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SHORT COMMUNICATION

Whole-blood immunoassay for γH2AX as a radiation biodosimetry assay with minimal sample preparation

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Abstract The current state of the art in high-throughput minimally invasive radiation biodosimetry involves the collection of samples in the field and analysis at a centralized facility. We have developed a simple biological immunoassay for radiation exposure that could extend this analysis out of the laboratory into the field. Such a forward placed assay would facilitate triage of a potentially exposed population. The phosphorylation and localization of the histone H2AX at double-stranded DNA breaks has already been proven to be an adequate surrogate assay for reporting DNA damage proportional to radiation dose. Here, we develop an assay for phosphorylated H2AX directed against minimally processed sample lysates. We conduct preliminary verification of H2AX phosphorylation using irradiated mouse embryo fibroblast cultures. Additional dosimetry is performed using human blood samples irradiated ex vivo. The assay reports H2AX phosphorylation in human blood samples in response to ionizing radiation over a range of 0–5 Gy in a linear fashion, without requiring filtering, enrichment, or purification of the blood sample.

Keywords Bioassay · Blood · Dosimetry · Radiobiology

Introduction

Common to every large-scale release of radioactive material is an urgent need to estimate the radiation dose received by a substantial number of individuals, where whole populations of people may have been exposed to unsafe levels of radiation. For both proper medical treatment and effective emergency management, this group must be quickly triaged into treatable, untreatable, and unaffected populations (Blakely et al. 2009; IAEA 2011). In the absence of a physical dosimeter, the post-exposure quantitation of radiation dose is accomplished through radiation biodosimetry, in which measured physiological changes are used to quantify previous radiation exposure.

Radiation biodosimetry has been developed using cytogenetic, gene expression, and metabolomic endpoints (Redon et al. 2011; Brengues et al. 2010). The most reliable of these methods is the dicentric chromosome assay (DCA), which scores chromosome replication errors in cultured lymphocytes. This method requires a trained practitioner, as the scoring is done by eye. DCA also requires a period of cell culture, which introduces additional time in analyses that are not compatible with the expediency required in an emergency. As many as 1000 metaphase spreads are analyzed per patient sample, although more recent efforts have demonstrated triage-level assay performance for a QuickScan DCA with as few as 20–50 scored spreads (Flegal et al. 2010; Ainsbury et al. 2011).

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Quantitative biodosimetry can also derive from acute cellular response to ionizing radiation. After exposure to ionizing radiation, a phosphorylation occurs in histone H2AX at positions 139 and 136 (Rogakou et al. 1998). The phospho-139 form is referred to commonly as γH2AX, and it occurs in response to and in proportion with double-stranded DNA breaks (Dickey et al. 2009). While the kinetics of this phosphorylation are rapid, with changes seen minutes after irradiation, phosphorylated plaques containing clusters of the modified histone peak in cultured fibroblasts and human blood cells within 30 min after irradiation (Rothkamm and Horn 2009). A whole-cell immunocytofluorescence assay can be used to quantify radiation-induced changes in the phosphorylation status of the histone 2A.X (H2AX) (Redon et al. 2009). This approach allows for radiation dose estimation from a measurement of γH2AX foci, as previously demonstrated in human cells and non-human primates (Redon et al. 2010; Ivey et al. 2009). In a typical biodosimetry assay, the number of these foci per cell is quantified using in situ fluorescent staining, where the density of γH2AX foci correlates linearly with recent radiation exposure (Redon et al. 2009). This technique obviates cell culture requirements to yield results more quickly than DCA (Rothkamm et al. 2013), and it can report bystander damage in healthy tissues (Sokolov et al. 2005; Redon et al. 2011).

In an effort to increase sample throughput and mitigate the need for highly trained operators, several strategies have been successfully developed for the automation of a dosimetric assay. Such strategies include automation of image processing for microscopy-based foci-counting techniques (Roche-Lefévre et al. 2010), as well as morphological cell selection and automatic scoring (Valente et al. 2011). Robotic systems have been developed for automated sample processing, including cell fixation and staining, to significantly increase assay throughput and decrease required human interaction (Chen et al. 2010; Garty et al. 2010). Sample preparation can be simplified through flow cytometry-based γH2AX quantification, which does not require cell fixation and further decreases assay turnaround time (Muslimovic et al. 2008). All of these techniques successfully increase throughput and decrease sample processing time. However, the requirement for complex optical hardware or the addition of significant robotic sample preparation limits the utility of these approaches for point-of-care radiation biodosimetry.

In this work, a simplified enzyme-linked immunosorbent assay (ELISA) is developed to provide γH2AX-based radiation dose assessment from a small volume of minimally prepared sample. The ELISA relies on direct antibody-based detection of cellular proteins from sample lysate immobilized on a solid substrate. The γH2AX response is quantified per sample, instead of measured on a per cell basis as seen in previous whole-cell staining and flow cytometry techniques. This method eliminates cell fixation and staining, and non-specific immobilization of cellular proteins obviates initial filtration of samples. Through a focus on simplified sample preparation, omitting filtering and centrifugation processes typically required in γH2AX assays, we aim to eliminate much of the instrumentation and expertise needed for existing biodosimetry techniques to increase portability. Additionally, the use of a chromogenic reporter in place of a fluorescent label simplifies optical quantification. Using this γH2AX immunoassay, we report that a linear proportionality between received radiation dose and phosphorylation of H2AX is measured from simple cell lysates of cultured cells and ex vivo irradiated human whole-blood. The γH2AX ELISA described here employs a small sample volume and minimal sample preparation, which make it ideally suited for future adaptation to portable platforms. After further development, it may be especially suitable for triage-level point-of-care dose assessment after radiation exposure.

Materials and methods

Human and animal rights

Research using blood samples of human origin was performed in accordance with applicable federal and state guidelines and approved by the Columbia University Medical Center Institutional Review Board (IRB). Animal research was performed in accordance with applicable federal and state guidelines and approved by the Animal Care and Use Committee of Columbia University Medical Center.

Sample irradiation

Samples were irradiated using a Gammacell 40 apparatus (Atomic Energy, Ontario, Canada) containing a ^{137}Cs source emitting gamma rays at 0.79 Gy/min. Samples were insulated in room temperature foam during transport and irradiation to limit the effects of temperature deviation from the 37 °C environment of the incubator.

Cell lines

Mouse embryo fibroblasts were generated as described previously (Young et al. 2012). Cells were cultured in DMEM (Cellgro, Manassas, VA) +15 % fetal bovine serum (Gemini, Sacramento, CA) with penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO) in a 37 °C humidified incubator and fed three times per week.

Whole-cell mouse embryo fibroblast lysates

Fibroblast cultures were incubated 30 min under standard culture conditions after irradiation and were then washed three times with chilled phosphate-buffered saline (PBS) and scraped off the plate into radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 50 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, Teknova, Hollister, CA) supplemented with protease inhibitor cocktail at the concentration recommended by the manufacturer (Sigma-Aldrich, St. Louis, MO). Cell lysates were allowed to extract for 30 min on ice before a 10-min spin at 16,000*g* in a refrigerated microcentrifuge. Centrifugally cleared supernatants were subsequently stored in aliquots at −80 °C prior to analyses.

Whole-blood lysates

Human blood from consenting volunteers was collected in EDTA-containing Vacutainer™ tubes (BD Biosciences, Franklin Lakes, NJ) to prevent clotting. Where necessary, plasma was obtained by centrifugation of whole-blood at 5000*g* × 7 min. Each de-identified blood sample was split into aliquots ranging from 25 to 200 μL, depending on the anticipated range of experiments, and stored for irradiation and incubation in 1.5 mL Eppendorf tubes (Fisher Scientific, Pittsburgh, PA). These aliquots were subject to irradiation or sham treatment as described. After 1-h incubation at 37 °C following irradiation or sham treatment, whole-blood lysates were made by supplementing the existing volume of blood with protease inhibitor-supplemented RIPA buffer in a 5:1 ratio (RIPA/blood). Identical proportions were used in creation of whole-blood lysates obtained in time courses with mice. For these smaller volumes, Eppendorf tubes were prefilled with 10 μL EDTA solution. Mice were handled according to protocols approved by the Columbia Institutional Animal Care and Use Committee. Adult C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) were irradiated and subsequently bled via tail prick at intervals. Whole-blood from the animals was supplemented with RIPA buffer at the same 5:1 ratio as above lysing all cells. Resultant lysates were then used in ELISA experiments. At the completion of the time course, animals were euthanized per protocol using approved methods.

Western blotting

Whole-cell lysates were resolved on Criterion™ gels using Tris 2-(N-morpholino)ethanesulfonic acid (MES) buffering (Kashino et al. 2001) with a commercially sourced kit (Bio-Rad, Hercules, CA). Resolved proteins were transferred to polyvinylidene fluoride membranes and then blocked with a solution of PBS +2 % (w/v) non-fat milk +0.1 % Tween (Sigma-Aldrich, St. Louis, MO). After blocking, membranes were subject to 1 h incubations in antibody-supplemented blocking buffer. Between antibody incubations, and prior to detection, the membrane was washed three times for 5 min with PBS with agitation. Primary antibodies employed against γH2AX were from AbCam (ab22551, Abcam, Cambridge, MA), and ascites fluid employed against actin (A-2547) was sourced from Sigma-Aldrich (St. Louis, MO). Secondary anti-mouse IgG horseradish peroxidase-conjugated antibodies were from Jackson ImmunoResearch (West Grove, PA) and diluted per manufacturers instructions to 0.8 mg/mL before subsequent 1:2000 dilution. Detection was carried out using a chemiluminescent detection kit (Pierce, Rockford, IL).

ELISA protocols

Cell lysates were diluted in a 100 mM sodium carbonate/bicarbonate coating buffer (pH 9.6), added to 96 -well microtiter plates (Nunc Maxisorp, Rochester, NY), and then incubated overnight at 4 °C. Adsorbed lysates were blocked by addition of a blocking buffer comprised of 0.05 % Tween 20 (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS-T) supplemented with 1 % (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Wells were blocked for 2 h at room temperature. After blocking, wells were washed with PBS-T before addition of primary monoclonal antibody against phosphorylated H2AX (ab22551, Abcam, Cambridge, MA) at a concentration of 0.5 μg/mL in blocking buffer. This detection antibody was incubated in wells for 2 h at room temperature and subsequently washed with PBS-T. Wells were then probed with a secondary horseradish peroxidase-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) at a concentration of 1.6 μg/mL in blocking buffer for 1 h at room temperature, followed by three washes in PBS-T. Detection of the bound secondary antibody was performed with a chromogenic substrate solution of 1 mM 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich, St. Louis, MO) in 70 mM citrate–phosphate buffer (pH 4.2) immediately following the addition of 1 μL of 30 % hydrogen peroxide per milliliter of substrate solution. Absorbance at 405 nm was read and recorded with a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA).

Results

Verification of a radiation sensitive phosphorylation signature of H2AX

The radiation sensitive phosphorylation signature was first verified using a commercial monoclonal antibody specific

for γH2AX using mouse embryo fibroblast (MEF) cells irradiated ex vivo. MEF cells in confluent cultures were irradiated and lysed in RIPA buffer 30 min after irradiation. Whole-cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with the anti-γH2AX monoclonal antibody. Resultant blotting demonstrated a dose-dependent increase in phosphorylation of the histone (Fig. 1a). Normalized gel loading was confirmed by additional staining for actin. For MEF cells receiving from 0 to 10 Gy ex vivo radiation, the concentration of γH2AX is seen to increase with higher radiation doses. This is qualitatively commensurate with results from literature establishing γH2AX as a viable marker of radiation exposure (Rothkamm and Horn 2009). This radiation sensitive signature was used to develop an immunosorbent assay to reliably quantitate a radiation dose dependent protein response in a simple solid phase format.

Development and optimization of γH2AX ELISA using ex vivo irradiated mouse embryo fibroblasts

Cultured MEF cells were used to find optimum conditions for an indirect enzyme-linked immunosorbent assay (ELISA) used to measure phosphorylation of H2AX. In this format, cell lysates were first incubated directly in well plates, where targeted and nontargeted proteins alike adhere non-specifically to a polystyrene surface. This arrangement obviates the need for a matched antibody pair as required by a sandwich assay. Immobilized proteins were detected using a primary monoclonal antibody specific to γH2AX followed by a secondary enzyme-linked antibody for chromogenic readout.

A series of pairwise dilutions was made in order to optimize the absolute concentrations of cell lysate, primary detection antibody, and secondary reporter antibody to maximize sensitivity and resolution. It should be noted that the strongest absolute signal, as measured by optical absorbance, did not correspond to the highest signal-to-background absorbance ratio. In many cases, a decrease in primary or secondary antibody concentration from maximum signal is required to maximize the signal-to-background ratio. A primary antibody concentration of 0.25–0.5 µg/mL and a secondary antibody concentration of 0.8–1.6 µg/mL yielded optimal assay performance (data not shown).

The optimized indirect ELISA was evaluated against ex vivo irradiated and sham-irradiated MEFs that were lysed 30 min after irradiation. The assay, via chromogenic substrate reaction, reported a linear increase in proportion to radiation dose over a range from 0 to 10 Gy (Fig. 1b).

Evaluation of γH2AX ELISA on ex vivo irradiated human whole-blood

The γH2AX assay was tested for its compatibility with blood as a sample matrix. Blood from five human donors was drawn under IRB-approved protocols and used for this evaluation. Assay tuning was performed to determine optimal whole-blood lysate concentration for the initial sample incubation. Starting lysate concentration from 0.05 to 50 % in coating buffer was tested, and it was found that maximum signal occurs at a 12.5 % lysate concentration. This corresponds to a 2.1 % concentration of initial whole-blood as prepared in a 1:5 (blood/RIPA buffer) volume

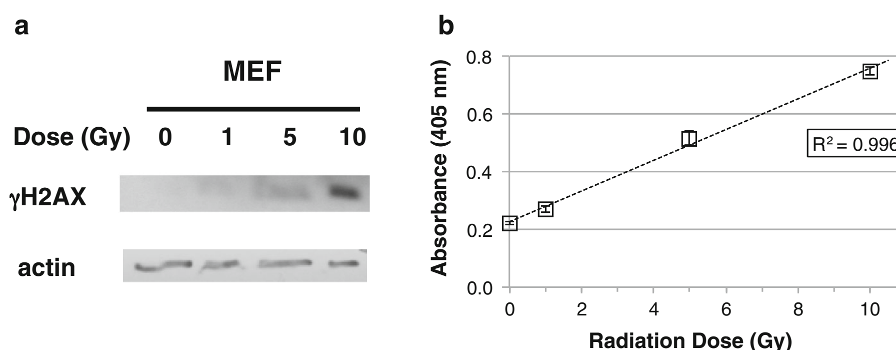

Fig. 1 Radiation sensitive γH2AX signal in lysates from mouse embryo fibroblasts irradiated ex vivo. **a** Biochemical assessment of gamma irradiated mouse embryo fibroblasts (MEF). MEFs were irradiated with gamma rays from a ^{137}Cs source at the doses shown and returned to culture for 30 min before cells were lysed. Whole-cell lysates were probed with monoclonal antibody specific for the phosphorylated form of H2AX and demonstrate a dose-dependent increase in the phosphorylation of the histone. **b** Quantitation of received radiation dose in irradiated MEFs using a well plate ELISA targeting γH2AX. MEFs were irradiated with gamma ray doses of 1, 5, and 10 Gy and processed alongside a sham-irradiated sample. Whole-cell lysates were immobilized in plastic well plates and probed with a monoclonal antibody specific to γH2AX, followed by readout with a secondary antibody and horseradish peroxidase chromogenic reaction. Final absorbance demonstrates a linear relationship with radiation dose with a correlation exceeding $R^2 = 0.995$. *Error bars* represent one standard deviation of technical triplicates

ratio, or approximately 2 μL of whole-blood for a standard 100 μL reaction volume.

An additional experiment was performed to assess variability in histone extraction in sample lysates derived from a fixed volume of blood. A blood sample from a single human donor was split into four volumes, each of which was lysed independently according to protocol. The four samples were analyzed using the developed ELISA protocol, in which an anti-H2AX antibody (ab11175, Abcam) was used for primary detection; this antibody reacts with the native, non-phosphorylated form of H2AX. ELISA results from the four independently processed sample lysates generated a mean of 2.07 absorbance units with a standard deviation of 0.06 for assay signal derived from non-phosphorylated H2AX. The largest percentage change among four samples observed was 5.3 % as compared to a percentage change of 150 % encompassing the dynamic range of 0–5 Gy that we observed in the assay. This verifies a repeatable histone extraction in normalized volumes of blood and further indicates that the anti-H2AX antibody could be used for normalization of histone content in a putative point-of-care appliance utilizing both markers in tandem.

To more clearly evaluate the potential for ELISA-based γH2AX biodosimetry in human applications, the assay was verified using human whole-blood that had been irradiated ex vivo. De-identified blood samples from the five human donors were each split into multiple volumes as described in the Methods. Each volume was subjected to irradiation at the specified dose or sham treatment of 0–5 Gy, incubated at 37 °C, and lysed using RIPA buffer before being stored at −80 °C for further analysis. These lysates, in our studies to date, seem to be capable of prolonged storage without degradation of signal in time spans of several months. No additional sample preparation was performed. Each sample lysate was analyzed in triplicate using the γH2AX assay, and the mean absorbance value for each donor and dose was used to assess biological variation among the five human donors. The obtained result is representative of 50 independent experiments. The developed assay resulted in a linear dose response curve with high correlation ($R^2 = 0.9997$; Fig. 2). This provides an initial exploration of biological variability among multiple human donors using the developed ELISA.

Evaluation of γH2AX ELISA using in vivo irradiated mouse whole-blood samples

Having established dose–response using blood as a sample matrix for the assay, the γH2AX ELISA was further tested for its ability to report an in vivo radiation exposure. Whole-blood samples were obtained from two mice at several time points after a 3 Gy exposure or

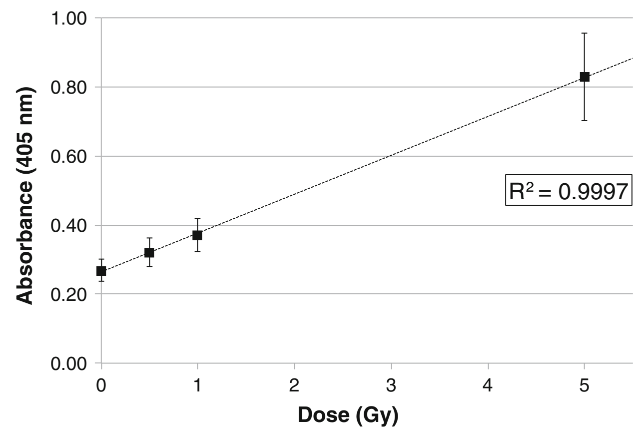

Fig. 2 Quantitation of absorbed radiation dose in ex vivo irradiated human blood samples from five donors. Whole-blood was drawn from five consenting adult donors and irradiated with gamma ray doses of 0.5, 1, and 5 Gy and processed alongside a sham-irradiated sample. Whole-blood was incubated after irradiation for 1 h, after which each sample was lysed by the addition of RIPA buffer. Lysates were studied for γH2AX formation using a custom ELISA protocol. The mean absorbance measured from all five donors is reported here and demonstrates a linear relationship with radiation dose with a correlation exceeding $R^2 = 0.999$. *Error bars* represent one standard deviation of mean absorbance across the five donors

sham treatment. These samples were lysed using RIPA buffer and stored at −80 °C until further analysis. No additional sample preparation was performed. A sham-irradiated mouse was used to provide control samples, mirroring the protocol used for ex vivo irradiated blood samples. Mouse whole-blood lysates were quantified for γH2AX using an adapted version of the γH2AX ELISA protocol. A rabbit-derived polyclonal primary detection antibody (ab1174, Abcam, Cambridge, MA) was used in place of the mouse-derived antibody described in the Methods, and a matched secondary antibody with conjugated horseradish peroxidase was employed. These protocol changes were used to mitigate cross-reactions between assay reagents and the mouse whole-blood sample matrix. The assay reported an increase in γH2AX following the 3 Gy exposure at 1 and 6 h after irradiation with signal returning to near background levels by 24 h (Fig. 3).

Signal validation testing

Components of the assay chemistry and blood sample fluid were assessed for inappropriate contribution to or attenuation of our acquired analytical signal. A control ELISA was used to analyze the proportion of chromogenic signal attributed to non-specific binding of the secondary reporter antibody. Both the normal assay protocol and a protocol omitting the primary detection antibody were run in parallel against lysates of ex vivo irradiated human whole-

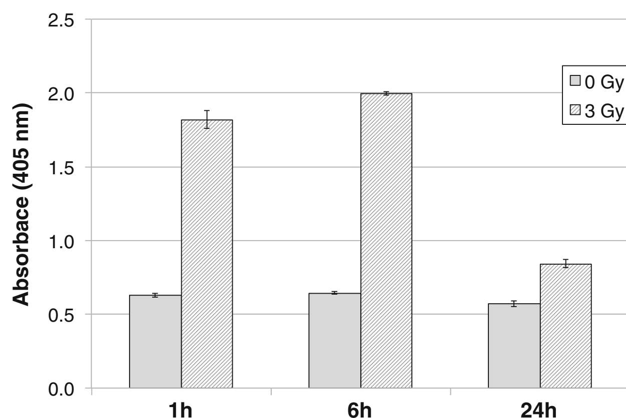

Fig. 3 γH2AX interrogation of in vivo irradiated mouse whole-blood. A live mouse was irradiated with 3 Gy of γ radiation and handled in parallel alongside a sham-irradiated mouse per approved protocols. Blood samples were drawn at 1, 6, and 24 h after irradiation, after which each whole-blood sample was directly lysed. Lysates were assessed for γH2AX phosphorylation using a variant of the developed ELISA protocol. *Error bars* represent one standard deviation of technical duplicates from each lysate

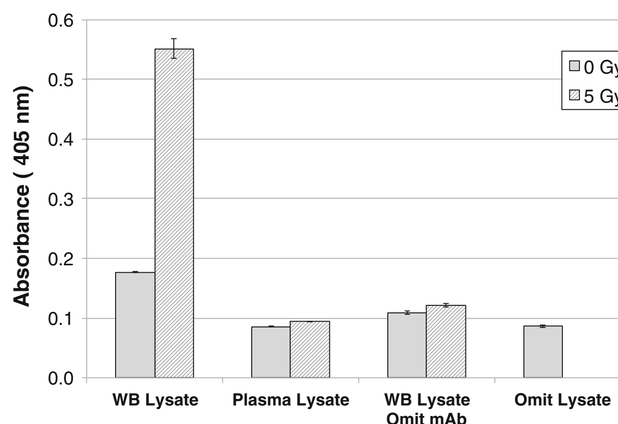

Fig. 4 Assay controls for γH2AX ELISA on ex vivo irradiated human whole-blood. Whole-blood (WB) was drawn from one consenting adult donor and irradiated 5 Gy of γ radiation and processed in parallel alongside a sham-irradiated 0 Gy sample. WB was incubated after irradiation for 1 h, after which the sample was lysed. A volume of the same irradiated WB was separated by centrifugation, and the plasma fraction was lysed independently. Lysates were assessed for γH2AX phosphorylation using the developed ELISA protocol. Removal of cells from blood in the plasma lysate or omission of the primary detection antibody induces a signal negation comparable to omission of sample lysate from the assay. Signal is therefore attributed primarily to specific binding of primary detection antibody with phosphorylated H2A.X within cells in blood. *Error bars* represent one standard deviation of technical triplicates for each assay variant

blood from a single human donor. The chromogenic signal was found to be attributable only to the presence of the modified histone and its cognate binding antibody (Fig. 4). The refined assay was also tested against a cell-free plasma. Here, blood was drawn from one consenting adult donor and irradiated with 5 Gy of gamma radiation and processed after 1-h incubation. The signal was present in the whole-blood lysates and was absent in acellular plasma fractions and sham-irradiated negative controls.

Discussion

We relate the refinement of a straightforward whole-blood immunoassay competent to report H2AX phosphorylation in response to ionizing radiation. Our results demonstrate the ability to measure a highly correlated linear relationship between radiation dose and phosphorylation of H2AX in a small volume of minimally prepared sample lysate. The assay has proven to be functionally competent on irradiated mouse embryo fibroblast cells, in vivo irradiated mouse whole-blood, and ex vivo irradiated human whole-blood samples. The linear range for the assay was demonstrated to be at least 0–5 Gy for human samples, and work in MEFs indicated a linear response over a larger 0–10 Gy dose range. At 1 h after exposure, the assay can resolve radiation dose into 1-Gy bins in the 0–5 Gy range using human samples. This resolving power is sufficient for clinical triage during a nuclear emergency response scenario (Blakely et al. 2009). Our human sample testing suggests that this window may extend up to 6 h after exposure, although extensive time course experiments using human whole-blood are not feasible, as extended incubation of whole-blood in vitro causes confounding DNA damage (Narayanan et al. 2001). The γH2AX signal has been shown to be elevated for 1–4 days following total-body irradiation in non-human primates at similar doses (Redon et al. 2010). In our own in vivo mouse experiment (Fig. 3), the H2AX phosphorylation signal is highest at 6 h following 3-Gy radiation exposure. The signal is diminished at 24 h, but still elevated in comparison with the control. Further investigation using a larger sample size is required to more fully establish the in vivo H2AX phosphorylation kinetics for mice.

The use of γH2AX as a radiation sensitive signature has been previously exploited and uses the phosphorylation of this histone as a proxy reporter for radiation dose via its assumed 1:1 stoichiometric association with double-stranded DNA breakage. Competing whole-cell immune staining techniques include fluorescence and in-cell ELISA approaches (Matsuzaki et al. 2010), which require complicating fixatives and washes. The ability to quantify H2AX phosphorylation status in simple whole-blood lysates as a surrogate marker for radiation damage represents a significant and unique improvement over existing techniques. Specifically, the assay described here does not require leukocyte purification, filtering, fixation, or centrifugation. Furthermore, our investigation of cell-free

plasma lysate (Fig. 4) strongly suggests that the measured γH2AX protein in whole-blood is not diminished by or attributable to blood-borne confounders. The refined γH2AX ELISA reported here uses approximately 2 μL of human whole-blood per reaction, which is compatible with a rapid and minimally invasive finger prick for sample collection.

The strengths of the assay include vastly simplified sample preparation of a very small initial volume of blood, cross-species functionality, and high fidelity of dose–response at early time points with relatively low biological variability in a small tested human cohort. Together, these features may extend the reach of such an assay beyond the clinical environment through adaptation to a portable, point-of-care platform. The limitations of this work include its modest cohort sizes and the limited quantitative resolution of the developed assay in the 0- to 1-Gy dose range. This modest resolution may limit the current assay format to triage applications in biodosimetry, where larger, more coarse dose-binning with straightforward sample processing is required.

Further investigation is required to assess the person-to-person variability of absolute γH2AX signal in lysed whole-blood. We attribute the bulk of γH2AX signal variation to this individual baseline variability and individual γH2AX response (Ismael et al. 2007), but validation of this claim requires increased sample numbers (Fig. 2). Furthermore, while initial results shown here indicate similar baseline and elevated signatures for a small cohort of donors, extension of the assay to many individuals may necessitate additional internal controls for absolute dose determination. Controls might include radiation insensitive markers or involve the use of antibodies specific to the non-phosphorylated H2AX for a ratiometric comparison. Such additional refinements in the context of this simplified assay have the strong potential to function in point-of-care triage use in emergency response scenarios.

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Conflict of interest The authors share the following competing interests: MLJ is a principal in Bialanx, Inc., which was previously awarded an NSF SBIR Phase I grant (#1314228). For the remaining authors, none were declared.

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