the PCC assay for the estimation of absorbed doses in case of radiation accidents.

However, the protocol's requirements of using blood sample volumes of 1-2ml for each person, Ficoll-Paque gradients for lymphocyte isolation and 15ml round-bottom culture tubes for cell fusion, PCC induction and chromosome preparation, introduce difficulties in applying it for mass-

casualty events. For the development of an automatable micro-PCC assay for rapid individualized risk assessments in large-scale radiological emergencies, the various steps of the above procedures were, therefore, adapted so that they could be applied to very small blood volumes of 100μl using 96-well plates of 2ml/well.

The 96-well plates are advantageous for high-throughput analysis since the various steps required by the protocol can be applied to all 96 wells simultaneously. Interestingly, the morphology of the lymphocyte PCCs obtained was practically identical to that obtained using the conventional PCC procedure, which requires much larger blood volumes, and it allows, therefore, the simultaneous processing and dose assessments for multiples of 96 blood donors. In fact, the use of only 1.5ml of hypotonic solution and the fixation of cells twice with 1.5ml of Carnoy's fixative in the 96-well plates offers high quality PCC images. More details for the development of such an automatable micro-PCC assay are presented in the Results section.

4.4 Centromeric and telomeric staining of lymphocyte PCCs with PNA probes

Staining of centromeres and telomeres in lymphocyte PCCs (C/T FISH staining) was performed using the Q-FISH technique with a FAM-labeled PNA probe, specific for telomere sequences (TelC-FAM), and a Cy3-labeled PNA probe, specific for centromere sequences (Cent-Cy3) (both from Panagene, Daejon, South Korea). Briefly, the slides were kept in an oven at 60^{o}C for at least 1h, washed in phosphate buffered saline (PBS) solution for 15min, fixed in formaldehyde 4% solution for 2 min, washed again in PBS twice for 5 min and digested in a pre-warm pepsin solution (1mg/ml) for 3 min at 37^{o}C. After three PBS washes, slides were washed and refixed, dehydrated with 70%, 90%, 100% ethanol and air dried. PNA probes for centromere and telomere staining were applied, co-denaturated for 3 min at 80^{o}C and incubated for 2 h in a humidified chamber at room temperature in the dark. After hybridization, slides were washed with 70% Formamide, 1% Tris 1M pH7.2, 1% BSA 10%, H2O, twice for 15 min, then in TBS/Tween 0.08% three times for 5 min each, dehydrated with 70%, 90%, 100% ethanol series, and finally

counterstained with DAPI (1µg/ml) and mounting medium.

4.5 Analysis and scoring criteria

Lymphocyte PCC spreads were located manually and their analysis was facilitated by the use of a semi-automated image analysis system (Ikaros, MetaSystems). Specifically, the analysis of excess PCC fragments in lymphocyte PCC spreads stained with Giemsa was greatly facilitated by the appearance of the PCCs, which are lighter stained than the CHO mitotic cells and, therefore, easily distinguished from the mitotic chromosomes of the CHO cells. In unirradiated lymphocytes, 45-46 elements were scored in PCCs spreads and, in order to calculate the frequency of excess PCC fragments, this number was subtracted from the one obtained in the irradiated lymphocyte PCCs. Generally, a number of 20-30 PCC-spreads was considered adequate for dose estimation following a single exposure. When C/T staining with PNA probes was applied, dicentrics plus centric ring chromosomes were quantified accurately on the basis of the detection of centromeric regions and telomeric sequences using the ISIS FISH-imaging software (MetaSystems). Only PCC spreads with 46 centromeres were analyzed.

4.6 Whole blood cell cultures for conventional dicentric chromosome analysis at metaphase

Peripheral blood from healthy individuals was drawn in heparinized tubes. Informed consent was obtained from each donor. Cultures were set up by adding 0.5 ml of whole blood to 5 ml of RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% phytohemagglutinin (PHA), 1% glutamine and 1% antibiotics (penicillin, streptomycin). Cultures were then incubated at 37^{o}C in a humidified atmosphere with 5% CO2.

After 48-50h at 37^{o}C, cell cultures were harvested, treated with hypotonic solution KCl (0.075 M) and fixed with methanol:glacial acetic acid (3:1, v/v), following standard cytogenetic procedures

with centrifugation at 250g. Chromosome spreads were prepared, slides were air-dried and stained with 3% Giemsa solution. Only metaphases with 46 centromeres were analyzed and chromosomal aberrations were scored for each experimental point using light microscopy. The analysis was greatly facilitated by image analysis systems (Ikaros MetaSystems).

5 Results

5.1 Development of an automatable micro-PCC assay for early triage biodosimetry

The original protocol for the conventional PCC assay uses 1-2ml blood sample volumes per experimental point, lymphocyte isolation by means of Ficoll-Paque or Biocoll gradients, as well as 15ml round-bottom culture tubes for cell fusion, PCC induction, and chromosome preparation. The various steps of the procedure were adapted, therefore, towards the development of an automatable micro-PCC assay using blood volumes of only 100µl, and multi-tube racks or 96-well plates of 2ml.

5.1.1 Protocol adaptation to the requirements of the micro-PCC assay

As a first step, lymphocytes were separated from 3ml of whole blood using Biocoll and, from the lymphocytes obtained, only the proportion corresponding to blood volumes of 100µl were used and successfully fused with CHO mitotic cells for PCC induction in 2ml round bottom safe-lock tubes. Subsequently, in order to comply with the requirements of a high-throughput blood sample collection based on small volumes of around 100µl, as a second step, we experimented to isolate efficiently lymphocytes from such small blood samples, using either Biocoll or ammonium chloride red blood cells lysing solution. Even though the isolation of lymphocytes with Biocoll in 2ml tubes was feasible, it was time-consuming and less efficient than that carried out with ammonium chloride.

5.1.2 Lysis of red blood cells with ammonium chloride allows induction of lymphocyte PCCs without the need of Biocoll gradient

As an alternative to the Biocoll gradient for separating lymphocytes from whole blood, the use of red blood cell lysing solution appeared to be more efficient and practical. Indeed, the entire procedure required a single 2ml tube, which was used for the 100µl blood sample collection, red

blood cell lysis, lymphocyte fusion to mitotic CHO cells, culturing of fused cells for PCC induction, and cell fixation for chromosome slide preparation for the analysis of lymphocyte PCC spreads. This improvement reduces the number of sample transfers, minimizes cell loss, and simplifies tracking operations. We demonstrated for the first time, therefore, that lymphocytes isolated from 100µl blood sample via ammonium chloride lysing solution in 2ml tubes can be fused with CHO mitotic cells.

5.1.3 Implementation of the micro-PCC assay in multi-tube racks and 96-well plates

The milestones achieved in the development of the micro-PCC assay were successfully applied in multi-tube racks and 96-well plates of 2ml. The 96-well plates proved to be more useful, as the various steps required by the protocol could be applied to all 96 wells simultaneously (Figure 13). Briefly, heparinized blood samples in volumes of 100µl were transferred to each well and 1.5ml of cold red blood cell ammonium chloride solution were added for 10 minutes. The red blood cell lysing solution was prepared from a 10X, 100ml stock solution containing 8.02g ammonium chloride, 0.84g sodium bicarbonate and 0.37g disodium EDTA. Following centrifugation at 250g for 6 min, lymphocytes and mitotic CHO cells were mixed in 1.5 serum-free RPMI-1640 medium in each well in the presence of colcemid.

After centrifugation at 200g for 6 min, the supernatants were discarded keeping the 96-well plates inverted on a paper towel for a few minutes in order to drain the pellets in the wells from excess liquid. In continuation, 100µl of 45% (w/v) PEG were injected to the cell pellet in each well and, subsequently, 1ml of PBS containing colcemid was slowly added, before centrifuging the plates at 200g for 6 min. All the supernatants were discarded at once and the cell pellet in each well was resuspended gently in 0.3 ml RPMI 1640 complete growth medium with colcemid and 1% phytohemagglutinin (PHA). After culturing for 75 min at 37°C, cells were treated with hypotonic KCL (1.5 ml in each well), and fixed twice with 1.5 ml per well methanol:glacial acetic acid (3:1 v/v), following standard procedures. Following centrifugation at 250g, chromosome spreads were prepared for each well. For this purpose, the cells in each well were resuspended

in 50μl of fixative and three microscope slides with chromosome spreads were prepared by dropping 15μl aliquots to each pre-cleaned slide. Finally, air-dried slides were stained with 3% Giemsa solution in Sorensen's buffer (pH 6.8) and covered with cover slips using Entellan mounting medium, before their microscopic analysis. A scheme of the various steps of the developed protocol is presented in Figure 14.

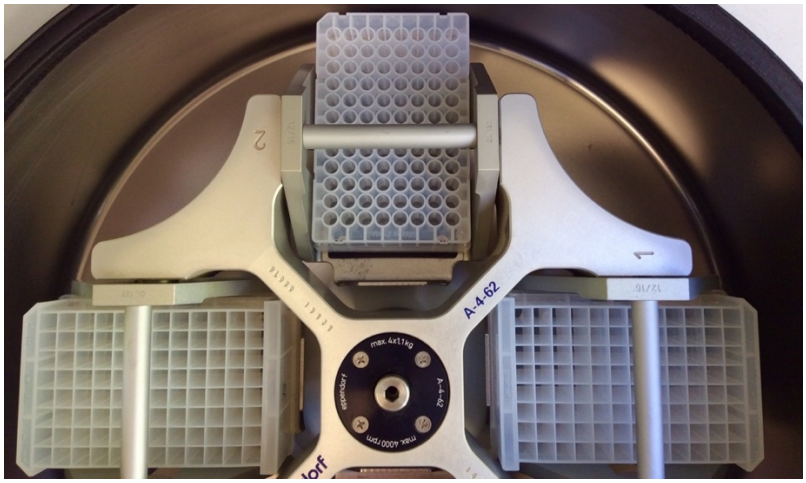

Figure 13. The 96-well plates used in the micro-PCC assay enable the simultaneous preparation of chromosomes for multiples of 96 individuals since the various steps required by the protocol can be applied to all 96 wells simultaneously.

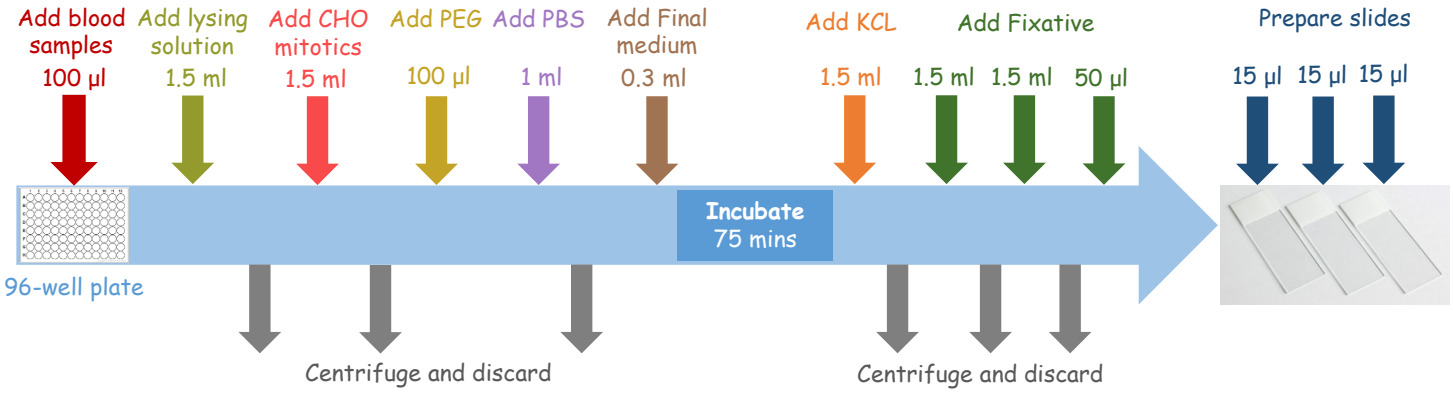

Figure 14. Scheme of the automatable micro-PCC assay for simultaneous analysis of 96 individuals. Volumes refer to each well of the 96-well plate.

5.1.4 Morphology of the lymphocyte PCCs obtained using the micro-PCC assay and Giemsa staining

The morphology of the lymphocyte PCCs obtained with the above micro-PCC procedure is practically identical to that obtained using the conventional PCC assay. It allows, therefore, the analysis of radiation induced excess PCC fragments stained with Giemsa, which is simple, rapid and cost-effective. Interestingly, the use of only 1.5ml of hypotonic solution and the fixation of cells twice with 1.5ml of Carnoy's fixative in the 2ml tubes offered high quality PCC images. Figure 15 (top) presents a typical image of a non-irradiated lymphocyte (46 PCCs), whereas Figures 15 (bottom left and right) show the impact of 0.5Gy (3 excess PCCs) and 2Gy (10 excess PCCs) of Co-60 γ-irradiation, respectively.

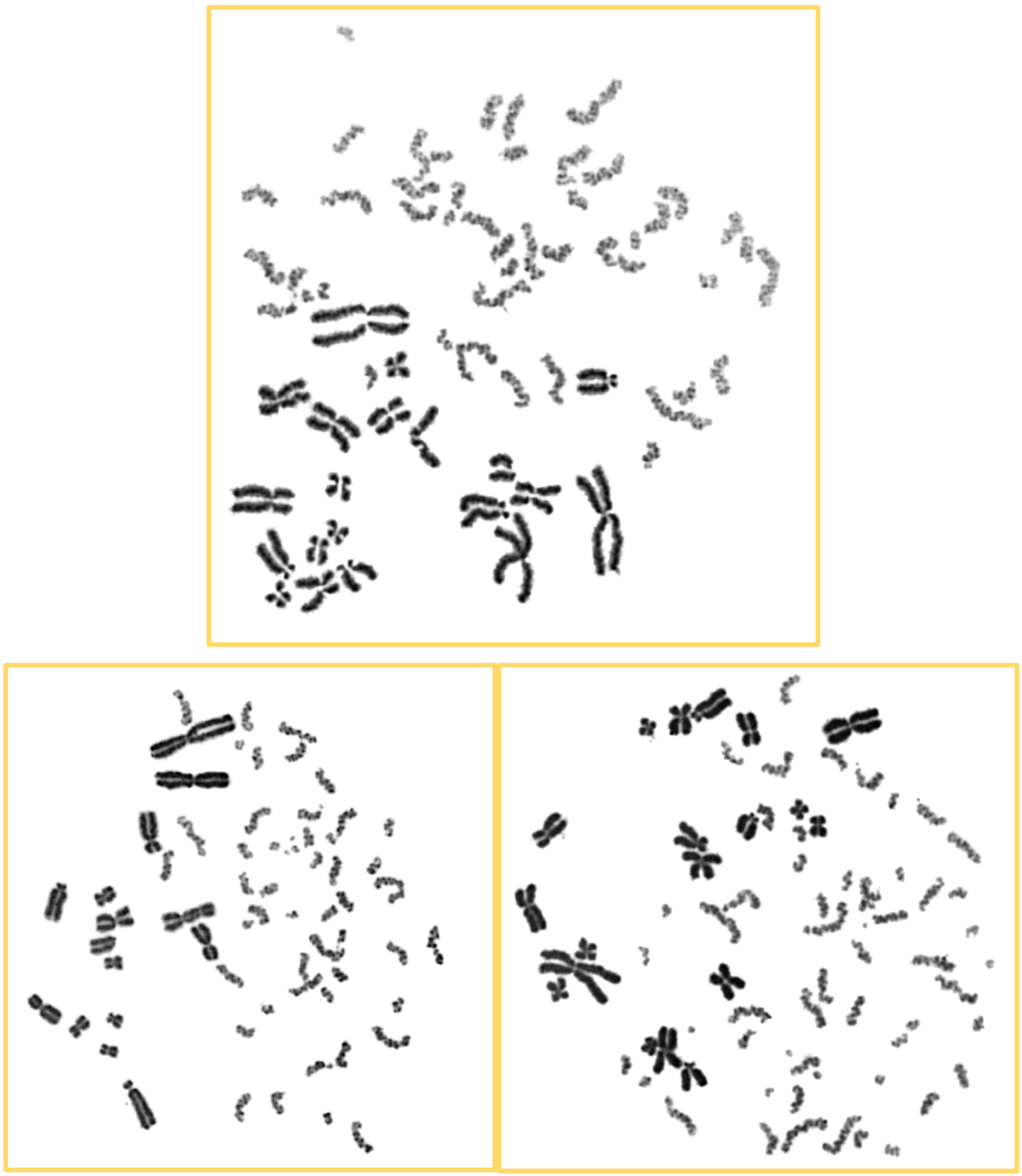

Figure 15. Giemsa stained PCCs, obtained by means of the micro-PCC assay, demonstrating 46 single chromatid chromosomes in a non-irradiated lymphocyte (top). Three excess (over 46) PCC fragments can be visualized in an irradiated lymphocyte with 0.5Gy of Co-60 γ-irradiation (bottom left). Ten excess PCC fragments can be scored following irradiation with 2Gy (bottom right).

5.2 Construction of dose response calibration curves adjusted to the conditions of the proposed micro-PCC assay for early triage

Following the protocol for the micro-PCC assay described above and based on our results so far, the analysis of 20-30 lymphocyte PCC spreads are sufficient to obtain a reliable dose estimate. Essentially, the analysis of the 15µl fixed cells dropped on a single slide provides the necessary amount of fusions to allow for the estimation of the dose received by exposed individuals. However, when blood samples of less than 100µl were used, more than one slide need to be analysed, depending of course on the number of lymphocytes/µl in the blood sample. Lymphocyte PCC spreads were located manually and their analysis was facilitated by the use of a semi-automated image analysis system (Ikaros, MetaSystems). In unirradiated lymphocytes, 46 chromosomes can be scored in PCC spreads and, in order to calculate the frequency of excess PCC fragments, this number was subtracted from the one obtained in the irradiated lymphocyte PCCs. Figures 16A, 16B and 16C present reference calibration curves constructed for dose estimation in case blood samples are received within 6, 12 or 24 hours post-exposure. A comparison of the dose-response curves obtained under the different post-exposure repair times is presented in Figure 16D.

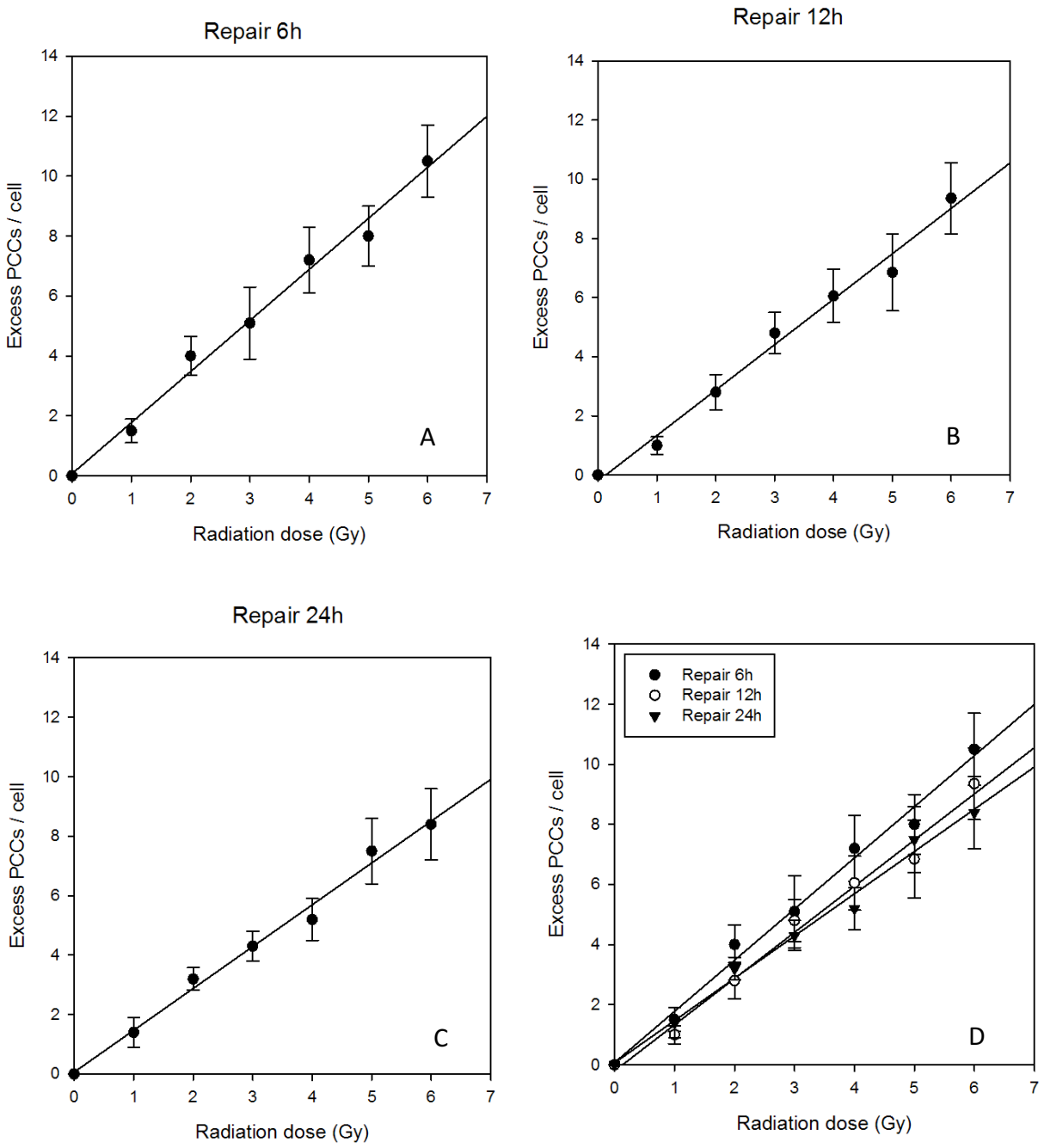

Figure 16. Dose-response curves are presented for Giemsa-stained excess lymphocyte PCC fragments per cell for post-exposure repair time of 6h (Linear, a_1=1.70 ± 0.08, y_{01}=0.075) (A), 12h (Linear, a_2=1.54 ± 0.08, y_{02} = -0.20) (B) and 24h (Linear, a_3=1.41 ± 0.06, y_{03} =0.06) (C). Figure D depicts a comparison of the dose-response curves obtained under the different post-exposure repair times.

5.3 Advantages of the micro-PCC assay compared to the DC assay for early triage biodosimetry

For the standardization of the micro-PCC assay, appropriate calibration curves were constructed as presented above, and its applicability and reliability for rapid dose assessment was compared to the DC assay through the evaluation of speed of analysis and minimum number of cells required for each method. For this purpose, a simulation of an accident was performed. Specifically, blood samples from healthy individuals were irradiated with doses of 0.5Gy, 1Gy and 2Gy of gamma irradiation, coded blindly and, subsequently, used for dose estimation by means of both assays. The micro-PCC assay as well as the DC assay were performed and air dried chromosome preparations were stained with Giemsa.

The yields of excess fragments in G_0-Lymphocyte PCCs were obtained scoring 10, 20 or 30 cells (Table 2), while the yields of dicentrics plus centric rings analysed at metaphase were obtained scoring 50, 100, 200 or 300 cells, with a delay of two days due to lymphocyte culturing (Table 3). By comparing the results of each method, it is evident that the analysis of only 20-30 cells by the micro-PCC assay offers dose estimates with an accuracy that would require the analysis of 200-300 cells by the DC assay.

Table 2. Simulated whole body exposure to 0.5, 1 and 2Gy and dose estimation using the micro-PCC assay for the analysis of Giemsa-stained excess PCC fragments by scoring 10, 20 or 30 PCC spreads in non-stimulated lymphocytes. Mean doses are shown with low- (LCL) and upper- (UCL) confidence limits.

	Analysis of 10 Cells					Analysis of 20 Cells					Analysis of 30 Cells				
			Estimated Dose (Gy)					Estimated Dose (Gy)					Estimated Dose (Gy)		
True Physical Dose (Gy)	Excess fragm.	Excess fragm. /cell	Dose	LCL	UCL	Excess fragm.	Excess fragm. /cell	Dose	LCL	UCL	Excess fragms	Excess fragm. /cell	Dose	LCL	UCL
0.5	5	0.5	0.31	0.15	0.49	13	0.65	0.42	0.25	0.60	19	0.63	0.41	0.24	0.58
1.0	11	1.1	0.74	0.56	0.93	23	1.15	0.77	0.59	0.97	42	1.40	0.95	076	1.16
2.0	24	2,4	1.66	1.44	1.90	57	2.85	1.98	1.75	2.23	91	3,03	2.11	1.87	2.36

Table 3. Simulated whole body exposure to 0.5, 1 and 2Gy and dose estimation using the Dicentric plus centric ring (Dic +CR) analysis at metaphase lymphocytes. Mean doses are represented with low- (LCL) and upper- (UCL) confidence limits.

	Analysis of 50 Cells				Analysis of 100 Cells				Analysis of 200 Cells				Analysis of 300 Cells			
		Estimated Dose (Gy)				Estimated Dose (Gy)				Estimated Dose (Gy)				Estimated Dose (Gy)		
True Physical Dose (Gy)	Dic+CR	Dose	LCL	UCL	Dic+CR	Dose	LCL	UCL	Dic+CR	Dose	LCL	UCL	Dic+CR	Dose	LCL	UCL
0.5	0	0	0	0.95	1	0.26	0	0.81	3	0.36	0.10	0.70	7	0.47	0.26	0.74
1.0	2	0.66	0.15	1.38	7	0.92	0.54	1.38	18	1.06	0.79	1.37	24	0.99	0.76	1.24
2.0	10	1.65	1.10	2.30	16	1.46	1.07	1.91	44	1.74	1.46	2.04	82	1.96	1.73	2.20

5.4 Combining C/T FISH staining with micro-PCC for the overexposed individuals

Once the overexposed individuals have been identified using the micro-PCC assay, further evaluation into treatment-level categories can be achieved by combining this assay with centromeric/telomeric (C/T) peptide nucleic acid (PNA) probes and FISH technique [28, 88]. As shown in Figure 17, dicentrics and centric ring chromosomes cannot be identified using Giemsa staining. Indeed, the arrow in this figure points to a probable dicentric chromosome but detection of the centromeric regions is necessary in order to confirm it.

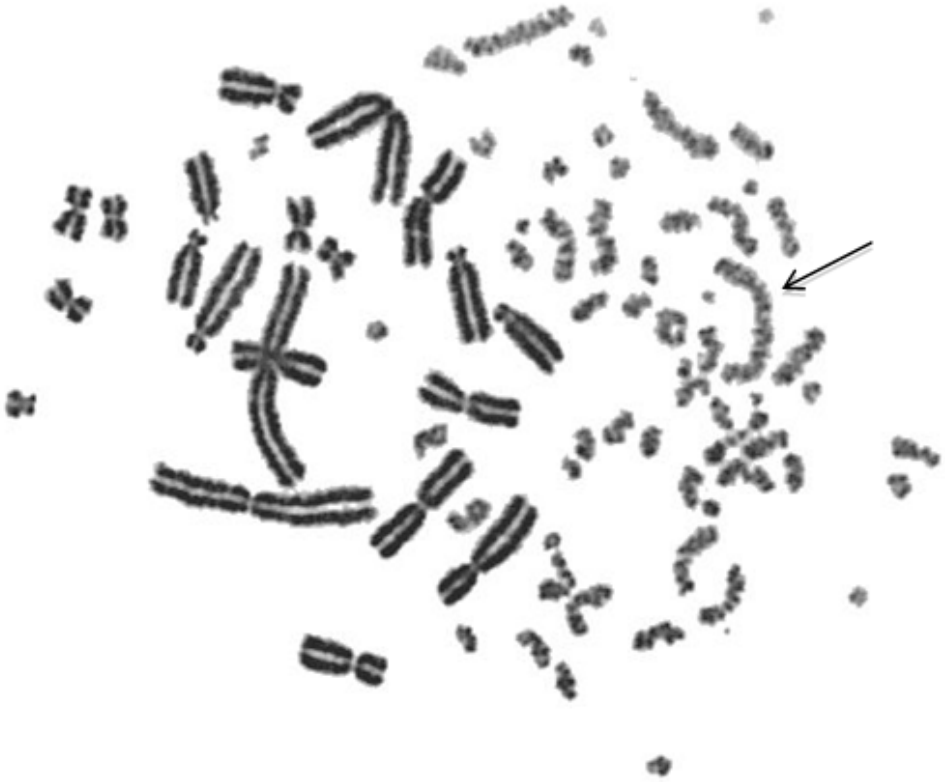

Figure 17. Using Giemsa staining, dicentrics and centric ring chromosomes cannot be identified. The arrow points to a probable dicentric chromosome but detection of the centromeric regions is necessary in order to confirm it.

The C/T FISH technique enables visualization of centromeric and telomeric regions (Figure 18) and quantification of radiation induced chromosomal aberrations in the lymphocytes PCCs as shown in Figure 19.

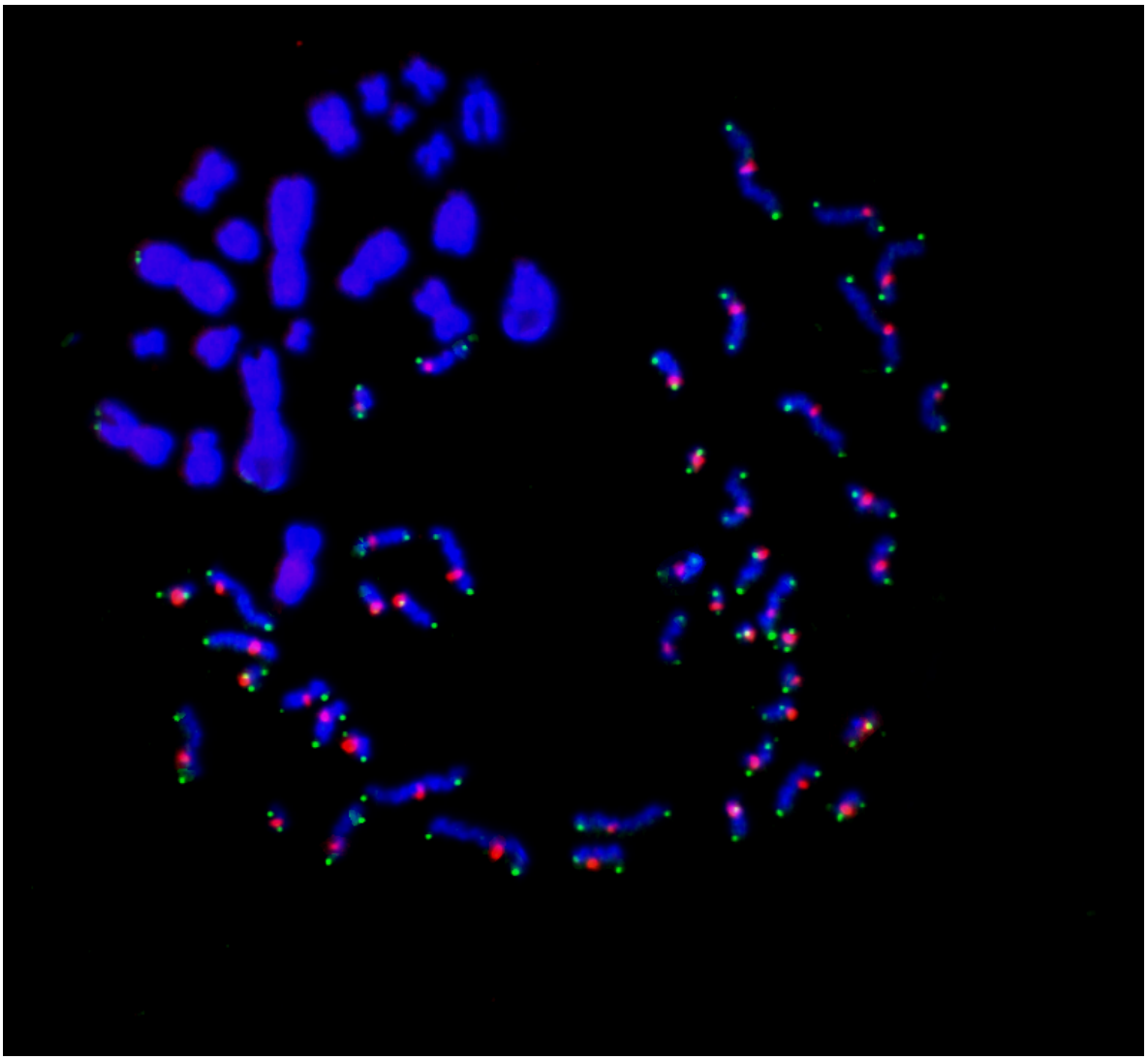

Figure 18. Lymphocyte PCCs visualized by means of C/T staining with PNA probes. In a non-irradiated lymphocyte, not only the 46 distinct single chromatid PCCs can be now visualized, but their centromeres and telomeres as well.

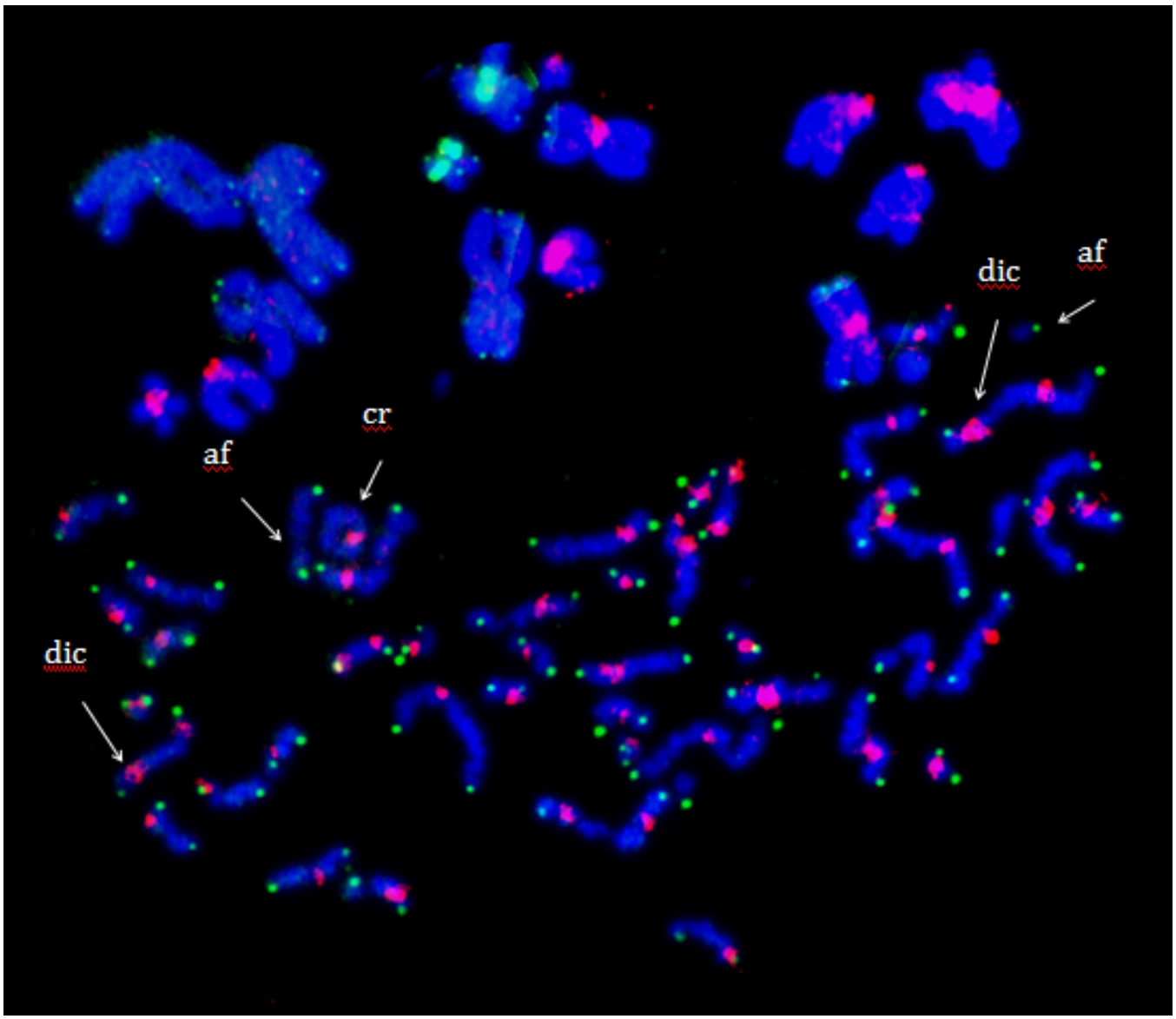

Figure 19. Dicentrics and centric ring chromosomes can be detected in non-stimulated lymphocytes with a level of accuracy and ease not previously achievable. Dicentrics and a centric ring chromosome are shown in a lymphocyte exposed to 8 Gy, following a 24 h post irradiation repair period.

As a result, it is then possible to obtain rapid dose and risk assessments based on the detection of centromeric and telomeric regions, which enables the accurate scoring of dicentric and centric ring chromosomes in G0-lymphocyte PCC spreads (Figure 20), and the calibration curve constructed for doses ranging from 0 up to 10 Gy γ-radiation (Figure 21).

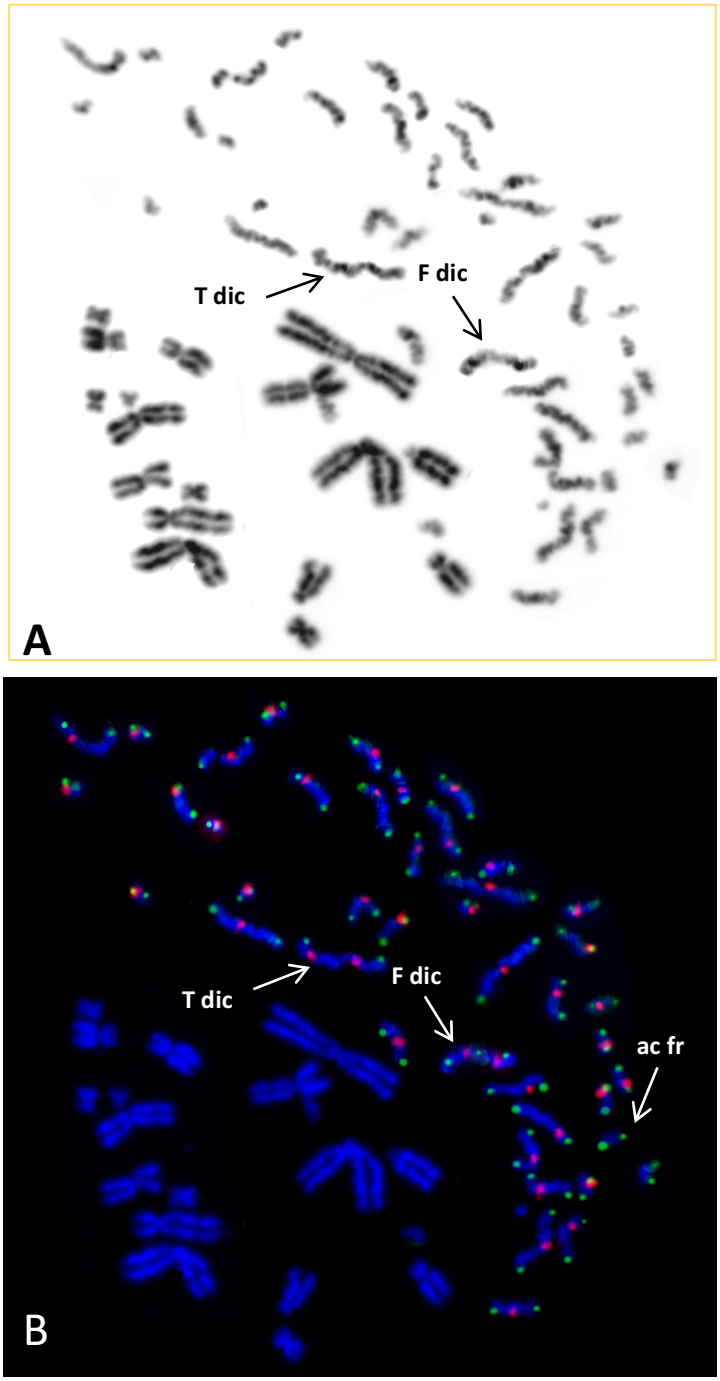

Figure 20. G0-lymphocyte PCCs visualized by means of inverted grey scale mode (ISIS-FISH software, MetaSystems) following a 24 h post irradiation repair period showing 2 possible dicentric chromosomes (A). True dicentric chromosomes (T dic) can be detected accurately only by means of C/T FISH staining with PNA probes (B). The presence of telomeric staining (Tel-FAM, green) between the 2 centromeres (Cent-Cy3, red), as shown in this figure (B), confirms undoubtedly a false dicentric (F dic).

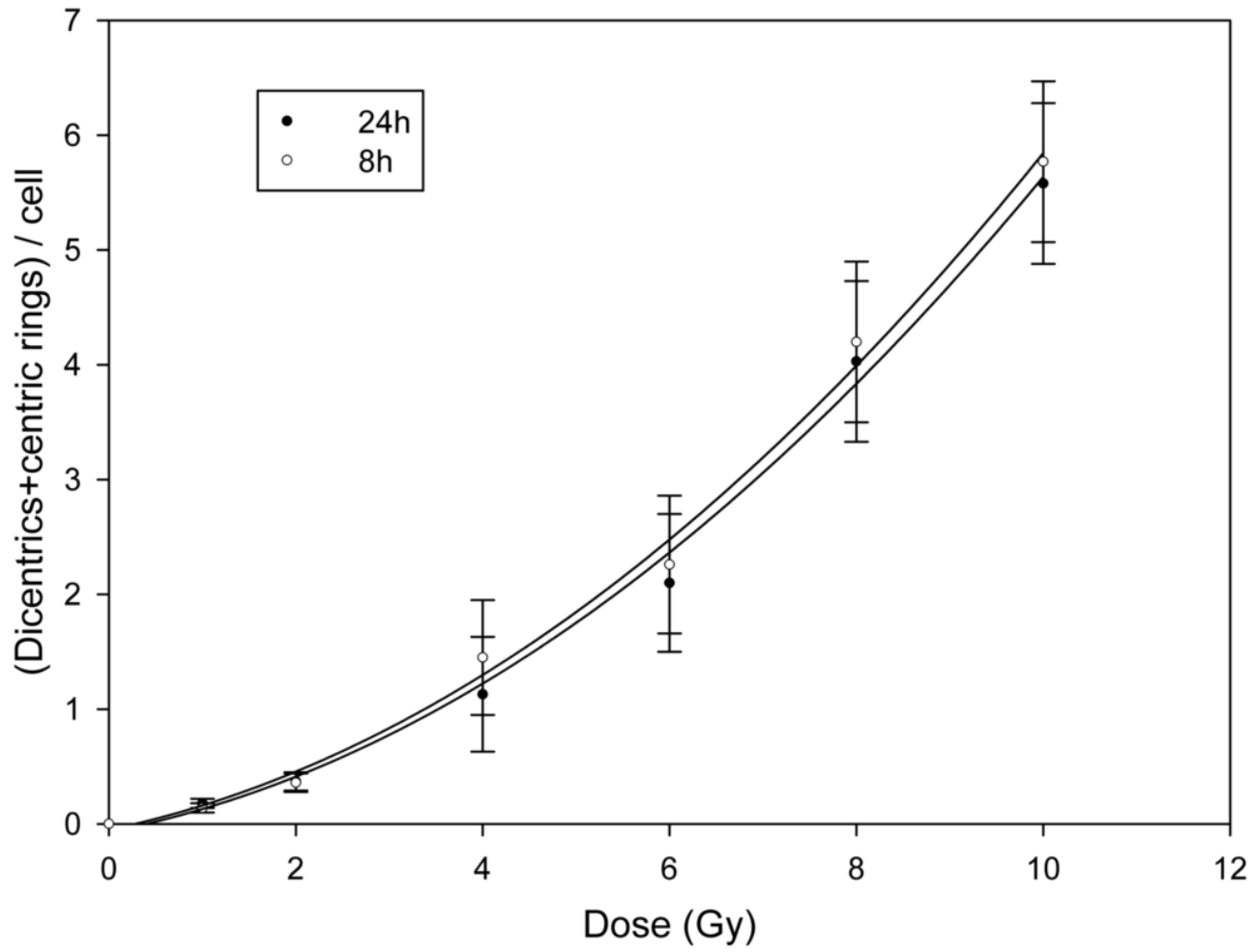

Figure 21. Absorbed dose estimates can be obtained based on the calibration curve constructed in our laboratory.

5.5 Work in progress towards the automation of the developed micro-PCC assay

Regarding the automated acquisition of PCC images and their automated analysis, we are currently collaborating with MetaSystems (Germany), the world-leader in computerized automated microscopic imaging. Specifically, we are considering a potential adaptation of their slide-scanning platform "*Metafer*" for the needs of automated acquisition of high-resolution images of CHO metaphases-Lymphocyte PCC spreads. Since the CHO metaphase chromosomes appear much darker than the light-Giemsa-stained human lymphocyte PCCs, it is easy to identify the lymphocyte PCC spreads enabling, thus, their automated acquisition and analysis required for dose estimation. To this end, a suitable image analysis software is being developed that will process the PCC images and automate their analysis.

With respect to the automation of all the individual steps of the micro-PCC assay for high-throughput biodosimetry, the *Centers for Medical Countermeasures Against Radiation* (CMCR) at *Columbia University Medical Center* is being consulted, given their experience with robotic platforms for blood sample handling and chromosome analysis. In addition, the Cytogenetic Biodosimetry Laboratory at the Radiation Emergency Assistance Center, Oak Ridge Institute for Science and Education, is being consulted as well, due to their interest in the development of high-throughput platforms for cytogenetic applications and novel biomarkers based on chromosomal rearrangements in lymphocyte PCC spreads as visualized by the mFISH technique (multicolor Fluorescence In Situ Hybridization).

6 Discussion

The main goal of biodosimetry is to utilize biological changes caused by ionizing radiation in an individual and use them as biomarkers of exposure in order to estimate the dose received and to predict its clinically relevant consequences. The development of rapid, accurate, and reliable biodosimetry tools has been primarily motivated by the potential need to confront large-scale radiological events. In such cases, it is crucial to be able to identify the exposed individuals who would benefit from receiving urgent medical care.

To this end, it is imperative to set a reasonable cut-off dose of absorbed radiation as a threshold that will allow the categorization of the exposed population. Specifically, for doses below such a cut-off value, countermeasures are not immediately needed since medical treatment would not be expected to impact mortality. On the other hand, for individuals with absorbed doses above the cut-off value, medical treatment would be necessary to improve survival rates. This cut-off is generally set at 2 Gy, yet this threshold could be set higher if the number of affected individuals is beyond the capacity of the available medical facilities [4, 7, 89-91]. Such biodosimetry screening enables the categorization of the exposed individuals into three categories: those who have suffered radiation injury, for whom immediate medical intervention is vital; those with intermediate exposure close to the threshold cut-off dose, for whom medical intervention is necessary to mitigate the short, medium and long term effects of exposure, and the “worried well” with probable low doses, for whom no deterministic effects are expected but long term monitoring may be required [92].

For timely biodosimetry dose assessments, the 48hour peripheral blood lymphocyte culture required for the standard DC analysis at metaphase remains the major obstacle for rapid dose estimation and the use of the dicentric assay for triage of a population after a mass exposure event. Alternatively, the conventional PCC fusion technique, which is based on the induction of premature chromosome condensation in unstimulated G_o-peripheral blood lymphocytes, allows a rapid visualization of radiation-induced chromosomal aberrations enabling their analysis.

However, the use of this technique for triage biodosimetry has been restricted so far, since it requires 1-2ml blood samples per exposed individual, so that fingerstick blood sampling, which is crucial for large population screening, cannot be utilized.

To overcome this obstacle, we developed in the present work, an automatable micro-PCC assay that is appropriately designed to be suitable for triage biodosimetry to obtain rapid individualized dose estimates in cases of large-scale radiological emergencies or accidental overexposures. Indeed, this new method has the potential to screen fingerstick derived blood samples of around 50µl, in order either to estimate past radiation exposure, or to sort a large number of individuals exposed above or below a pre-set cut-off dose. Compared to the dicentric chromosome assay, which at present is the standard technique for biological dosimetry, our results demonstrate that the micro-PCC assay is quicker as well as reliable and cost effective for early triage biodosimetry. Indeed, Giemsa-stained excess PCC fragments can be visualized for scoring within 2 hours from the moment blood samples are available and dose estimations can be obtained subsequently using the appropriate calibration curves that we have constructed for standardization purposes of this assay.

Specifically, we have introduced for the first time the use of blood samples of 100µl for PCC induction in 96-well plates. Interestingly, the morphology of the lymphocyte PCCs so obtained is practically identical to that obtained using the conventional PCC assay. This innovation in the PCC protocol has two main advantages for its use in early triage of radiation emergencies involving large populations, as such very small amounts of blood need to be sampled together with the use of the 96-well plates, allowing thus a fast and reliable triage of many individuals simultaneously. Indeed, the analysis of only 20-30 lymphocyte PCC spreads are sufficient to obtain reliable dose estimates, as presented in Table 2. This is because the analysis is based on radiation-induced chromosomal fragments in excess of 46 PCCs. This number of 46 PCCs constitute the human genome, which is remarkably stable in healthy individuals. This allows to link every single excess fragment above 46 to radiation exposure. In contrast, using the conventional DC analysis at metaphase, radiation-induced dicentric chromosomes are rare, especially at low doses, and

therefore many more cells must be analysed for reliable dose assessment, as shown in Table 3. Therefore, the micro-PCC technique has the potential to deliver data for dose assessment in a significantly shorter period of time than any other biological assay being used currently, as only few lymphocyte PCC spreads need to be analysed in order to detect exposed individuals. Indeed, by comparing the results of each method in Table 2 and Table 3, it is evident that the analysis of only 20-30 cells by the micro-PCC assay offers dose estimates with an accuracy that would require the analysis of 200-300 cells by the DC assay.

While overexposed individuals are identified using the micro-PCC assay through the analysis of Giemsa stained excess lymphocyte PCCs, centromeric and telomeric staining can be also applied in lymphocyte PCCs using PNA probes and the FISH technique (C/T-PCC-FISH) for the accurate scoring of dicentric and centric ring chromosomes. This additional analysis permits the reliable detection of all unstable chromosomal aberrations in lymphocyte PCCs with high level of precision and sensitivity as explained in Figure 20. Therefore, it can be carried out in order to confirm doses in the overexposed individuals only, given that it is quite an expensive procedure. Moreover, the C/T-PCC-FISH method may be also of use for the detailed categorization of the exposed individuals.

Further development of the micro-PCC assay is in progress in order to pave the way to the automation of assay for early triage biodosimetry in order to increase throughput and scoring objectivity. Indeed, we are currently collaborating with the company MetaSystems (Germany) and the Centers for Medical Countermeasures Against Radiation (CMCR, USA), world leader in computerized automated microscopic imaging and experienced in robotic platforms for blood sample handling and chromosome analysis, respectively. Moreover, the outgoing collaboration with the Cytogenetic Biodosimetry Laboratory (CBL) at the Radiation Emergency Assistance Center, Oak Ridge Institute for Science and Education, will allow additional applications of the micro-PCC assay to develop new radiation exposure biomarkers and investigate the mechanisms underlying chromosomal instability based on chromosomal rearrangements as visualized by the multicolor Fluorescence In Situ Hybridization (mFISH) technique[93-96].

To conclude, the micro-PCC assay developed in this work has the potential to deliver data for dose assessment in a significantly shorter period of time than any other cytogenetic assay currently being used, as it does not require lymphocyte culture and only a few cells need to be analysed in order to detect exposed individuals. In addition, this automatable assay has the potential to discriminate between whole- and partial-body exposure, which is of great importance for designing the treatment of individuals exposed to life threatening doses of radiation. Moreover, the analysis of Giemsa-stained excess PCC-fragments is a simple and cost-effective alternative to the C/T-PCC-FISH method and it enables a rapid estimation of absorbed doses within 2-3hours. Furthermore, its automation could potentially increase throughput and scoring objectivity of the PCC-assay, and it remains a challenge for the near future.

7 References

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