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Integration of new biological and physical retrospective dosimetry methods into EU emergency response plans – joint RENEB and EURADOS inter-laboratory comparisons

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ABSTRACT

Purpose: RENEB, 'Realising the European Network of Biodosimetry and Physical Retrospective Dosimetry,' is a network for research and emergency response mutual assistance in biodosimetry within the EU. Within this extremely active network, a number of new dosimetry methods have recently been proposed or developed. There is a requirement to test and/or validate these candidate techniques and inter-comparison exercises are a well-established method for such validation.

Materials and methods: The authors present details of inter-comparisons of four such new methods: dicentric chromosome analysis including telomere and centromere staining; the gene expression assay carried out in whole blood; Raman spectroscopy on blood lymphocytes, and detection of radiation-induced thermoluminescent signals in glass screens taken from mobile phones.

Results: In general the results show good agreement between the laboratories and methods within the expected levels of uncertainty, and thus demonstrate that there is a lot of potential for each of the candidate techniques.

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Conclusions: Further work is required before the new methods can be included within the suite of reliable dosimetry methods for use by RENEB partners and others in routine and emergency response scenarios.

Introduction

Biological and physical retrospective dosimetry is a recognised technique to assist in triage of suspected exposed individuals following an accidental or deliberate radiation exposure involving potentially large numbers of casualties. The state of the art in terms of biodosimetry techniques is represented by the achievements of the EU FP7-funded 'Realising the European Network of Biodosimetry and Physical Retrospective Dosimetry' (RENEB) project. Development during the project focused on establishing a working network for biological and physical dosimetry emergency preparedness and response within the EU (Kulka et al. 2015, 2016). While the funded part of the RENEB project came to a close at the end of 2015, RENEB remains an active network bringing together members from 23 institutions from 16 EU countries with expertise in preparedness and management of radiological or nuclear incidents, biological and physical methods of retrospective dosimetry analysis, and many other related competencies. Furthermore, many members of the biological and physical retrospective dosimetry communities are also active in the European Radiation Dosimetry Group (EURADOS) Working Group 10 (Rühm et al. 2016).

Within the fields of biological and physical retrospective dosimetry, there are a number of established techniques including the dicentric chromosome aberration assay, premature chromosome condensation (PCC), the micronucleus assay (MN), the fluorescence in situ hybridisation translocation assay (FISH), gene expression (GE), assessment of gamma-H2AX signals, electron paramagnetic resonance spectroscopy (EPR) and optically stimulated luminescence (OSL) techniques (Kulka et al. 2015, 2016; International Atomic Energy Agency [IAEA] 2011). However, EURADOS and RENEB network members represent many active university departments and national government and public radiation research institutes and further collaborate with many partners worldwide, for instance through IAEA and WHO initiatives (Maznyk et al. 2012; IAEA 2011). As such, new developments within this field which could be exploited for biodosimetry are a constant occurrence. One of the key outputs of RENEB was a quality manual which outlines a framework for quality assurance and quality management within the operational network (Gregoire et al. 2016). When a new dosimetry technique is proposed, it is necessary to develop, test and then validate the method before it can be said to reach a sufficient standard for inclusion as a fully operational dosimetry method. Inter-comparison exercises are a recognised method for validating tools and techniques within the field of biological and physical retrospective dosimetry (Badie et al. 2013; Romm et al. 2013; Ainsbury et al. 2014; Abend et al. 2016).

The authors here present the results of a recent inter-comparison involving three new techniques for biological and physical retrospective dosimetry: the gene expression assay carried out in whole blood (GE), Raman spectroscopy on blood lymphocytes (RS), and detection of radiation-induced thermoluminescence signals in glass displays taken from smart phones (TL). In addition, three well established and two relatively new laboratories to the RENEB consortium carried out the 'gold standard' dicentric chromosome aberration assay (DCA) as the reference technique for comparison with the results of the new assays. Moreover we performed an inter-comparison with a new approach for the detection of dicentric chromosomes by the introduction of telomere and centromere (TC) staining. This approach allows for better scoring of dicentrics in metaphases improving the dose response relationship (M'kacher et al. 2014). The resulting curve is more reliable and robust than those based on the classical dicentric assay considered to be the gold standard in the field of the biodosimetry.

The organisation of the inter-comparison and the materials, methods and results for the DCA and RS assays are here presented in detail; the results of the GE and TL assays are given only in brief with the detailed materials, methods and results presented in two additional papers (GE: Manning et al. 2016; TL: Woda et al. in preparation).

Materials and methods

Inter-comparison partners

For the DCA, five laboratories took part in the exercise: PHE, BfS, IRSN, IRBA and RSC (NB: See the author list for definitions of the participant acronyms). For DCA detected with telomere and centromere probes (DCA-TC), 17 laboratories took part in the exercise: BFS, UULM, CEA, ENEA, ICHTJ, INSP, IRSN, IST/ITN, LAFE, NCRRP, NCSRD, NRIRR, PHE, SERMAS, UAB, UNITUS, US.

For the GE assay, five laboratories participated: PHE, BIR, INCT, SCK-CEN and FzJ. One laboratory, DIT, carried out RS. A total of 11 laboratories – four RENEB partners (IRSN, ISS, HMGU, PHE) and seven EURADOS partners (SURO, DUR, LU, SCK-CEN, UNIMI, OSU, KAERI) – took part in the TL inter-comparison. Thus a total of 30 partners participated in the exercises as a whole.

Sampling, irradiation and distribution

For the biological assays DCA, GE and RS, blood samples were taken by venipuncture at PHE from four healthy volunteers with informed consent in accordance with local ethics procedures (Berkshire REC 09/H0505/87). Whole blood was irradiated using the Medical Research Council Co-60 gamma irradiation facility (Harwell Campus, Didcot, Oxfordshire, UK), with a horizontal geometry, at an acute dose rate of 0.7 Gy min^{-1}. The irradiation system is calibrated and traceable to national standards, to give dose to water within a

Perspex block holding the 10 ml blood tubes and all doses were delivered to within an accuracy of 5%. Irradiation took place at room temperature but samples were kept at 37 °C prior to irradiation and for 2 h for DCA and RS and 24 h for GE following irradiation.

For DCA-TC, the analysis was performed on images sent to the participants from slides prepared during the first RENEB dicentric intercomparison exercise (Oestreicher et al. 2016) with doses of 0, 0.94 and 3.27 Gy simulated whole body exposure and a dose of 4.75 Gy simulated to 50% of the body (mixed 1:1 with unirradiated blood from the same donor). Telomeres and centromeres were stained using the Q-FISH technique with a Cy-3-labelled PNA probe specific for TTAGGG for telomeres and a FITC-labeled PNA probe specific for centromere sequences (both from Panagene, Daejon, South Korea), as described in detail in M'kacher et al. (2014). Images of metaphase cells were acquired using automated acquisition module Autocapt software (MetaSystems, version 3.9.1). Image galleries were sent to each partner involved in the inter-comparison.

For RS and GE, it was first necessary to create calibration curves, thus samples were irradiated to doses of 0, 0.16, 0.41, 0.70, 1.43, 2.92 Gy and shipped to the partners in order for calibration curves to be created within each laboratory. Following establishment of the GE and RS calibration curves, four blind samples of at least 2 ml each were shipped to each partner for the DCA, RS or GE assays in December 2015. The doses given were 0, 0.44, 1.08 and 1.89 Gy.

In all cases, the samples were packed according to IATA packaging instructions 650 applied to UN 3373 Biological Substance Category B and shipping followed PHE's standard operating procedures. Shipment occurred at ambient temperature for the DCA and RS samples; the blood for GE was shipped frozen on dry ice. Each package of DCA and RS samples also contained a temperature logger, which revealed that during shipment the DCA and RS samples remained below 20 °C at all times. The majority of partners received the samples within 24 h of posting, but the partner carrying out RS received the sample after 45 h.

For TL on glass, training on sample preparation and measurement protocol was carried out at HMGU in November 2015. All RENEB partners and three EURADOS WG 10 members attended. Intact mobile phone samples were irradiated at IRSN using a 4 MV linear accelerator, with irradiation taking place in air kerma conditions and with the screen facing towards the source. Three samples were irradiated per participating laboratory, the nominal doses being 0.6, 1.5 and 2.5 Gy. Full details will appear in Woda et al. (in preparation).

Analysis and reporting of results

Dicentric assay

Upon receipt, each partner processed the samples and scored aberrations according to their own standard operating procedures with minor variations between the laboratories, but all according to the recommendations of the IAEA (2011) and refined during the MULTIBIODOSE project (Romm et al. 2013) and the two quality controlled exercises in the frame of RENEB (Oestreicher et al. 2016). In brief, whole blood was mixed with a suitable medium (either F10-Ham, RPMI, PB-Max or MEM – various suppliers e.g. Fisher Scientific, Loughborough, UK; Biochrom, Berlin, Germany; Life-Technologies, Cergy Pontoise, France), supplemented as required with 10–20% heat inactivated foetal calf serum, 1% PHA (phytohaemagglutinin), 100 units ml^{-1} penicillin plus 100 μg ml^{-1} steptomycin and 2 mM L-glutamine (all from Fisher Scientific, Loughborough, UK). Cells were then cultured for 48–50 h at 37 °C with or without 5% CO_2. The two laboratories using fluorescence plus Giemsa (FpG) staining added 5-bromodeoxyuridine (e.g. Sigma-Aldrich, Gillingham, UK; Sigma, Steinheim, Germany; Sigma-Aldrich, France) at a concentration of 20–50 μM ml^{-1}. Colcemid (e.g. Sigma-Aldrich, Gillingham, UK; Roche, Mannheim, Germany; Sigma-Aldrich, France) was added for 2–24 h at a concentration of 0.08–10 μg ml^{-1} before termination of the cultures. Metaphases were then harvested by a standard hypotonic treatment in 0.075 M potassium chloride for 7–15 min at 37 °C followed by three changes of 3:1 methanol:acetic acid fixative. Fixed cells were dropped onto clean microscope slides, air dried and stained with Giemsa (e.g. VWR International, Lutterworth, UK; Merck, Darmstadt, Germany; Life-Technologies, Cergy Pontoise, France), when cell cycle control was achieved with a long Colcemid treatment, or the FpG technique to distinguish 1st division cells.

The analysis was then performed in either manual mode using a standard light microscope or using the automatic scoring system Metafer 4 by MetaSystems (Altlussheim, Germany). Following automated metaphase finding (MSearch), cells were either scored manually or dicentric candidates were detected by the DCScore software from high resolution images at 63× magnification (with oil, AutoCapt) and then assessed by a scorer. Each participants' own preferred classifier was used (Vaurijoux et al. 2009; Romm et al. 2013). Standardised scoring sheets were provided for both manual and semi-automated scoring modes, and standard scoring procedures were also applied. The only notable difference was that lab DCA_1, scored both dicentrics and centric rings in manual mode, whereas all the other labs scored only dicentrics. All labs scored only complete cells with exactly 46 centromeres in manual scoring mode but for semi-automatic mode, scoring was carried out according to each labs' chosen classifier, according to standard practice (Romm et al. 2014). For statistical analysis of the dicentric results, the variance to mean ratio and u-test (the normalised unit of the standardised normal distribution) were applied to test for departure of the distribution of aberrations from Poisson and the Chi-squared test was used to give an initial indication of the homogeneity of scoring results between laboratories. To analyse the difference between the estimated doses with test doses and the homogeneity of the dosimetry results between laboratories, the z-test based on robust statistics was applied according to ISO 13528, comparing applied test sample dose to estimated dose within each dose group, separately for manual and automated scoring. Finally, general linear model analysis of variance (ANOVA) was applied to assess the impact of the experimental variables of: scoring method (semi-automatic vs. manual); dose and laboratory on the

deviation of the test dose and actual dose for each measurement. Post hoc testing (Tukey's test and Dunnett's test) was applied to further investigate significant variables.

Dicentic assay with telomere and centromere staining

Each partner scored dicentrics, centric and acentric rings and different types of acentric chromosomes. Acentrics with four telomeres resulted from a fusion event generally accompanying the formation of a dicentric or centric ring. Acentrics with two telomeres were scored, representing terminal deletions or unfused acentrics associated with a ring or dicentric as well as acentrics without telomeres representing interstitial deletions.

For a male donor, the centromere of chromosome Y had a very weak signal and the DAPI image (strong heterochromatin) was used to identify this chromosome.

The dose estimations were performed with the free software CABAS V2.0 (Deperas et al. 2007). The dose effect curve published in M'kacher et al. (2014) was used.

Raman spectroscopy

After receipt of the samples, peripheral blood mononuclear cells (PBMC) were isolated. A total of 3 ml of Dulbecco's modified phosphate buffered saline (DPBS) (Sigma) was added to 3 ml of blood, mixed by gentle inversion and overlaid over 3 ml of Histopaque. Samples were then centrifuged at 400 *g* for 30 min at room temperature. The PBMC layer was removed and washed three times. Finally, cells were centrifuged at 250 *g* for 5 min at room temperature. The cell pellet was then resuspended in 1 ml of full media (RPMI +12.5% (v/v) FBS +2 mM L-glutamine (Sigma) supplemented with 2.5% (v/v) phytohaemagglutinin (PAA Laboratories). One ml of cell suspension was transferred to a T25 flask containing 4 ml of full media and incubated for 24 h at 37 °C, 5% CO_2 to allow separation of lymphocytes and monocytes by plastic adherence.

The cells were fixed using 2% paraformaldehyde in phosphate-buffered saline. From the suspension, 40 µl was drop cast onto calcium fluoride (CaF_2) slides. The slides were then washed in deionised H_2O and the samples were allowed to dry before Raman spectroscopic measurements.

Raman spectroscopy was performed using a Horiba Jobin Yvon Labram HR800 UV system, equipped with a 660 nm solid-state diode laser delivering 100 mW of power to the sample. A spectrum of silicon, 1,4-Bis (2-methylstyryl) benzene and NIST SRM 2245 were recorded for calibration purposes. Multiple calibration spectra were recorded before recording a sequential group of cellular spectra. Spectra were recorded from 30–50 cells per sample. The cells were ~8–12 µm in size and each spectrum was recorded from individual cells using a 4 × 4 µm raster scan of the cell including both signal from its nucleus and cytoplasm. Spectra were recorded with a 20-sec integration time and averaged across three integrations per spectrum. Spectra were recorded using a diffraction grating ruled with 300 lines/mm giving a spectral resolution of ~2.1 cm^{-1}. The confocal hole was set to 100 µm with the grating centered at 1450 cm^{-1}. All spectra were recorded within 1–2 weeks of slide preparation. Slides were stored in a desiccator until measurement.

Raman spectral post processing and multivariate statistical analysis

Spectra were imported into Matlab vR2015b (The Mathworks Inc.) and were subjected to processing procedures detailed elsewhere (Maguire et al. 2015), including wavenumber correction, vector normalization, removal of cosmic ray spikes and background removal. First-order derivative spectra were also taken using a Savitzky-Golay derivative (with a 7-point window fitting a 5th order smoothing function) prior to analysis.

Multivariate statistical analysis was conducted using the R statistical software package (http://www.r-project.org) and using sparse Partial Least Squares Regression (SPLSR) within the *caret* package (Kuhn 2008).

GE and TL

For the GE assay, five established gene expression laboratories each used their own specific platforms for analysis. The materials and methods are described in Manning et al. (2016).

TL measurements were carried out following the protocol described in Discher and Woda (2013) and Discher et al. (2013), using a single dose calibration point at 1 Gy. Each participant prepared one glass aliquot and, if feasible, a second one that was additionally treated with HF (Discher et al. 2013). Full details of the materials and methods for the TL assay will appear in Woda et al. (in preparation).

Results

Dicentric assay

Of the five participating laboratories, two laboratories carried out only automated analysis and three laboratories carried out both manual and automated analysis. The curves used by the five participating laboratories are illustrated in Figure 1. Four laboratories used their own calibration curves and one laboratory, lab DCA_1, used calibration curves from the literature (IAEA [2011] for the manual curve DCA_1_m and Vaurijoux et al. [2015] for the automated curve DCA_1_a). The calibration curves were all created using either Cs-137 or Co-60 gamma irradiation with acute dose rates ranging from 0.27–0.5 Gy min^{-1} and irradiation at 37 °C or room temperature. As expected, Figure 1 shows differences in the calibration responses between the scoring modes, but good agreement between the laboratories within the automated (_a) or manual (_m) scoring modes.

The scoring and dosimetry results for the dicentric chromosome aberration assay are given in Table 1.

The u-test statistic and variance to mean ratio results indicate departure from Poisson for six of the reported results – given in bold in Table 1.

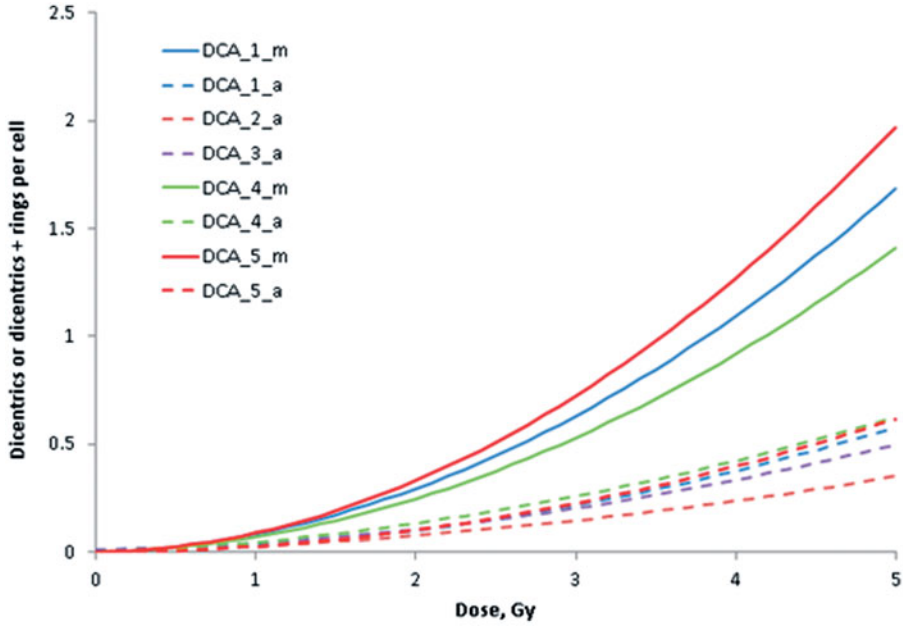

Figure 1. Calibration curves used by the five laboratories participating in the DCA assay: DCA_1 to DCA_5 in either manual (_m – solid lines) or automated (_a – dashed lines) scoring modes.

Table 1. Dicentric scoring results for the blind test dose samples from the participating laboratories.

								Dicentric distribution							
Scoring method	Test dose (Gy)	Lab	Cells	Dics	CR	y	SEy	0	1	2	3	4	d	SEd	u
Automated	0.44	DCA_1	3449	46	–	0.013	0.002	3403	46	0	0	0	0.99	0.02	−0.55
		DCA_2	4613	71	–	0.015	0.002	4544	67	2	0	0	**1.04**	**0.02**	**1.99**
		DCA_3	3745	43	–	0.011	0.002	3702	43	0	0	0	0.99	0.02	−0.49
		DCA_4	2076	24	–	0.012	0.002	2052	24	0	0	0	0.99	0.03	−0.37
		DCA_5	1839	14	–	0.008	0.002	1825	14	0	0	0	0.99	0.03	−0.22
	1.08	DCA_1	3143	211	–	0.067	0.005	2935	205	3	0	0	0.96	0.03	−1.53
		DCA_2	3660	179	–	0.049	0.004	3490	163	6	0	1	**1.09**	**0.02**	**3.67**
		DCA_3	3662	174	–	0.048	0.004	3498	154	10	0	0	**1.07**	**0.02**	**2.91**
		DCA_4	2101	109	–	0.052	0.005	1999	96	5	1	0	**1.10**	**0.03**	**3.11**
		DCA_5	2183	77	–	0.064	0.007	2109	71	3	0	0	1.04	0.03	1.43
	1.89	DCA_1	3006	344	–	0.114	0.006	2676	316	14	0	0	0.97	0.03	−1.27
		DCA_2	3619	552	–	0.153	0.006	3103	481	34	1	0	0.98	0.02	−0.78
		DCA_3	3099	260	–	0.084	0.005	2854	231	13	1	0	1.04	0.03	1.56
		DCA_4	2624	323	–	0.123	0.007	2322	282	19	1	0	1.01	0.03	0.49
		DCA_5	1586	143	–	0.090	0.008	1449	131	6	0	0	0.99	0.04	−0.16
	0	DCA_1	3021	4	–	0.001	0.001	3017	4	0	0	0	1.00	0.02	−0.05
		DCA_2	3374	1	–	0.000	0.000	3373	1	0	0	0	1.00	0.00	0.00
		DCA_3	3420	10	–	0.003	0.001	3411	8	1	0	0	**1.20**	**0.02**	**8.60**
		DCA_4	1567	7	–	0.004	0.002	1562	4	0	1	0	**1.85**	**0.03**	**25.80**
		DCA_5	2862	5	–	0.002	0.001	2857	5	0	0	0	1.00	0.02	−0.06
Manual	0.44	DCA_1	500	14	0	0.028	0.007	486	14	0	0	0	0.97	0.06	−0.43
		DCA_4	500	14	0	0.028	0.007	486	14	0	0	0	0.97	0.06	−0.43
		DCA_5	500	10	0	0.020	0.006	490	10	0	0	0	0.98	0.06	−0.30
	1.08	DCA_1	500	81	0	0.162	0.016	421	77	2	0	0	0.89	0.06	−1.76
		DCA_4	500	60	2	0.120	0.015	444	52	4	0	0	1.02	0.06	0.25
		DCA_5	500	46	2	0.092	0.014	457	40	3	0	0	1.04	0.06	0.65
	1.89	DCA_1	500	153	0	0.306	0.021	363	121	16	0	0	0.91	0.06	−1.51
		DCA_4	500	138	15	0.276	0.023	380	103	16	1	0	1.00	0.06	0.02
		DCA_5	500	114	4	0.228	0.021	400	88	10	2	0	1.05	0.06	0.87
	0	DCA_1	500	1	0	0.002	0.002	499	1	0	0	0	1.00	0.00	0.00
		DCA_4	500	0	0	0.000	0.000	500	0	0	0	0	1.00	0.00	0.00
		DCA_5	500	2	0	0.004	0.003	498	2	0	0	0	1.00	0.05	−0.05

Dics: dicentrics; CR: centric rings; y: yield of aberrations per cell; SEy: standard error on y; d: dispersion coefficient, the variance to mean ratio (which should be ∼1 for the Poisson distribution); SEd: standard error on d; u: u-test statistic (with a value between −1.96 and +1.96 indicating no departure from Poisson).

The Chi-squared test revealed no evidence of any departure from homogeneity of scoring within any of the dose groups (Chi p all >0.89). The robust z-test results are given in Table 2. The data presented demonstrate a highly satisfactory set of results – with only two dose estimates out of the thirty two results (5 labs in automated mode ×4 samples, plus 3 labs in manual mode ×4 samples) being significantly different from the actual dose according to the robust z-test. Figure 2 illustrates the cell scored by lab DCA_4 as containing 1 dicentric and 1 tricentric.

Table 2. Dose estimation results for the blind test dose samples from the participating laboratories.

			Whole body dose estimate			Robust statistics	
Scoring method	Test dose (Gy)	Lab	Dose (Gy)	LCL	UCL	z score	z result
Automated	0.44	DCA_1	0.62	0.478	0.753	1.134	Satisfactory
		DCA_2	0.68	0.57	0.79	1.519	Satisfactory
		DCA_3	0.46	0.24	0.73	0.129	Satisfactory
		DCA_4	0.33	0.17	0.48	−0.722	Satisfactory
		DCA_5	0.49	0.30	0.67	0.290	Satisfactory
	1.08	DCA_1	1.60	1	1.767	2.130	**Questionable**
		DCA_2	1.41	1.29	1.54	1.369	Satisfactory
		DCA_3	1.29	1.01	1.61	0.869	Satisfactory
		DCA_4	1.06	0.87	1.26	−0.079	Satisfactory
		DCA_5	1.14	0.92	1.37	0.252	Satisfactory
	1.89	DCA_1	2.13	1.934	2.331	0.366	Satisfactory
		DCA_2	2.71	2.54	2.89	1.750	Satisfactory
		DCA_3	1.83	1.52	2.20	−0.359	Satisfactory
		DCA_4	1.89	1.67	2.12	−0.206	Satisfactory
		DCA_5	1.87	1.55	2.18	−0.268	Satisfactory
	0	DCA_1	0.06	0	0.232	0.828	Satisfactory
		DCA_2	0.00	0.00	0.09	0.000	Satisfactory
		DCA_3	0.10	0.00	0.35	1.315	Satisfactory
		DCA_4	0.14	0.00	0.28	1.788	Satisfactory
		DCA_5	0.17	0.00	0.35	2.288	**Questionable**
Manual	0.44	DCA_1	0.505	0.33	0.68	0.773	Satisfactory
		DCA_4	0.57	0.38	0.76	1.545	Satisfactory
		DCA_5	0.42	0.26	0.58	−0.214	Satisfactory
	1.08	DCA_1	1.438	1.256	1.62	1.432	Satisfactory
		DCA_4	1.14	1.11	1.54	0.232	Satisfactory
		DCA_5	1.01	0.85	1.16	−0.288	Satisfactory
	1.89	DCA_1	2.038	1.85	2.22	0.510	Satisfactory
		DCA_4	2.12	1.90	2.34	0.783	Satisfactory
		DCA_5	1.64	1.49	1.79	−0.862	Satisfactory
	0	DCA_1	0.031	0.00	0.197	0.369	Satisfactory
		DCA_4	0.00	0.00	0.02	0.000	Satisfactory
		DCA_5	0.14	0.00	0.30	1.678	Satisfactory

LCL: lower 95% confidence limit; UCL: upper 95% confidence limit; z-score is calculated using the robust standard deviation according to Algorithm A of ISO 13528 (2008) and z result is based on the classification system of satisfactory scores for $z <= 2$; questionable scores for $2 < z <= 3$ and unsatisfactory scores for $z > 3$.

In addition to the formal z-test, ANOVA was applied to assess the impact of the experimental variables of: scoring method (semi-automatic vs. manual); dose and laboratory on the variation between the test dose and actual dose for each measurement. As expected, dose was highly significant ($p < 0.001$) and there was no evidence of a difference between the automatic and manual data ($p = 0.834$). There was a significant effect of laboratory ($p = 0.002$) however post-hoc testing revealed that this discrepancy was due to laboratory DCA_1, as evidenced by Dunnett's test for comparison of the laboratory 1 results with the results from the other labs (p DCA_1 vs. DCA_2: 0.002; p DCA_1 vs. DCA_3: 0.032; p DCA_1 vs. DCA_4: 0.005; p DCA_1 vs. DCA_5: 0.008; All other pairwise comparison p values >0.70) and Figure 3.

Dicentic assay with telomere and centromere staining

Figure 4 illustrates the scoring methodology for the DCA-TC method. In total results from 17 labs were available however in one lab galleries were scored by two scientists and in two labs, galleries were scored by three scientists. Thus the intercomparison was performed on 22 sets of data.

The DCA-TC scoring method provided highly homogeneous results: With each reported dose being within 20% of the actual dose for both the whole body exposure samples (0.94 Gy and 3.27 Gy). Each partner correctly identified the zero dose point and each partner also identified the simulated partial body irradiation with a mean estimate of 67 ± 4%. The results for 3.27 Gy are shown in Figure 5.

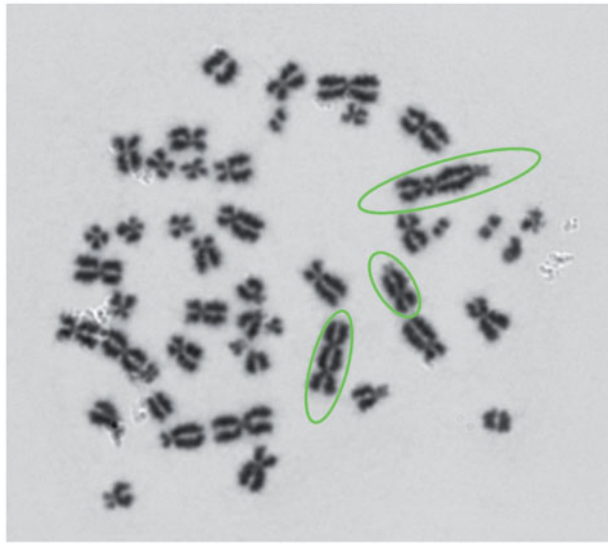

Figure 2. A cell from a control, 0 Gy, exposed sample identified by the DCScore software (automated scoring mode) as having three dicentrics; subsequently checked by eye and confirmed as containing 1 tricentric and 2 dicentrics (circled) – one of which was ignored according to the automated scoring protocol of laboratory DCA_4.

Raman spectroscopy

A model was trained using all of the Raman spectra from the training samples (with doses of 0, 0.25, 0.5, 1, 2, 3 and 4 Gy), and optimized for complexity using ten-fold cross-validation within the training set. A dose prediction was then generated for each unseen test spectrum by passing it through the model. The mean dose prediction plus its standard deviation were then calculated as:

- Test dose: 0.44 Gy; dose estimate: 0.65 ± 0.19 Gy;
- Test dose: 1.08 Gy; dose estimate: 0.93 ± 0.45 Gy;
- Test dose: 1.89 Gy; dose estimate: 0.73 ± 0.21 Gy;
- Test dose: 0 Gy; dose estimate: 0.13 ± 0.41 Gy.

GE and TL

Additional data and fully detailed results for the GE assay will appear in Manning et al. (2016) and for the TL assay will appear in Woda et al. (in preparation). In brief, the GE results are given in Table 3, which is reproduced from Manning et al. (2016).

For the TL measurements, we here report that the majority of measured doses agree with the nominal dose for all three dose categories within the 95% confidence interval. No significant dose underestimation was observed, implying that the tested measurement protocol successfully corrected for signal fading and optical bleaching due to mobile phone use.

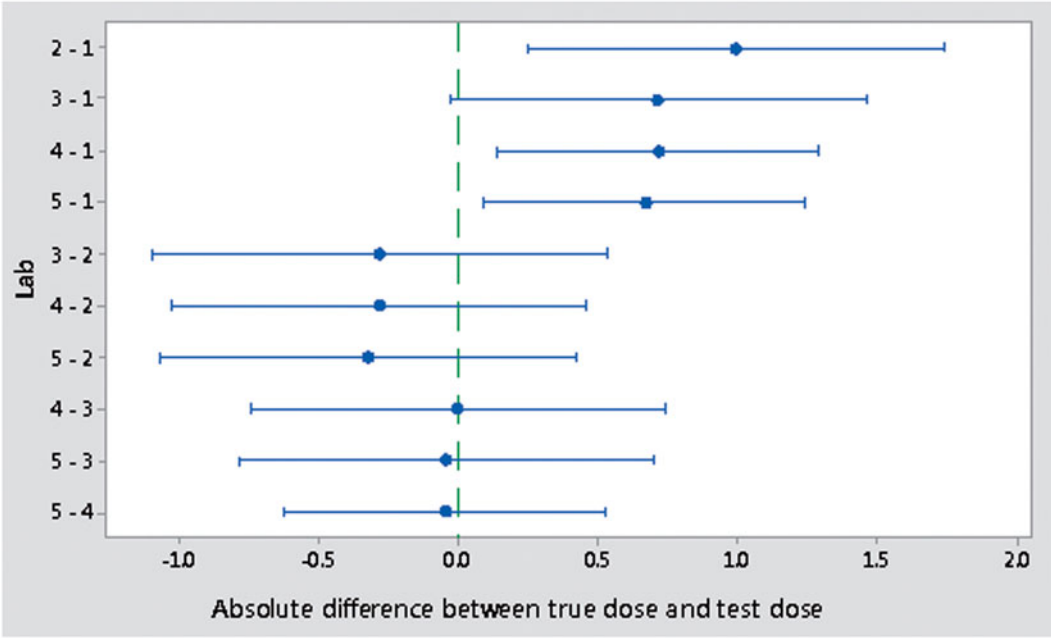

Figure 3. Tukey's simultaneous 95% confidence limits on the absolute differences between the true dose and the mean dose. If an interval does not contain zero, the corresponding means are significantly different.

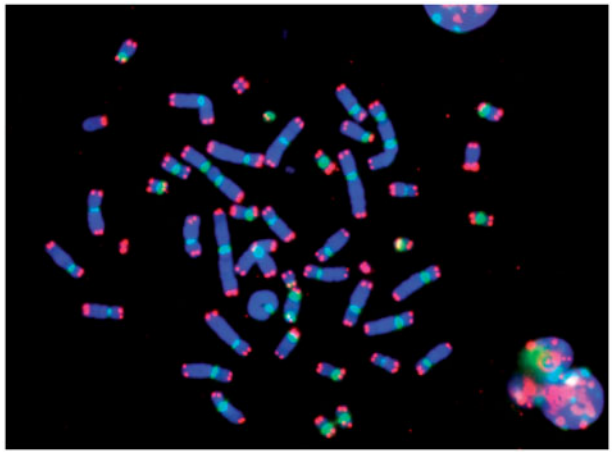

Figure 4. Detection of unstable chromosome aberrations after telomere (red; fluorescence staining at the ends of the chromosomes) and centromere (green; fluorescence staining at the centre of the chromosomes) staining. A dicentric chromosome corresponds to two green centromere signals and four red telomere signals at the ends of the chromosome. A chromosome ring corresponds to a circular chromosome with a green centromere signal and without red telomere signals. An acentric chromosome corresponds to a chromosome fragment without a green centromere signal with or without red telomere signals.

Discussion and conclusions

Biological and physical retrospective methods of dose assessment are highly useful methods to assist medical personnel in triage of exposed or suspected exposed individuals following a radiation accident or incident. The newly renamed Running the European Network of Biological Dosimetry and Physical Retrospective Dosimetry (RENEB) network represents the state of the art in terms of emergency readiness. However, the field of radiation biomarkers is quickly developing, so in order to maintain the status quo, newly developed methods must be assessed and integrated into the RENEB 'toolkit' as appropriate.

The aims of this inter-comparison were:

- To test the performance of DCA-TC labelling in an inter-comparison exercise;
- To test performance of the new method of GE on whole blood against the benchmark of the dicentric assay in a quality controlled dosimetry exercise;
- To test performance of the new method of TL on glass;
- To evaluate performance of the completely new technique of RS in a blind inter-comparison exercise.

The dicentric assay is still recognised as the international gold standard method for biological dose assessment (Oestreicher et al. 2016), and as such is well placed to act as the validation standard in this inter-comparison. Detailed analysis of the results is also justified. The u-test statistic and variance to mean ratio results indicate departure from Poisson for six of the reported results – given in bold in Table 1. In this exercise, all the samples simulated whole body irradiation, and as such this result is somewhat unexpected. It is notable that the overdispersed results all come from automated analyses. The fact that lab DCA_4 observed one cell with 1 tricentric and 2 dicentric (Figure 2) for the control sample is somewhat unexpected: in automated mode (DCScore) 1 dicentric and 1 tricentric chromosome was detected (recorded in Table 1 as 3 dicentrics). Interestingly, Table 1 shows that lab DCA_3 also observed a multiaberrant cell, with two dicentrics, in the control sample taken from the sample individual. 'Rogue cells' are observed in both exposed and unexposed populations and there are several potential causes (Rozgaj et al. 2002, M'kacher et al. 2010; Druzhinin et al. 2016). The history of this volunteer was reassessed in response to this observation but no recent radiation exposures (X-ray, CT or otherwise) or other potential contributing factors were identified. However, the probability of observing

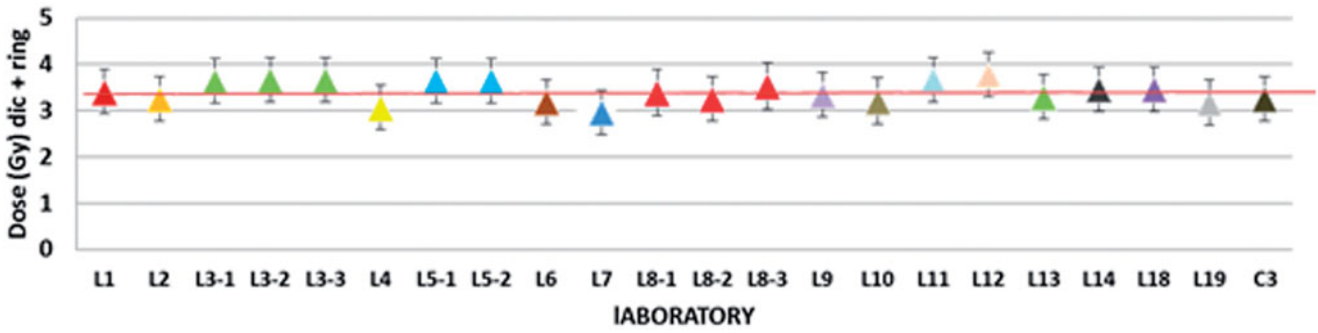

Figure 5. Doses estimated using the DCA-TC method (Dic + ring) from each of the partners compared to the physical dose of 3.27 Gy (red line). The error bars give the Poisson standard error on the estimated dose.

Table 3. Reported dose estimates from laboratories running QRT-PCR or microarrays for each of the samples irradiated with a known (true) dose.

		True dose for each sample (Gy)						True dose for each sample (Gy)					
		0.0	0.4	1.1	1.9	MAD (Gy)	No. of measurements out of ±0.5 Gy	0.0	0.4	1.1	1.9	MAD (Gy)	No. of measurements out of ±0.5 Gy
	approach: curve fit/gene(s)	**Diluted blood** reported dose estimates (Gy)						**Whole blood** reported dose estimates (Gy)					
Lab 1	FDXR	0.0	0.6	0.8	1.2	**0.3**	**1**	0.0	0.5	0.4	1.3	**0.4**	**2**
Lab 2	log FDXR	0.0	0.4	2.0	2.1	**0.3**	**1**	0.0	0.3	1.3	2.3	**0.2**	**0**
Lab 3	Hierarchical clustering	0.0	0.2			**0.1**	**0**	0.0	0.4	1.4	2.9	**0.3**	**1**
	Regression analysis												
	6 (6) genes	0.0	0.8	2.5	2.4	**0.6**	**1**	0.0	0.8	1.4	2.1	**0.2**	**0**
	4 (5) genes	0.0	0.7	2.5	2.3	**0.5**	**1**	0.0	0.5	1.3	2.0	**0.1**	**0**
	2 (3) genes	0.0	0.5	2.3	2.4	**0.4**	**1**	0.0	0.5	1.2	1.9	**0.1**	**0**
	K-nearest neighbour	0.0	0.5	2.0	2.0	**0.3**	**1**	0.0	1.0	1.0	2.0	**0.2**	**1**
Lab 4	Integrated over 12 (17) genes	0.0	0.6	1.5	2.4	**0.3**	**0**	0.0	0.7	2.0	2.8	**0.5**	**2**
Lab 5	Integrated over 8 genes	0.0	0.5	1.1	5.6	**0.9**	**1**	0.1	0.2	0.5	0.9	**0.5**	**2**
					Mean	**0.4**	**0.8**				**Mean**	**0.3**	**0.9**
					SEM	**0.08**	**0.15**				**SEM**	**0.05**	**0.31**

Numbers in bold refer to values used for descriptive statistics (mean and SEM calculations). Underlined numbers represent values lying outside the ±0.5 Gy uncertainty interval. Reproduced from Manning et al. (2016). MAD: mean of the absolute differences.

seven dicentrics in total in 1567 cells given the Poisson distribution is around 1.4%, so it is possible that this observation was simply due to chance. Indeed, the larger number of cells assessed in automated analyses naturally increases the chance of observing total numbers of aberrations as well as multiaberrant/rogue cells. Nevertheless, the observation of a tricentric in an unexposed individual is unexpected and, in this case, remains unexplained. Going forward, automatic scoring of chromosomal aberrations, which facilitates collection of data from large numbers of cells, could be used to progress knowledge in the prevalence of atypical cells

The Chi-squared test revealed no evidence of any departure from homogeneity of scoring within any of the dose groups (Chi p all >0.89). However, as most laboratories used their own calibration curves, the quantities of interest within this part of the inter-comparison are the homogenetity of the dose estimations and the variation between the dose estimates and the true doses given to the test samples. The ISO standard for proficiency testing through inter-laboratory comparisons 13528 (ISO 2005) recommends a number of methods for comparing results from two or more laboratories. The robust z-test has previously been usefully applied in biodosimetry (Di Giorgio et al. 2011; Wilkins et al. 2015) and thus is used again here. The results, which are presented in Table 2, demonstrate satisfactory agreement across the laboratories for the vast majority of the data. This is further evidenced by the ANOVA results, which show that only laboratory DCA_1 gave results significantly different from the mean. Of the two 'questionable' robust z-test results, one might be explained as it comes from one of the newer RENEB members, who used a calibration curve from the literature rather than their own curve. It is thus recommended that this laboratory produce their own manual and automatic calibration curves as soon as possible and then participate in a further inter-laboratory comparison to ensure that their results are homogeneous. The second questionable result comes from a much more experienced laboratory, so the result cannot be explained by the need for further training or similar. However, the result comes from one of the control samples, for which the uncertainties are recognised to have the greatest impact.

Further, the observed number of dicentrics was 5 in 2862 cells, which falls within the expected normal background of 0–2 in 1000 (IAEA 2011) and the reported lower 95% confidence limit on the dose does encompass the true value of 0 Gy. As such, this dose estimate can still be defined as acceptable according to the IAEA (2011) and ISO 13528 (2005) guidelines. ANOVA further revealed that there was no difference between the automated and manual results, again demonstrating that the semi-automated methodology can be applied for dosimetry purposes (e.g. Romm et al. 2013; Vaurijoux et al. 2015). Indeed, Gruel and colleagues demonstrated that semi-automated analysis can also be used in emergency response, with dose estimates based on as few as 50 cells (Gruel et al. 2013).

The dicentric assay is still recognised as the international gold-standard method for biological dosimetry and classification of genotoxic agents. The introduction of telomere and centromere staining not only renders the scoring of dicentric chromosomes and centric rings more reliable and robust but also permits the detection of different types of acentric chromosomes leading to the precise calculation of the number of DNA double-strand breaks (DSB). Manual scoring following TC staining in metaphase revealed a significantly higher frequency of dicentrics ($p < 10^{-3}$) (up to 30%) and estimated DSB ($p < 10^{-4}$) compared to uniform staining due to improved detection of unstable chromosomal aberrations leading to an improved dose response curve (M'Kacher et al. 2014).

The results of this intercomparison, illustrated in Figure 5 for the highest dose (3.27 Gy) whole body exposure sample, where the greatest variation is thus expected, confirm the accuracy of the DCA-TC methodology. However, it should be noted that unlike the DCA assay, for the DCA-TC inter-comparison the labs scored the same images, so the positive result is not unexpected. The next step for this assay will be for laboratories to score slides they have made and stained themselves and then to use their own calibration curve to produce a dose estimate. Interestingly, telomere and centromere staining can also be performed in non-stimulated lymphocytes following premature chromosome condensation in which it permits dicentrics identification which is not possible by conventional staining (M'Kacher et al. 2015). Further inter-comparisons will also be needed to confirm the reproducibility of the scoring.

Raman spectroscopy is a validated technique for molecular biochemical profiling which has recently been proposed as a radiobiological dosimetry technique (Maguire et al. 2015). The results of this inter-comparison show that the 0 and 1.08 Gy test samples corresponded to the dose estimates provided, within the quoted standard deviations. The results for the 0.44 Gy test sample were just outside the lower limit of the resulting standard deviation, but inside the 95% confidence limit, which can thus be counted as a successful comparison according to ISO standard 13528 (2005). Indeed, for rapid categorization purposes, this result is satisfactory as it would result in correct categorisation as a dose of <1 Gy.

The remaining result, corresponding to the 1.89 Gy test sample, was given as 0.73 ± 0.21 Gy. This is much lower than the real dose and the confidence limits do not include the true dose. It should be noted that the sparse PLS model revealed that most of the variables within the spectra were required for the optimisation of the regression (even using a sparse model 475 from 632 variables were selected). This suggests that most of the spectral features change with dose, as seen previously (Meade et al. 2010, 2016; Maguire et al. 2015). There was, however, much more spectral variability observed here than in previous studies. Raman spectroscopy is very sensitive to sample preparation and the higher spectral variability may be due to the transport, culture and other experimental conditions. Thus, while the lower dose result represent a success for Raman spectroscopic analysis as a biodosimeter, it is recommended that further work is necessary to fully develop and validate the methods for the full range of doses for which biodosimetry is likely to be applied.

In addition to the established physical techniques of EPR and OSL, TL on glass has recently been proposed as an additional fortuitous dosimeter for radiation emergency response (Discher et al. 2013). For TL in this inter-comparison, the results are very encouraging, with the reported dose for all three dose categories within the 95% confidence interval of the test doses. No systematic difference in the quality of results could be seen for laboratories that participated in the training and laboratories that received detailed written instructions on sample preparation and measurement only. This is different to the results obtained for the inter-comparison using OSL on surface mount resistors in mobile phones, where misidentification of the right component was the main source of error for untrained participants (Bassinet et al. 2014; Trompier et al. 2016). In a few cases (four out of 30) a systematic overestimation of the given dose was observed. The reason for this is currently not entirely clear, preliminary results indicate the possibility of the existence of a pronounced intrinsic, non-radiation induced signal. This will be further investigated in Woda et al. (in preparation).

Transcriptional techniques are also fast becoming recognised as being highly applicable to assessment of individual radiation responses (Badie et al. 2013; Abend et al. 2016). For GE, the full results will be reported in the parallel publication (Manning et al. 2016); however, the main conclusions of this exercise are that: irrespective of the analytical approach, comparable dose estimates of blinded samples were obtained by all five laboratories and that the use of diluted or whole blood does not affect dose estimations. Thus the use of whole blood for identification of transcriptional biomarkers can be further explored. It was also shown that an already existing historical calibration curve can be used to provide similar dose estimates.

It should also be noted that this exercise was designed as an inter-comparison rather than a timed exercise, and as such participants applying biological dosimetry analysis were asked to evaluate doses using their chosen techniques to the best of their ability over a timescale of several weeks, rather than to use rapid analyses. For TL, the results were requested to be returned to the coordinator within one week.

Overall, the DCA, DCA-TL, GE, TL and RS results were comparably reliable within the uncertainties associated with each assay – however, for all the new assays there is more work to

be done. The RENEB and EURADOS platforms are the obvious choice for such development. The next stage for validation of the new methods would be to run a timed exercise, in order to ensure that the methods can be applied in 'rapid categorization' so that they could produce useful information in a mass casualty scenario, complementary to that already available from the existing RENEB tools.

Finally, it is recommended that a true biological and physical retrospective dosimetry inter-comparison exercise is carried out within the next few years, in order to compare all the working assays in a more realistic exposure scenario. Such an exercise would require a great deal of planning, however it would prove crucial to demonstrating the operational capabilities of the platforms to respond during a large scale radiation emergency.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are response for the content and writing of the paper.

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