Abstract:

Title: SYSTEMS AND METHODS FOR HIGH-THROUGHPUT, MINIMALLY-INVASIVE RADIATION BIODOSIMETRY

(57) Abstract: The disclosed subject matter provides a method for obtaining a sample, determining the sample's metabolomic signature, comparing that signature to at least one known metabolomic signature, and quantifying the radiation exposure of the sample. Embodiments of the disclosed subject matter can utilize samples such as blood, blood plasma, sweat, urine, sebum, saliva, or cells, or a combination thereof. Embodiments of the disclosed subject matter can utilize samples obtained non-invasively. Certain embodiments of the disclosed subject matter are capable of high throughput, for example rates of 50, 100, 1000, 10,000, or more samples per hour. Embodiments of the disclosed subject matter can utilize samples derived from mice, humans, or other mammals. Some embodiments of the disclosed subject matter utilize chromatography, mass spectroscopy, or radio-frequency differential ion mobility spectrometry analysis, or a combination thereof. Embodiments of the disclosed subject matter employ software for comparing the metabolomic signature of the sample with at least one known radiation exposure metabolomics signature.
SYSTEMS AND METHODS FOR HIGH-THROUGHPUT, MINIMALLY-INVASIVE RADIATION BIODOSIMETRY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Application Serial No. 60/840,245 filed on August 25, 2006; U.S. Provisional Application Serial No. 60/942,090 filed June 5, 2007; and U.S. Provisional Application Serial No. 60/954,499 filed August 7, 2007, each of which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made in part with Government support under Department of Health and Human Services Grant U19A1067773-01. The Government has certain rights in the invention.

FIELD OF THE INVENTION


BACKGROUND

[0004] The need for high throughput rapid biodosimetry can be well illustrated by reference to the 1987 radiation incident in Goiania, Brazil, a city with about the same population as Manhattan. In the first few days after the incident, approximately 130,000 people required radiation exposure screening. In response to a radiological dispersal device (RDD) event in a U.S. city, one would anticipate a similar scenario. Tens or possibly hundreds of thousands of individuals will need to be screened for radiation exposure within a few days of any incident.

[0005] Mass radiological triage will be critical after a large-scale event because of the need to identify, at an early stage, those individuals who will benefit from medical intervention, and those who will not. Eliminating and reassuring those patients who do not need medical intervention will, of course, be crucial in what will certainly be a resource-limited scenario.

[0006] Regarding those who do need medical intervention, the best estimate for the LD50/60 in humans is in the 3.5 to 4.5 Gy range (Anno GH, Young RW, Bloom RM, Mercier JR. Dose response relationships for acute ionizing radiation lethality. Health Phys 2003;84:565-75.), but human tolerance for radiation exposure can be roughly doubled by the use of antibiotics, platelet and
cytokine treatment (Id.), so it is crucial that individuals who actually received whole-body doses above, for example, 1.5 Gy are identified and treated. Some individuals who are in this dose range will be clearly identifiable through early nausea, vomiting, and acute fatigue. For example, worker "C" at the 1999 radiation accident at Tokai-mura received a best-estimate whole-body equivalent dose of more than 3 Gy (Snyder, A.R. (2004) Review of radiation-induced bystander effects. Hum Exp Toxicol, 23, 87-89; Shao, C., Folkard, M., Michael, B.D. & Prise, K.M. (2004) Targeted cytoplasmic irradiation induces bystander responses. Proc Natl Acad Sci U S A.), was initially almost entirely asymptomatic, yet developed bone marrow failure (Hirama T, Tanosaki S, Kandatsu S, Kuroiwa N, Kamada T, Tsuji H, et al. Initial medical management of patients severely irradiated in the Tokai-mura criticality accident. Br J Radiol 2003;76:246-53.). Thus accurate biodosimetry is crucial in this dose range.

[0007] At higher doses, for example between 5 and 12 Gy, there is also a critical need for biodosimetry. This is because there is only a narrow dose window (approximately 7-10 Gy) in which bone-marrow transplantation is a useful option (below 7 Gy, survival rates are good solely with medication, while above 10 Gy patients will generally have lethal gastrointestinal damage) (Hall EJ. Radiobiology for the radiologist. 5th ed. Philadelphia: Lippincott, Williams & Wilkins; 2000). Thus it is important to ascertain, through biodosimetry, whether a patient's dose is within this dose window, such that a bone-marrow transplant would be a useful option.

[0008] It should be noted that the dose estimates discussed above are for adults; Children are likely to be more sensitive to radiation than adults in terms of their LD50 (8-10), so it is desirable that biodosimetric information should also be obtainable at lower doses in children.

[0009] After a large-scale radiological event, there will be a major need to assess, within a few days, the radiation doses received by tens or hundreds of thousands of individuals. Because current "high throughput" biodosimetry can, at best, assess a few hundred individuals per day, methods and devices suitable for very high throughput, minimally-invasive biodosimetry are clearly needed.
The range of potential biodosimeters have been reviewed by several groups. Table 2, adapted from Amundson et al. (Amundson SA, Bittner M, Meltzer P, Trent J, Fornace AJ, Jr. Biological indicators for the identification of ionizing radiation exposure in humans. Expert Rev Mol Diagn 2001;1:21 1-9.) gives a brief summary of current biodosimeters. It can be immediately seen that relatively few biodosimeters have the potential for very high throughput.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ease of assay</th>
<th>Baseline variability</th>
<th>Detection limit (Gy)</th>
<th>Radiation specificity</th>
<th>Post-exposure longevity</th>
<th>High throughput potential?</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR/EPR</td>
<td>Difficult</td>
<td>High</td>
<td>0.1</td>
<td>Good</td>
<td>years</td>
<td>No</td>
</tr>
<tr>
<td>Blood counts</td>
<td>Simple, but multiple repeats required</td>
<td>Low</td>
<td>1</td>
<td>Good</td>
<td>weeks</td>
<td>No</td>
</tr>
<tr>
<td>Somatic Mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsp by flow cytometry</td>
<td>Simple</td>
<td>Moderate</td>
<td>1-2</td>
<td>Good, but only for ½ of population</td>
<td>years</td>
<td>Yes</td>
</tr>
<tr>
<td>hprt- T-cell clonig</td>
<td>Simple</td>
<td>Moderate</td>
<td>1-2</td>
<td>Moderate</td>
<td>months</td>
<td>No</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicentrics / translocations</td>
<td>Moderate</td>
<td>Moderate</td>
<td>0.3</td>
<td>Good</td>
<td>years for translocations</td>
<td>No</td>
</tr>
<tr>
<td>Micronuclei</td>
<td>Simple</td>
<td>Moderate</td>
<td>0.3</td>
<td>Good</td>
<td>days</td>
<td>Yes</td>
</tr>
<tr>
<td>PCC</td>
<td>Simple</td>
<td>Moderate</td>
<td>0.3</td>
<td>Good</td>
<td>days</td>
<td>Yes</td>
</tr>
<tr>
<td>DSB (γ-H2AX)</td>
<td>Simple</td>
<td>Moderate</td>
<td>0.3</td>
<td>Good</td>
<td>days</td>
<td>Yes</td>
</tr>
<tr>
<td>Genomics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td>Potential to simplify</td>
<td>Low?</td>
<td>0.2</td>
<td>Low-Moderate</td>
<td>days</td>
<td>Yes</td>
</tr>
<tr>
<td>Gene Profiles</td>
<td>Potential to simplify</td>
<td>Low?</td>
<td>0.2</td>
<td>Good</td>
<td>days</td>
<td>Yes</td>
</tr>
<tr>
<td>Metabolomics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Profiling</td>
<td>Potential to be very simple</td>
<td>Low?</td>
<td>?</td>
<td>?</td>
<td>days?</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2

aberrations in human lymphocytes for biological dosimetry. Radiat Res 1997;148:S39-S44), were ruled out as not having the potential for high throughput analysis. Of the various somatic mutation endpoints, only the gpa (glycophorin A) assay appears amenable to a high throughput approach: in this assay in M/N constitutional heterozygotes, variant red blood cells expressing only one allele can be quantified through flow cytometry (Langlois RG, Nisbet BA, Bigbee WL, Ridinger DN, Jensen RH. An improved flow cytometric assay for somatic mutations at the glycophorin A locus in humans. Cytometry 1990;1:151-31); a critical drawback of this approach, however, is that only 50% of the population are M/N heterozygotes.

[0012] Although compatible with certain aspects of the inventions described herein, the PCC (premature chromosome condensation) technique is not thought to be well suited to the application, because a high-throughput PCC assay (Prasanna PG, Blakely WF. Premature chromosome condensation in human resting peripheral blood lymphocytes for chromosome aberration analysis using specific whole-chromosome DNA hybridization probes. Methods Mol Biol 2005;291:49-57; Prasanna PG, Escalada IsID, Blakely WF. Induction of premature chromosome condensation by a phosphatase inhibitor and a protein kinase in unstimulated human peripheral blood lymphocytes: a simple and rapid technique to study chromosome aberrations using specific whole-chromosome DNA hybridization probes for biological dosimetry. MutatRes2000;466:131-41.) requires the use of chromosome-specific fluorescent in-situ hybridization (FISH). Extensive experience with chromosome-specific FISH (Hande MP, Azizova TV, Geard CR, Burak LE, Mitchell CR, Khokhryakov VF, et al. Past exposure to densely ionizing radiation leaves a unique permanent signature in the genome. Am J Hum Genet 2003;72:1 162-70), suggests that the quite complex protocols involved are not well suited to automation. However, a fully automated device may be able to harness the potential of FISH-based PCC analyses.

[0013] The emphasis is therefore on four other approaches: micronuclei, γ-H2AX (DNA DSB assay), gene-profiling, and metabolomic profiling. These four approaches vary in their level of current development.

[0014] The radiation-induced micronucleus assay has been exceptionally well characterized and, since 1998, there has been an ongoing international collaborative study (the Human Micronucleus Project) on the use of the micronucleus assay for measuring DNA damage in humans (Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The Human MicroNucleus Project-An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res 1999;428:271-83.). However, current systems, whether based on flow cytometry or in-situ analysis, have limited throughputs of, at most, a few hundred samples per day (Offer T, Ho E, Traber MG, Bruno RS, Kuypers FA, Ames BN. A simple assay for frequency of


[0016] The γ-H2AX assay, which is a marker for DNA double stand break yields, was developed by William Bonner at the NCI in 1998 (Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 1998;273:5858-68). Its mechanistic basis has been extensively studied, and it has been suggested (Pilch DR, Sedelnikova OA, Redon C, Celeste A, Nussenzweig A, Bonner WM. Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem Cell Biol 2003;81:123-9), though not characterized, as a radiation biodosimeter because a) it is simple and rapid to automate, and b) the γ-H2AX yield is linear with dose over an extremely wide dose range.

[0017] Accordingly, the present application describes devices and methods for high-throughput minimally- or non-invasive radiation biodosimetry, which all take advantage of commonly available biological samples. A metabolomic signature of radiation exposure is identified and utilized to develop a very fast non-invasive biodosimetric device based on either urine, saliva or sweat. The biomarker should have appropriate specificity, i.e. the measured response should be specific to radiation, as opposed to a more general stress response, or a chemical or biological agent response.

SUMMARY

[0018] The disclosed subject matter provides systems and methods for obtaining a sample, determining the sample's metabolomic signature, comparing that signature to at least one known metabolomic signature, and quantifying the radiation exposure of the sample. Embodiments of the disclosed subject matter can utilize samples such as blood, blood plasma, sweat, urine, sebum, saliva, or cells, or a combination thereof. Embodiments of the disclosed subject matter utilize samples obtained non-invasively. Certain embodiments of the disclosed subject matter are capable of high throughput, for example rates of 50, 100, 1000, 10,000, 100,000, or more samples per hour. Embodiments of the disclosed subject matter can utilize samples derived from mice, humans, or other mammals. Some embodiments of the disclosed subject matter utilize chromatography, mass spectroscopy, or radio-frequency differential ion mobility spectrometry analysis, or a combination thereof. Embodiments of the disclosed subject matter employ software for comparing the metabolomic signature of the sample with at least one known radiation exposure metabolomics signature.

DETAILED DESCRIPTION

[0019] As genomics and proteomics have become established, a new field has emerged from the application of cutting-edge analytical chemical and statistical methodologies for the study of the so-called metabolome. The critical scientific contributions and impacts on human health that are expected to arise from this field are underscored by the prominent place of metabolomics on the NIH Roadmap (Zerhouni, E. (2003) Medicine. The NIH Roadmap. Science, 302, 63-72). Metabolomics is a set of procedures that seek to define the qualitative and quantitative natures of the cellular, or the complete organism's, complement of small molecules (those not covered by genomics, transcriptomics and proteomics), together with the definition of their physiological and pathophysiological fluxes and responses to external stimuli or genetic modification. In short, metabolomics is metabolic profiling of cell and tissue content, or of body fluids. The principal
analytical techniques employed include liquid chromatography-coupled tandem mass spectrometry (LCMS and LC-MS/MS), ultra-high performance liquid chromatography-coupled mass spectrometry (UPLC-MS), gas chromatography coupled mass spectrometry (GCMS) and nuclear magnetic resonance spectroscopy (NMR).

[0021] The ideal metabolomic solution will have (i) a high resolution (separate hundreds/thousands of individual constituents), coupled with (ii) a high signal-to-noise ratio and analytical sensitivity. Additionally, an idealized metabolomic solution would (iii) provide accurate assignment of the chemical nature of constituents, coupled with (iv) chemometric software that could analyze the resultant large data sets and provide an evaluation of the effect of an external stimulus on the metabolic fluxes within non invasively obtained samples, such as urine, sweat or saliva. Such a metabolomic solution will be required for the development of robust biomarkers of radiation exposure. The classical NMR approach fulfills criteria (iii) and (iv), but fails on criteria (i) and (ii). The solution lies in a combination of chromatography and mass spectrometry that would satisfy all four criteria when linked to the appropriate chemometric software.

[0022] A specific and coherent metabolomic solution has recently been developed by Waters Corporation, i.e. Ultra Performance LC™ (UPLC™) coupled to time-of-flight mass spectrometry (UPLCMS(TOF)) (Plumb, R.S., Stumpf, C.L., Granger, J.H., Castro-Perez, J., Haselden, J.N. & Dear, G.J. (2003) Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. Rapid Commun Mass Spectrom, 17, 2632-2638; Wilson, I.D., Plumb, R., Granger, J., Major, H., Williams, R. & Lenz, E.M. (2005) HPLC-MS-based methods for the study of metabonomics. J Chromatogr B Analyt Technol Biomed Life Sci, 817, 67-76). In a recent study of rats given a low dose of the model nephrotoxin mercuric chloride, it was reported that two negative ions of mass 178.0492 and 212.001 1 m/z (m, mass; z, charge number) were increased after dosing. These were readily identified as hippurate and indican, respectively. Error in mass measurement was merely 36 ppm. Eleven other biomarkers decreased in urine after dosing, seven of which were unequivocally identified. Likewise, 7 and 16 positive ions increased and decreased, respectively, after dosing, of which 12 were identified from their derived empirical formulae (47). It is this cutting-edge technology that is employed to define the metabolomic signature for radiation exposure, first in mice (Aims 1 and 2) and then in human subjects (Aim 3). One of the first such instruments in the US (Waters AQUITY Ultra Performance LC™ system with a Waters Micromass® QTOF Premier™ mass spectrometer and MassLyte™ and MarkerLynx™ software) has just been installed at the Laboratory of Metabolism, NCI, NIH. The members of this consortium will direct work on this instrument in Bethesda.


[0024] MEMS technology has also been applied to micro-GC design, resulting in the "GC", which is ~1 cm3 in size and can separate and measure mixtures of volatile organics in the ppb range (Lu, C-J., Tian, W.-C, Steinecker, W.H., Guyon, A., Agah, M., Oborny, M.C, Sacks, R.D., Wise, K.D., Pang, S.W. & Zellers, E.T. (2003) Functionally integrated MEMS micro gas chromatograph subsystem. 7th International Conference on Miniaturized Chemical and Biochemical Analysis Systems, 411-415.). A tiny GC system that can sit on a penny has also been developed, but only limited performance data have been published (Tian, W.-C, Pang, S.W., Lu, C-J. & Zellers, E.T. (2003) Microfabricated preconcentrator-focuser for a microscale gas chromatograph. J Microelectromech Sys, 12, 264-272). Moreover, a miniature GCMS has been reported (Shortt, BJ., Darrach, M.R., Holland, P.M. & Chutjian, A. (2005) Miniaturized system of a gas chromatograph coupled with a Paul ion trap mass spectrometer. J Mass Spectrom, 40, 36-42.), for NASA applications, with limits of detection around 1 ppb, for a broad range of organic compounds.

[0025] MEMS devices are already employed to protect homeland security, for example at US airports for the high-throughput screening of checked-in luggage and hand baggage, both for
explosives and narcotics. Such devices detect chemical signatures of a wide range of explosives and drugs using a miniaturized gas chromatograph (Scintrex Trace Corp. NDS-2000 handheld drug detector) or the microDMx™ chip (Thermo Electron Corporation EGIS™ Defender portable lightweight desktop explosives/narcotics detection system). The microDMx™ chip had its origins as a high-field asymmetric waveform-ion mobility spectrometer. It was able to detect benzene, toluene and acetone at the 100 ppb level (Miller, R.A., Nazarov, E.G., Eiceman, G.A. & King, A.T. (2001) A MEMS radio-frequency ion mobility spectrometer for chemical vapor detection. Sensors and Actuators A, 91, 301-312). Then a first-of-a-kind MEMS radio-frequency ion mobility spectrometer was developed with a limit of sensitivity for some volatile organics as low as 1 ppb (Miller, R.A., Nazarov, E.G., Eiceman, G.A. & King, A.T. (2001) A MEMS radio-frequency ion mobility spectrometer for chemical vapor detection. Sensors and Actuators A, 91, 301-312). This chip-based instrument operates like a gas chromatograph, insomuch as a carrier gas (air) is employed in the separation, but it is ions that are separated and detected, as in a mass spectrometer. However until now there has existed a long-standing need for a miniaturized platform for high-throughput minimally-invasive biofluids screening of the metabolomic signature in potentially radiation-exposed populations.

[0026] Biological assays using mass-spectrometry often require time-consuming pre-concentration steps, and HPLC pre-separation, in order to enhance minor components and to reduce chemical noise. Mass spectrometric analysis of biomarkers for specific conditions or radiation exposure can easily be overwhelmed by concentrations of normal metabolic components. Simple, elegant, designs for DMS-MS pre-filters, are able to reduce chemical noise by factors of 50 to 100 or more for a range of analytes. For instance, caffeine may be detected in a mixture of PEG (polyethylene glycol) and Na-PEG species, where ions of interfering species were completely suppressed. Using this new DMS-MS pre-filter technology, the same level of performance with radiation-exposure-biomarkers may be achieved. Metabolomics (the study of global metabolite profiles) has the most potential to provide an extremely rapid non-invasive radiation biodosimeter, based on a completely non-invasive assay from biofluids such as urine, serum, saliva, or sweat. Application of radiation metabolomics is described in studies in mice as reported in Project 2. Early studies with human subjects are also described, which are consistent with measured dose-dependent increases of 8-epi-PGF2α in human urine, serum, plasma (Wolfram RM, Budinsky AC, Palumbo B, Palumbo R, Sinzinger H. Radioiodine therapy induces dose-dependent in vivo oxidation injury: evidence by increased isoprostane 8-epi-PGF(2 alpha). J Nucl Med 2002;43:1254-8), and saliva (Wolfram RM, Palumbo B, Chehne F, Palumbo R, Budinsky AC, Sinzinger H. (Iso) Prostaglandins in

[0027] Provided herein is a novel device which for the first time analyzes the metabolomic signature of radiation exposure in animals, and is applicable to both mouse models and human subjects. These metabolic data are used for a high-throughput miniaturized instrument suitable for rapid deployment to sites of radiological incidents for screening and triage of large populations. Such "drive through" technology is highly desirable for efficiently identifying individuals who display the metabolic effects of radiation injury and exposure to significant radiation doses, and referring these individuals for clinical intervention and/or follow-up. An embodiment of the disclosed subject matter emphasizes extremely high throughput (many thousands of samples per day per machine), in contrast to current technologies which can analyze at most a few hundred samples per day per machine (Offer T, Ho E, Traber MG, Bruno RS, Kuypers FA, Ames BN, A simple assay for frequency of chromosome breaks and loss (micronuclei) by flow cytometry of human reticulocytes. Faseb J 2004; Styles JA, Clark H, Festing MF, Rew DA. Automation of mouse micronucleus genotoxicity assay by laser scanning cytometry. Cytometry 2001 ;44:153-5).

[0028] Embodiments of the disclosed subject matter are less invasive than current biodosimetry practices. The term "invasive biodosimetry" refers to procedures that require a qualified health professional, such as the drawing of peripheral blood through venipuncture. Such procedures are very tirae-consuming, in that a health professional can at most draw blood from 15 to 25 individuals per hour. Thus, minimally invasive procedures, such as a capillary blood finger stick, or non-invasive approaches like the use of exfoliated cells from a mouthwash, or from urine, are generally preferred.

[0029] Embodiments of the disclosed subject matter also include completely self-contained readily-deployable biodosimetry kits.

[0030] Embodiments of the disclosed subject matter provide a positive control for each individual, so that the effects of inter-individual variability in radiosensitivity can be taken into account. Previously, a concern with regard to biodosimetry was that of inter-individual variability in radiation sensitivity. Specifically, it would be highly desirable to be able to recognize individuals with high radiation sensitivity, because they would constitute a high-risk group which might warrant different follow-up procedures, and furthermore because at particularly at high doses (> 2Gy) the uncertainty in biodosimetrically-based dose estimates will predominantly be due to inter-individual differences (Thierens H, Vral A, de Ridder L. Biological dosimetry using the micronucleus assay for lymphocytes: interindividual differences in dose response. Health Phys 1991;61:623-30), The
current application describes responding to this issue by splitting the biodosimetry sample in half, with one of the two samples being irradiated to a known dose, before being analyzed.

[0031] Embodiments of the disclosed subject matter provide methods for evaluating effects of lower-dose radiation exposure, which presents difficulties for current practices. High-throughput products will be useful down to doses of about 0.5 Gy, significantly below a life-threatening dose, but one that is likely to increase long-term carcinogenic risk. Thus in the event of a large-scale radiological event, the dosimetric data generated with the products developed here could form the basis for long-term epidemiological studies.

[0032] Embodiments of the disclosed subject matter provide methods for minimally-invasive sample collection. Previously, a key issue in high-throughput biodosimetry had been invasiveness. Though venipuncture is an excellent source of peripheral blood, this procedure requires the expertise of a trained professional. In a scenario where one might need to assess hundreds of thousands of individuals in a few days, such a procedure would clearly limit efficiency, in that a health professional can at most draw blood from 15 to 25 individuals per hour. Thus, minimally invasive procedures, such as a capillary blood finger stick, or non-invasive approaches like the use of exfoliated cells from a mouthwash, or from urine, are generally preferred.

[0033] Embodiments of the disclosed subject matter provide methods for rapid evaluation of dosage. For example, an appropriate first level of triage might be a very rapid yes/no answer as to whether a given dose of, say, 2 Gy had been exceeded. As discussed above, there are other situations where an actual dose estimate is also important.

[0034] While all the disclosed biodosimeters can be calibrated over a wide dose range, some biodosimeters are more appropriate for lower doses, some for higher doses, and some are useful over a very wide range of doses. For example, a micronucleus assay, gene-profiling, DSB (γ-H2AX), or a metabolomics approach can be more informative. Furthermore, biodosimeters, such as micronuclei in lymphocytes, are very stable with time, over a period of many weeks. Some biodosimeters are practical for use only within limited time periods after the radiation incident. For example, the γ-H2AX biodosimeter, which reflects the presence of DNA double strand breaks, will be most useful in the first 36 hours after a radiation event, while micronuclei in blood reticulocytes will be most useful from about 24 to 60 hours after radiation exposure.

[0035] A rapid, non-invasive radiation biodosimetry device based on metabolomic analysis is provided. A signature of radiation exposure arising from analysis of metabolic markers is identified and utilized based for a very fast non-invasive biodosimetric device using body fluids such as urine, blood, saliva or sweat.
Identification of reliable metabolomic markers is required to assess radiation exposure and extent of radiation injury. In one embodiment, a UPLC-MS(ToF) is used with a body fluid to identify biomarkers indicative of radiation exposure. For example, mass spectrophotometry has been used to analyze the mouse and urinary metabolome and will be useful for analyzing the human urinary metabolome. The same analysis is performed to describe and quantify the metabolome of, for example, blood plasma, sweat and saliva, to determine the optimal metabolomic signature for each chosen matrix.

Analysis of over 2,600 mouse and human urine samples spanning a dose range from 0.1 to 11 Gy using a metabolomic profiling approach and the Waters UPLC-MS(ToF) (ultra performance liquid chromatograph + time-of-flight mass spectrometry) has uncovered a variety of radiation-responsive metabolites as potential biomarkers. From detailed informatic analyses and dose-response studies, five (thymidine, hexanoylglycine, citrate, isocitrate, and azelaic acid) were selected as initial candidate radiation metabolomic signature for optimization on the first Sionex ESI-DMS biodosimetry prototype. These potential biomarkers appear to be informative at times between 1 and 11 days after radiation exposure.

Potential detectable radiation-responsive molecules in mouse urine have been identified and human urine sample collection is underway for testing. The signal molecules are those that demonstrate dose-dependent differences in level after exposure to radiation. For example, potential radiation-responsive molecules in mouse and human urinary metabolome will display variations in level between 1 and 8 days after irradiation with doses from about 0.5 to 13 Gy (ranges more limited for human data).

The need to detect DNA damage by radiation requires specific markers that can be easily seen and quantified, and γ-H2AX foci formation is one such event that can be used in this scenario. It is known that H2AX phosphorylation is specific to sites of DNA damage and is also indicative of amount of DNA damage. However, in order to use γ-H2AX as a quick screening tool, it must be optimized for sensitivity and rapidity.
The first aspect that the present disclosure addresses is the image quality of foci in cells as it is important for uses in testing or as a diagnostic marker. Certain parameters need to be used in order to test the efficacy of the γ-H2AX and the best procedure for producing the images. Light intensity ratios of foci being one such parameter, can be optimized through antibody concentrations during chemiluminescence. The goal is to achieve the sharpest image possible and also to record the relationship between radiation level and foci counts.

For the first experiments, to determine the γ-H2AX induction, MEF cells in culture were used. It was seen that there was an increase of foci with increasing doses (Fig. 2) of radiations in the fibroblasts. Experiments were also performed to measure which concentration of antibodies yielded the best image quality based on the contrast between the cell background and fluorescence signal given by the γ-H2AX foci. Cells that exhibited the largest intensity ratio were deemed the best for viewing for clarity and with the most distinction between foci and cellular background. Cells were treated with antibodies using various concentrations of 1-100, 1-500, 1-1000, and 1-2000 dilution of both the primary and secondary antibodies. Cell were then imaged and compared. It was found that
the 1:100 concentrations for the primary antibody and the 1:500 for the secondary antibody yielded the best intensities ratios so far (Fig. 3). A comparison was also done using different kinds of blocking agents, and it was found that even though Superblock (Pierce Biochemicals) yielded faster results, NFDM (Non fat dried milk) provided clearer pictures of the foci.

![Intensity ratio graph](image)

**Fig 3: Relationship of concentration of primary antibody to intensity ratio of γ-H2AX in MEF cells.**

**Examples**

[0042] The following non-limiting examples are provided to further illustrate the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

**Example 1: Irradiation of Mice**

[0043] In initial irradiation experiments with mice, 614 daily urine samples were collected and processed, representing a dose range from 0.1 to 6 Gy. Data-mining approaches including principal components analysis and supervised analysis using partial least squares discriminant analysis were applied to this data, resulting in the identification of a set of at least 13 ions discriminating between mice exposed to 0.1 and 6 Gy of radiation at day 1. Candidate biomarkers in this experiment included sulfate conjugates, including a sulfate conjugate of either o-, m-, or p-cresol. Other potential biomarkers from this experiment are currently being identified, and their behavior with respect to dose and time since exposure is being mapped.
Confounding variables associated with cage stress have also been identified and controlled. "Stress molecules" have been identified, including stachydrine (proline betaine), that appear to be unrelated to radiation. These molecules appear to be excreted as a result of housing of animals in the metabolism cages, and may persist for up to 6 days. The protocol has been modified to allow longer conditioning of the mice to the metabolic cage environment prior to irradiation, thus limiting the effects of cage stress. Biomarkers that appear after allowing for conditioning are believed to be a reliable indicator of the effects of radiation, especially when they follow a time course.

Alternate metabolomes including blood plasma, sweat, saliva are all suitable for the analysis, and are expected to lead to identification of a maximum set of molecules that will be indicative of radiation exposure.

This device combines metabolomics and stress-signaling analysis with sensor-chip technology (Sionex Corporation), to provide instrumentation for rapid non-invasive assessment of radiation exposure and injury using metabolic markers. Irradiation in vivo triggers the expression of many genes involved in intercellular signaling, whose proteins can have wide-ranging effects on cellular metabolism. Data from a modern metabolomics approach indicate that these changes are reflected in alterations in the spectrum of metabolites in urine and sputum. Such metabolomic analyses offer several key advantages including simple, non-invasive collection, and thus the potential for a very high-throughput biodosimeter screening. Also contemplated is use of a metabolomic signature in sweat, which increases throughput still further.

Expression profiling and metabolite analyses are carried out in mouse model systems to determine the tissues and signaling pathways which are reflected in metabolomics changes. Informatics analyses are used to select thoroughly characterized metabolomics markers to develop an optimal radiation metabolomics signature. Translational studies extend these signatures into humans using samples from patients having total body irradiation.

The urinary metabolome of the mouse is examined to characterize changes occurring in a time- and radiation dose-dependent manner. Other metabolomes (blood serum, sweat, and saliva) can also be analyzed. Studies are done largely on a UPLC-MS(ToF) (ultra performance liquid chromatograph + time-of-flight mass spectrometry) machine (available at NCI). Putative radiation biomarkers are validated through studies of population variation, reproducibility, and assessment of the impact of potential confounding factors.

Mouse mutant models with altered radiation injury responses at the critical sites of hematopoietic (including lymphoid) and GI failure are used to study the impact of these specific
injuries on both metabolomic and gene expression signatures. These studies are to identify biomarkers for the severity of individual injury within the context of the biodosimetric signatures.

**Example 2: Irradiation of Human Subjects**

[0050] The urinary metabolome of human patients undergoing total body irradiation for bone marrow transplantation is also studied and compared with analyses of other metabolomes (blood serum, sweat and saliva), and with mouse data and with functional genomic data from the same patients.

[0051] While specific genotoxic stress responses demonstrate distinct features (Amundson, S.A., Grace, M.B., McLeland, C.B., Epperly, M.W., Yeager, A., Greenberger, J.S. & Fornace, Jr, AJ. (2004) Human in vivo radiation-induced biomarkers: gene expression changes in radiotherapy patients. Cancer Res, 64, 6368-6371), considerable overlap has been observed in general cell injury and inflammatory responses. The responding cytokines, such as TNFα, IL-1β, IL-8, and TGFβ, are implicated in cell injury responses and radiation bystander effects (2-6). Such cytokines can have a wide variety of effects on cellular metabolism and hence impact on the profile of metabolites, which can now be monitored with modern metabolomic approaches (Urbanczyk-Wojniak, E., Luedemann, A., Kopka, J., Selbig, J., Roessner-Tunali, U., Wilhitzer, L. & Fernie, A.R. (2003) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. EMBO Rep, 4, 989-993; Steuer, R., Kurths, J., Fiehn, O. & Weckwerth, W. (2003) Observing and interpreting correlations in metabolomic networks. Bioinformatics, 19, 1019-1026). There has been little attempt, however, to characterize and quantitate the effects of radiation exposure on metabolism. As a consequence, the metabolic outcomes of radiation exposure are largely unknown. With the application of a program of focused discovery and performance testing, however, the profiling of low molecular weight metabolites offers a novel and practical approach to radiation biodosimetry. An added advantage to this approach is that metabolomic profiles can be developed using non-invasively sampled biofluids, such as sweat, saliva and urine. A modern high-throughput instrumentation is provided, which delivers a new platform for high-throughput minimally- or non-invasive radiation biodosimetry suitable for large-scale screening.

[0052] The concept of defining low molecular weight chemical signatures of radiation exposure has grown out of work carried out in the fields of molecular radiobiology, stress signal transduction, and stress functional genomics. Organisms have evolved complex molecular responses to genotoxic stresses, such as ionizing radiation. These include changes in gene expression as well as post-translational modifications of many key signaling proteins that may have a myriad of effects on cellular metabolism. The complexity of these responses is highlighted by the large number of


[0054] Proximal gene expression effects can be linked to distal metabolomic consequences. For example, in an animal model for multiple sclerosis, microinjection into rat brain of recombinant adenovirus expressing either TNFα or EL-1α cDNA resulted in elevated urinary excretion of succinate and citrate (TNFα) or leucine, isoleucine, valine and myoinositol (IL-1β) (Griffin, J.L., Anthony, D.C., Campbell, S.J., Gauldie, J., Pitts, F., Styles, P. & Sibson, N.R. (2004) Study of cytokine induced neuropathology by high resolution proton NMR spectroscopy of rat urine. FEBS Lett, 568, 49-54). This report demonstrates a metabolomic effect resulting from altered gene expression, while also providing a basis for distinguishing between TNFα- and IL-1β-induced inflammatory brain lesions.

[0055] Several other studies have reported alterations in metabolites following in vivo irradiation. The urinary excretion of cAMP, cGMP, PGE2 and TxB2 was determined over 10 days in mice exposed to between 2 and 5 Gy whole body neutron irradiation. This study concluded that only PGE2 might be suitable for development as a biomarker of whole body or renal exposure to neutron fields (Schneiderkraut, M.J., Kot, P.A., Ramwell, P.W. & Rose, J.C. (1986) Regional release of cyclooxygenase products after radiation exposure of the rat. J Appl Physiol, 61, 1264-1269). Rats exposed to between 2 and 20 Gy of whole body γ-radiation also displayed a dose-related increased urinary excretion of TxB2 and 6-keto-PGFα (Schneiderkraut, M.J., Kot, P.A., Ramwell, P.W. & Rose, J.C. (1984) Thromboxane and prostacyclin synthesis following whole body irradiation in rats. J Appl Physiol, 57, 833-838). These authors and others concluded that COX-2 induction in the kidney was responsible for the elevated 6-keto-PGFα excretion, whereas extrarenal COX-2 induction led to the enhanced excretion of TxB2 (20). This underlines the importance of understanding the tissue-specific contributions of injury response to the observed metabolomic signature.
Only a small number of studies have taken similar approaches using human radiotherapy patients. One group has reported that, after $^{131}$I radiotherapy, both for hyperthyroidism and cancer, there is a significant dose dependent increase in prostaglandin 8-epi-PGF2α in serum and urine (Fig. 1) (Wolfram, R.M., Budinsky, A.C., Palumbo, B., Pahimbo, R. & Sinzinger, H. (2002) Radioiodine therapy induces dose-dependent in vivo oxidation injury: evidence by increased isoprostane 8-epi-PGF(2 alpha). J Nucl Med, 43, 1254-1258.) and significant dose dependent increases in 8-epi-PGF2α TXB2 and 6-keto-PGF,α in saliva (Fig. 2) (Wolfram, R.M., Palumbo, B., Chehne, F., Palumbo, R., Budinsky, A.C. & Sinzinger, H. (2004) (Iso) Prostaglandins in saliva indicate oxidation injury after radioiodine therapy. Rev Esp Med Nucl, 23, 183-188). This clear dose dependence for elevation of these metabolites following radiation exposure of humans is clear proof-in-principle for the metabolomics approach to biodosimetry in humans. By contrast, another group has reported that radiotherapy of the prostate did not elevate the urinary excretion of either 8-epi-PGF2α or 15-keto-dihydro-PGF2α (Camphausen, K., Menard, C., Sproull, M., Goley, E., Basu, S. & Coleman, CN. (2004) Isoprostane levels in the urine of patients with prostate cancer receiving radiotherapy are not elevated. Int J Radiat Oncol Biol Phys, 58, 1536-1539).

Fig 1 Concentration of 8-epi-PGF2α in human urine as a function of dose and time, after different radiation doses to the thyroid (22)
While this apparent discrepancy may reflect specificity for the site of radiation damage, it is clear that the relationships between time and dose of radiation exposure, the discrete elements of the inflammatory cascade, and the resulting urinary metabolome require rigorous investigation in both human and animal models. Results for radiotherapy patients, where a significant increase in two urinary prostaglandins is observed, 24 h after exposure, are reported herein.


Example 3: Effect of Radiation on the Inflammatory Cascade and Cell Signaling

Military Med., 167, 13-15; Amundson, S.A., Patterson, A., Do, K.T. & Fornace, Jr, A.J. (2002) A nucleotide excision repair master-switch: p53 regulated coordinate induction of global genomic repair genes. Cancer Biol Ther, 1, 145-149.), the induction of genes encoding numerous cytokines has been observed. This work has been extended to primary cells using ex vivo irradiation of human peripheral blood lymphocytes (PBL) (17), and in vivo using blood from patients undergoing total body irradiation (TBI) (1), and tissues from irradiated mice (Fig. 3). When radiation-responsive genes were surveyed for those with potential roles in intercellular signaling, inflammation, cell adhesion, and the acute phase response, a large number of genes were identified from both human and murine in vivo microarray experiments. Considering that smaller microarrays were used for the murine studies, the number of such genes is probably substantially greater. Genes encoding many cytokines, cytokine receptors, cell membrane and gap junction proteins were altered in response to ionizing radiation. Genes with roles in cell injury and inflammatory responses are also frequently altered, and include acute phase proteins, tissue proteases, and many cytokine-associated proteins. As already discussed, IL-1β expression has been shown to impact on the metabolic profile in rat urine (Griffin, J.L., Anthony, D.C., Campbell, S.J., Gauldie, J., Pitossi, F., Styles, P. & Sibson, N.R. (2004) Study of cytokine induced neuropathology by high resolution proton NMR spectroscopy of rat urine. FEBS Lett, 568, 49-54). The complex pattern of cytokine and other intercellular signaling genes induced by radiation would be expected to impact on metabolism at many levels.

Considering the broad and robust responsiveness of cytokine-related genes, rodent metabolomic results are likely applicable to determining a strategy to monitor for human radiation exposure with metabolomic markers. While there are only limited reports of radiation-induced metabolomic effects in rodents, Yamaoka et al (1998) reported that prostaglandins E2 and isoprostane 8-epi-PGF2α are increased in mouse urine after whole-body radiation exposure (Yamaoka, K., Obata, T., Iriyama, K., Iwasaka, T. & Hoshi, Y. (1998) Simultaneous quantitative analysis of prostaglandins and thromboxane after low-dose X irradiation. Radiat Res, 149, 103-106.).

Human urine was studied to determine whether there is a corresponding radiation-induced increase in these prostaglandins in human urine after a single 2 Gy fraction of radiotherapy. Six patients donated urine immediately before, 30 minutes after, and 24 hours after the first radiation treatment, and prostaglandin concentrations were measured in the urine using ELISA for isoprostane 8-epi-PGF2α and prostaglandin E2.
Both of these prostaglandins were significantly elevated at 24 h, consistent with the data from the mouse studies (Yamaoka, K., Obata, T., Iriyama, K., Iwasaki, T. & Hoshi, Y. (1998) Simultaneous quantitative analysis of prostaglandins and thromboxane after low-dose X irradiation. Radiat Res, 149, 103-106), the results for the isoprostane 8-epi-PGF$_{2\alpha}$ being shown here. Clinical results from Columbia are consistent with those of Wolfram et al who observed consistent dose-dependent increases of 8-epi-PGF$_{2\alpha}$ in urine, serum, plasma, and saliva after 131 I thyroid therapy (see Background Section, above), though Camphausen et al. did not see an increase in 8-iso-PGF$_{2\alpha}$ when assaying urine one to six weeks after the start of prostate radiotherapy.

**Example 4: Effect of Radiation on the Mouse Urinary Metabolome**

[0065] To assess the effects of radiation on metabolism, studies were carried out at the NCI. Analyses of urine were chosen because there is a concentration of metabolites over a defined period rather than a snapshot at a specific time, such as for blood. Mice were placed in metabolic cages for urine collection and were allowed to acclimatize to the cage for one day before a pre-irradiation sample of urine was collected over a 24 h time period. Groups of three C57B1/6 mice (12 week old males) were $\gamma$-irradiated with either 1.5 Gy or 6 Gy. Pooled urine samples for each group of 3 animals were collected just before and just after irradiation, and at 1, 5 and 8 days post-radiation. Gas chromatography-coupled mass spectrometry (GCMS) was used in these initial experiments to investigate the urinary metabolomes after various extraction, acid-hydrolysis and derivatization procedures, which permit detection and quantitation of organic acids, phenolic acids and phenols, the
end-products of intermediary metabolism. Approximately 800 peaks were detected in a 55 min chromatogram in total ion mode (TIM). Pair-wise overlays of TIM chromatograms of urines with respect to treatment (0, 1.5, and 6 Gy) and duration (0, 1, 5, and 8 days) revealed differences in the urinary excretion profiles. Variant peaks were first identified by comparison of their mass spectra with a spectral library and then were quantitated using single ion detection (SIM) mode, which has the virtue of greatly enhanced signal-noise ratio. Similar experiments were performed with unhydrolyzed urines, which also contain various sugars and amino acids in the TIM chromatograms.

[0066] Increases in urinary metabolites may represent changes in the rate of protein and nucleic acid turnover, which could reflect increased catabolism or repair. Monitoring the ion 261 m/z revealed that urea, as its tris(trimethylsilyl) (TMS) derivative was elevated 30-50% in urine on days 1, 5, and 8. This may be due to increased protein turnover, amino acid metabolism and urea cycle activity, i.e. nitrogen catabolism. Monitoring the ion 241 m/z revealed that uracil, as its bis-TMS derivative, was transiently increased by 3.6-fold, on day 1 post-irradiation with 6 Gy (Figure 4). This was not seen at 1.5 Gy. Interestingly, monitoring 264 m/z for urinary adenine (bis-TMS derivative) revealed no temporal changes, at either radiation dose, in the excretion of this base. Taken together, the uracil and adenine findings support increased uracil DNA glycosylase activity, rather than increased turnover of nucleic acids per se.
Changes in the levels of cysteine and lactate are consistent with a metabolic response to oxidative stress. Monitoring ion 220 m/z for urinary cysteine (tris-TMS) showed no changes after the 1.5 Gy dose but a fall to 55-67% of control levels after 6 Gy. This may represent increased oxidation of cysteine to cystine or be a general indicator of oxidative stress. Monitoring ion 117 m/z for lactate (bisTMS) revealed an interesting phenomenon. Mice irradiated with 1.5 Gy showed a transient 68% increase in lactate excretion on day 5 only.
However, after the 6 Gy dose, there was a 11.5-fold increase in lactate excretion on day 8 (Fig. 5, left). This elevation was so dramatic that it was clearly visible in the less sensitive TIM chromatogram (Fig. 5, right). Lactate produced by erythrocytes and muscle is converted to pyruvate by hepatic lactate dehydrogenase. This is the only metabolic option for lactate. The reversibility of this reaction is determined by the ratio of NAD+/NADH, i.e. the redox state of the cell. A high ratio drives the reaction to pyruvate, while excess NADH promotes lactate formation. Under conditions of radiation-induced oxidative stress, it would be expected that ROS would lead to an increase in lactate production. It has been reported that black-spined toads treated with 3.5 or 7 Gy of y radiation showed similar dose- and temporally-related patterns of lactate excretion (Mishra, J., Mittra, B. & Mittra, A. (2002) Effect of whole body gamma radiation on hepatic LDH activity, lactate, pyruvate concentration and rate of oxygen).

As already discussed, over expression of inflammatory cytokines has been reported to increase the urinary excretion of particular metabolites. Therefore, the excretion of the Krebs’ cycle intermediates succinate (bisTMS; 247 m/z) and citrate (tris-TMS; 273 m/z) were monitored. At a 1.5 Gy radiation dose, succinate and citrate increased in parallel to approximately 6-fold by day 8 (Figure 6).
These acids represent metabolomic biomarkers of radiation exposure that are correlated with expression of proinflammatory cytokines. As has been mentioned, TNFα overproduction leads to increased excretion of succinate and citrate in rats, while IL-1β overproduction led to an increased excretion of leucine and isoleucine (19). The excretion of these two amino acids was monitored, together with norleucine, (bis-TMS derivatives; 158 m/z) in mouse urine using SIM GCMS. All three amino acids showed a temporally increased excretion after 1.5 Gy, but, as for succinate and citrate, not after 6.0 Gy. Maximum increases of 305% (day 5) for norleucine, 468% (day 8) for isoleucine, and 156% (day 8) for leucine were observed (Figure 7).
[0071] This inverse-dose relationship may simply serve to illustrate the complexity of the organism's response to radiation at both a genomic and metabolomic level. Yet these data show conclusively that metabolic profiling of the mouse urinary metabolome by GCMS can be used to identify biomarkers of radiation exposure. The elevation of certain of these biomarkers (succinate, citrate, leucine, isoleucine) is consistent with the known effects of the proinflammatory cytokines TNFα and IL-1β (19).

[0072] Results using GCMS to monitor for changes in urinary metabolites are summarized in Table 1.
A fuller complement of radiation exposure biomarkers can be determined from additional studies of various mouse metabolomes, especially with using more powerful technologies, such as UPLC-MS(ToF).

Example 5: Mouse Models in the Dissection of Radiation Tissue Injury Responses

Considering the complexity of signal transduction pathways in vivo, a genetic approach offers a systematic approach to dissect mechanistically those pathways mediating significant injury responses to radiation, and to correlate them with downstream metabolomic effects. Radiation responsive genes show substantial tissue specific patterns of expression, which would be expected to impact on the metabolomic profile. In the case of the radiosensitive tissues spleen and thymus, the majority of their immediate transcriptional responses to radiation were comparable in wildtype and p53-deficient mice in spite of the different phenotypes discussed earlier. Thus, efforts can be made to identify tissue-specific profiles as well as the subset(s) associated with tissue-specific radiation injury as discussed in Fig. 5, 10, and 11. Analysis of in vivo metabolomic and functional genomics results will are made to discern signatures defining radiation exposure as distinct from unrelated injuries and disease states.

p38 has tumor suppressor properties and signals to other tumor suppressor pathways including p53 and Rb (31; 66; 67). While deletion of p38α, the major isoform of p38 in many tissues, is embryonic lethal (68), a dominant negative knock-in mutant of p38α (unpublished) which blocks much, but not all p38 signaling so that the heterozygotes are viable, has been generated.


Accordingly, a set of metabolites is identified candidate markers for radiation biodosimeters. Mouse model systems are first used to identify and to evaluate potential metabolomics signatures. Candidate markers and potential metabolomics signatures are then evaluated in humans.

Initial testing is performed using a large UPLC MS(TOF) instrument available at the NCI. The testing is later transferred to a benchtop device - an ion-mobility spectrometer. Appropriate sampling systems are can be selected and detection of previously identified metabolites is demonstrated. A breadboard device is then built according to specifications and attributes identified herein. Testing is accomplished with controlled samples first without interferants, and then with a variety of interferants. The breadboard device is then tested on human samples. Handheld devices are contemplated.

To evaluate, blinded studies are undertaken, and also can assess the effects of age, gender, and smoking status on the metabolomic signature.

Urine samples are collected from individual mice (n=3 to 5), rather than from groups of three mice. Radiation doses of 0.5, 1.5, 6 and 13 Gy are given, to cover the dose range relevant for GI failure, hematopoietic failure, and potential long-term effects in mice, which are more radioresistant than humans. Two doses (1.5 and 6 Gy) are a repeat of earlier studies. Urine will be collected at 6 hours after irradiation, and then at daily intervals from 24 hours to 8 days. Both native urine and urine hydrolyzed for 1h at 100°C with 6M HCl (75) will be analyzed by GCMS (automated Agilent
6890/5973N GCMS) after derivatization with BSTFA/TMCS reagent. This procedure has long been employed to determine urinary organic acid profiles in neonates (76) for the diagnosis of a wide range of inborn errors of metabolism that lead to abnormal urinary metabolomes. Irradiation of mice with 1.5 or 6 Gy leads to elevated urinary excretion of urea, uracil, lactate, succinate, citrate, leucine, and isoleucine. This list is not exhaustive and these studies are designed to reveal additional biomarkers. Hydrolyzed mouse urines will be screened for the approximately 150+ organic acids that are monitored by GCMS for neonatal diagnosis (Tanaka, K. & Hine, D.G. (1982) Compilation of gas chromatographic retention indices of 163 metabolically important organic acids, and their use in detection of patients with organic acidurias. J Chromatogr, 239, 301-322).


[0082] When novel biomarkers of radiation exposure are uncovered by the GCMS protocol, their presence and fold-elevation over controls are confirmed using LC-MS/MS (Applied Biosystems API 2000 with electrospray interface) by comparison with calibration curves of authentic standards. A comprehensive metabolomic analysis of the mouse urines generated in this project will be established using the Waters AQUITY UPLC system interfaced with a Waters Micromass QTOF Premier time-of-flight mass spectrometer (MSTOF). This metabolomics analytical solution is equipped with software designed to apply chemometric analysis to the UPLC-MSTOF chromatograms containing hundreds of constituent peaks. This is one of only three such instruments currently in the U.S. It is
anticipated that this instrument which, unlike the GCMS, inspects the entire urinary metabolome, will define principal components that co-vary (Plumb, R.S., Stumpf, C.L., Granger, J.H., Castro-Perez, J., Haselden, J.N. & Dear, GJ, (2003) Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. Rapid Commun Mass Spectrom, 17, 2632-2638; Wilson, L.D., Plumb, R., Granger, J., Major, H., Williams, R. & Lenz, E.M. (2005) HPLC-MS-based methods for the study of metabonomics. J Chromatogr B Analyt Technol Biomed Life Sci, 817, 67-76; Plumb, R., Castro-Perez, J., Granger, J., Beattie, L., Joncour, K. & Wright, A. (2004) Ulitrap erformance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. Rapid Commun Mass Spectrom, 18, 2331-2337) as a result of radiation exposure in the mouse. Excretory products such as glucuronide and sulfate conjugates, together with free sugars, that are not readily visible on GCMS, will also be detected. Many of the elevated urinary biomarkers will be anonymous, but the MSTOF has the advantage of producing exact mass estimates on pseudomolecular ions, which then permits generation of empirical formulae and analyte identification, subject to the intervention of the human expert system to ensure biologically meaningful structural assignments. As with GCMS, the identity of biomarkers and their fold-elevation will be confirmed by LC-MS/MS. All chromatography experiments will be performed at NCI and data analysis will be carried out in Prague after electronic transfer of raw data to an ftp server.

[0083] Preliminary studies reveal both dose- and time-dependence of specific biomarker elevation. For instance, uracil and lactate were raised, particularly at the highest dose (6 Gy) while succinate and citrate were elevated only at the lower dose (1.5 Gy). In addition, lactate was elevated only after 8 days, and not at earlier times (1 and 5 days post-irradiation). Such temporal and dose linkages can reveal possible underlying mechanisms. The diversity of apparent biomarker response suggests a metabolomic-based biodosimetry approach that is specific for the time after exposure.

Example 6: Alternative Mouse Metabolomes - Blood, Sweat, and Saliva

[0084] Radiation doses that produce a urinary metabolomic signature will be used in mouse experiments to investigate whether or not other mouse metabolomes also describe a radiation signature. Although urine analysis is considered the "gold standard" (Kidwell, D.A., Kidwell, J.D., Shinohara, F., Harper, C, Roarty, K., Bernadt, K., McCaulley, R.A. & Smith, F.P. (2003) Comparison of daily urine, sweat, and


[0086] Finally, blood (25-100 μl) will be sampled from the suborbital vein at 24 h intervals in a final set of irradiation experiments in C57BL/6 mice. Blood will be added to acetonitrile and
centrifuged to provide deproteinized samples (Yuen, P.S., Dunn, S.R., Miyaji, T., Yasuda, H., Sharma, K. & Star, R.A. (2004) A simplified method for HPLC determination of creatinine in mouse serum. Am J Physiol Renal Physiol, 286, Fill 6-9.) for chromatographic analysis by GCMS, LC-MS/MS, and UPLC-MSTOF. The data produced will assist in better understanding the biochemical changes elicited by radiation exposure. The skin swab and saliva data will help to direct the human studies, by suggesting the most potentially informative metabolomes on which to focus. Informatic analysis of dose and time dependence of these alternative metabolome signatures will also be undertaken.

**Example 7: Specificity Evaluation**

[0087] As a metabolomic signature begins to emerge for radiation-exposed mice, having examined urine, sweat, and skin swabs in detail using a number of mass spectrometric technologies, it will also be necessary to examine the reproducibility and specificity of the findings. For example, inbred mouse strains and not a wild population, as is Homo sapiens, will be employed. Lack of reproducibility in inbred mice of the metabolomic signature within reasonable limits, say D10%, may be due to a combination of stochastic effects and experimental error. The greatest variable in the measurement of the biological response to radiation is likely to be biological variability. Major 'natural' biological variables may potentially confound metabolomic radiation biodosimetry. Such potential confounding factors include age, gender and strain.

[0088] Experiments are made to evaluate reproducibility. Repeat experiments will permit insights into biological variability and multiple analyses of the sample set will yield data regarding analytical error. These data will provide criteria for detecting estimating radiation doses which will be set and tested experimentally. 95% confidence intervals for the candidate biomarkers will be calculated so that, in a subsequent analysis of 'blinded' samples, it will be possible to estimate the robustness of the initial metabolomic signature. The preliminary mouse urinary metabolomic signature can be tested to determine variability for different strains, ages and gender. In addition to C57BL/6, also routinely used are C57BL/6xI29 and AKT mice, and limited studies will be carried out to monitor for strain differences. Highly variable biomarkers will be eliminated from the signature, or assigned to specific sub-populations, for instance, markers only informative in females.

triggered by infection, chronic inflammatory conditions or xenobiotics must be avoided. Furthermore, a radiologic or nuclear event would be expected to involve victims with mixed injuries as well as individuals with unrelated underlying pathologic processes, all of which could potentially complicate dosimetry based on biological responses. While many of these factors will also be addressed in the human studies, the effect of potentially confounding stressors on the mouse urinary metabolome, where experimental variables can be more closely controlled, will be examined.


[0091] The metabolomic profiles resulting from these treatments will be compared with those from the radiation treatments using informatics tools. Because it has been shown that radiation can be distinguished from other stress agents in vitro by its gene expression signature, it is expected that radiation-specific metabolomic profiles will also be distinguishable from other stress factors. Thus, the specificity of our metabolomic signature of radiation exposure can be refined by eliminating markers indicative of general stress.
Example 8: Repeat Metabolomic Studies in Genetic Mouse Models with Altered Radiation Sensitivity

[0092] Metabolomic studies of genetic mouse models will be repeated, in either the hematopoietic system (p53-null and Bax-null) or the GI system (Asmase-null) to identify markers of specific injury that may occur in sensitive individuals below the normal dose-range where they would be expected.

[0093] Studies have been performed that define the cell-signaling pathways that respond to radiation. Insights from such studies can be harnessed to understand the basis of the metabolomic signature of radiation. For instance, some of the genes that are regulated by radiation code for enzymes, and the products of their enhanced enzymatic reactions may be detectable in the metabolomic compartments of the mouse, i.e. blood, urine, sweat/skin swab, and saliva. For example, cytochrome P450 2C29 is upregulated by radiation in the mouse. This is one of several murine cytochromes (P450 2C23, 2C29, 2C37, 2C38, 2C39, 2C40, and 2C44) that metabolize arachidonic acid to different products (DeLozier, T.C., Tsao, CC, Coulter, SJ., Foley, I, Bradbury, J.A., Zeldin, D.C. & Goldstein, J.A. (2004) CYP2C44, a new murine CYP2C that metabolizes arachidonic acid to unique stereospecific products. J Pharmacol Exp Ther, 310, 845-854; Luo, G., Zeldin, D.C, Blaisdell, J.A., Hodgson, E. & Goldstein, J.A. (1998) Cloning and expression of murine CYP2Cs and their ability to metabolize arachidonic acid. Arch Biochem Biophys, 357, 45-57.). Is there a relationship between this observation and the appearance of elevated prostaglandin excretion in mice and humans exposed to radiation? Can links be made between specific metabolites and radiation injury to specific tissues? Informatic pathway analysis of functional genomic experiments will help to relate molecular physiology to the metabolomic response to radiation, providing a more solid scientific basis for a metabolomic biodosimetry test.

[0094] Gene expression profiling in wt C57BL/6 mice irradiated with 0.5, 1.5, 6, and 13 Gy will be done to complement the metabolomic studies. Focus will be on hematopoietic (including lymphoid) and GI tissues and possibly more limited analysis of other major organs. Mice will be euthanized and tissues snap frozen for RNA and other analyses. Tissues of interest are spleen, thymus, liver, intestine, kidney, lung, and heart. Blood obtained by cardiac puncture will be stored for RNA analysis and confirmation of cytokine expression. Labeled cDNA probes will be prepared using the aminoallyl protocol as previously described (Amundson, S.A. & Fomace, AJJ. (2004) Microarray approaches for analysis of cell cycle regulatory genes. Methods Mol Biol, 241, 125-141). Initial studies will use healthy young (2 to 4 month) male mice and 3 mice per data point will be employed. Mice will be sacrificed 6 and 24 hr after irradiation in parallel with the metabolomic studies. For controls, RNA from each organ will be prepared from 10 unirradiated mice and pooled. These results will also be compared with gene expression profiles from patients undergoing total body irradiation.

[0095] As discussed, the most important targets for acute radiation injury are the hematopoietic and, at somewhat higher doses, GI systems. Molecular signatures with specificity for these critical toxicities would be valuable in assessing radiation exposure and injury. As summarized in Table 2, there are mutant mouse models where hematopoietic or GI radiosensitivity have been altered by genetic disruption of key regulatory proteins.

<table>
<thead>
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<th>Table 2. Radiation injury and stress signaling models¹</th>
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<td><strong>radiosensitive</strong></td>
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<td><em>Ppm1d/-</em></td>
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</table>

¹ Genotypes of mouse models currently in use. Highest priority models are designated with an asterisk*. ² Hematopoietic and Lymphoid

[0096] While the Bax-deficient protective phenotype is less impressive than p53 deficiency, the inclusion of Bax-null mice may help delineate metabolomic (and gene expression) markers in wt mice that reflect hematopoietic injury responses. For example, reduced responsiveness in thymus or spleen in p53-null mice involves a wide variety of genes, a subset of which are functionally linked with the radiation apoptosis phenotype. As the GI radiosensitivity present in p53 deficient mice (34) has not been reported in Bax-deficient mice, inclusion of these mice will help define specific markers associated with this type of injury.
Irradiation studies with the high priority mutant mouse models will be performed (Table 2) using the same doses and time-points as the initial metabolomic studies with wild-type mice. The focus will be on the urinary metabolome, but studies will be extended to other metabolomes (blood plasma, sweat, sputum) that have emerged from our initial studies as strongly informative. Limited genomic profiling will also be undertaken in the specific tissues of interest; e.g. in spleen and thymus for the Bax-null mice. Analysis of this gene expression data will help delineate tissue-specific signaling responses that mediate hematopoietic and GI injury. Informatic analysis will be performed in order to define metabolomic markers specific for injury to the hematopoietic and GI systems. The addition of such injury-specific markers to the biodosimetric signature may help to identify unusually sensitive individuals who have suffered an organ-specific radiation injury disproportionate to that which would be expected based solely on their dose.

**Example 9: Total Body Irradiation (TBI) Patients**

Blood serum, skin swabs, saliva and urine specimens will be provided from 25-50 TBI patients / yr, before and after irradiation at UPMC, Pittsburgh. The urine samples will be treated as those from the mice in Example 1, i.e. subjected to GCMS, LCMS/MS, and UPLC-MSTOF analyses. Peaks due to medications and their metabolites will be recognized by the MarkerLynx software of the UPLC-MSTOF, and will be eliminated from the data set. Armed with the experience of the mouse radiation responses, it will be straightforward to define a human radiation metabolomic signature in the urine of these TBI patients using informatic analysis tools as described elsewhere.

Since a genetic approach is not feasible and tissue access is limited, expression profiling in patient blood samples and metabolomic signatures will benefit from results obtained with our mouse models. The spectrum of radiation-responsive genes as well as induction of prostaglandins have many similarities between human and mouse. Insight into responses correlating with hematopoietic toxicity with the genetic approach in mice can be applied to similar responses in patients.

**Example 10: Robustness and Reliability Evaluation**

Gene expression profiles from blood of wt and mutant mouse lines will be compared to gene expression profiles in human blood. A similar approach will be carried out for metabolomic markers. Based on similarities between human and mouse blood as well as urine, predictions can be made for the relative contribution of specific tissue-type injury responses as well as particular signaling pathways.
occurrence of false-positives (FP), false-negatives (FN), true-positives (TP) and true-negatives (TN). A set of 25 pre-radiation and 25 post-radiation urines will be coded for the purposes of blinding and subjected to analysis of the metabolites in the signature developed above. Informatics analysis will then classify each sample as "radiation-positive" or "radiation-negative". These data, once unblinded, will be used to calculate the sensitivity [TP/(TP+FN)], specificity [TN/(TN+FP)], and efficiency [(TN+TP)/50] of the radiation metabolomic signature. These findings may mandate further refinement of the metabolomic signature by additional laboratory experiments, until satisfactory sensitivity, specificity, and efficiency of detection of radiation exposure is obtained. Additional factors, such as gender, age, smoking and disease status must also be considered. As larger numbers of patients are added to the metabolic profile database, an increased understanding of the impact of these variables will allow us to modify the classification algorithms applied to specific sub-populations as appropriate to obtain improved detection of radiation exposure. The 50 human urines from the blinded study may then be recategorized using this additional information, such as the age and gender of a given individual.

00102 Other important variables that could confound the testing of exposure include the presence of chronic inflammatory disease, acute infection or neoplasia. As an example, the last of these can be examined, because all patient samples from UPMC will be from patients suffering from neoplasia. The NCI laboratory has frozen 80 urine samples from anonymous healthy volunteers, which will be compared to the samples from the patients prior to TBI. The resultant metabolomic data should reveal if there are differences due to being healthy vs. having neoplastic disease. Additional variables, such as specific diagnosis of the patients will be similarly analyzed, to search for possible confounders.

Example 11: Alternative Human Metabolomes

00103 As with the mouse experiments in Example 1, skin swabs, saliva and blood plasma from the TBI patients will also be evaluated. There is considerable knowledge regarding testing of human skin wipes, sweat and saliva for their chemical constituents. This is done routinely in US airports to assure homeland security, with the view of detecting explosive and/or illicit drug residues on the parts of luggage that have been handled by the owner. Regarding skin swabs, HSPH will provide NCI with 25-50 skin swabs taken before and after TBI at the University of Pittsburgh Medical Center (UPMC). These will be analyzed by UPLC-MSTOF and LC-MS/MS for the presence of the metabolomic signature as for the mouse samples. Forensic scientists have reported that drug levels found on swabs of the fingers and forehead and on skin patches (PharmChek™) can mirror urinary excretion patterns (Kidwell, D.A., Kidwell, J.D., Shinohara, F., Harper, C., Roarty, K., Bernadt, K., McCaulley, R.A. & Smith, F.P. (2003) Comparison of daily urine, sweat, and skin swabs among cocaine users. Forensic Sci Int, 133, 63-78; Follador, M.J., Yonamine, M., de Moraes Moreau,


of the maximum steady state of lactate (MLSS) in saliva: an alternative to blood lactate determination. Jpn J Physiol, 49, 395-400; Segura, R., Javieire, C., Ventura, J.L., Lizarraga, M.A., Campos, B. & Garrido, E. (1996) A new approach to the assessment of anaerobic metabolism: measurement of lactate in saliva. Br J Sports Med, 30, 305-309.). It is clear that saliva represents a real opportunity for the noninvasive determination of the metabolomic signature and saliva samples from TBI patients at UPMC will be studied in this regard by UPLC-MSTOF and LC-MS/MS. Similarly, serum separated from blood samples obtained from the same patients before and after TBI will be profiled using GCMS and LC-MS/MS and subjected to metabolomic analysis by UPLC-MSTOF. These studies will lead to the definition of metabolic perturbations produced by radiation exposure and will lead to the final definition of the human radiation metabolomic signature and identification of the most informative biofluids to be targeted for radiation biodosimetry.

[00105] Current technology has not supported the type of large-scale population screening that will be needed in the wake of a large-scale radiological event. Provided herein is a portable screening device capable of delivering on-site analysis, to dramatically streamline screening procedures.

[00106] The technology underlying this device is radio-frequency differential ion mobility spectrometer (DMS) that operates like a gas chromatograph, in that a carrier gas (air) is employed in the separation, but it is ions that are separated and detected, as in a mass spectrometer. This chip is the underlying technology currently used both for online detection of sulfur odorants in natural gas (Varian CP4900 DMD), and in the ThermoFisher EGISTM Defender trace explosive and drug detection system, used in US airports for both baggage and passenger screening.

[00107] The microDMS developed by Sionex Inc (Eiceman, G.A., Nazarov, E.G., Miller, R.A., Krylov, E.V. & Zapata, A.M. (2002) Micro-machined planar field asymmetric ion mobility spectrometer as a gas chromatographic detector. Analyst, 127, 466-471; Eiceman, G.A., Krylov, E.V., Nazarov, E.G. & Miller, R.A. (2004) Separation of ions from explosives in differential mobility spectrometry by vapor-modified drift gas. Analytical Chemistry, 76, 4937-4944), is a novel detector for chemical and biological sensing applications. DMS is quantitative and has extremely sensitive detection limits, down to the parts-per-trillion range. The DMS method uses the non-linear mobility dependence of ions on high strength RF electric fields for ion filtering, and operates in air at atmospheric pressure. This novel method enables the rapid detection and identification of substances. The DMS scales down well, allowing miniaturization of the analytical cell using microelectromechanical (MEMS) fabrication, while preserving sensitivity and resolution. This makes DMS attractive as a quantitative detector that is potentially portable and sufficiently low in cost to be practical for use in our application.
Conceptually, the operating principle of the DMS spectrometer is similar to that of a quadrupole mass spectrometer, with a key difference that it operates at atmospheric pressure, so it measures ion mobility rather than ion mass. Mobility is a measure of how easily an ion travels through the air in response to an applied force, and it is dependent on the size, charge and mass of the ion. A DMS spectrometer acts as a tunable ion filter: to perform a measurement, a gas sample is introduced into the spectrometer, where it is ionized, and the ions are transported through an ion filter towards the detecting electrodes (Faraday plates) by a carrier gas, as shown in the Figure.
The ion filter is tuned by adjusting the electric fields applied between the parallel ion filter electrodes. Two fields are applied: an asymmetric waveform electric field which alternates between a high strength and low strength field, and a low strength DC compensation electric field. The amplitude of the asymmetric field is kept constant, while the compensation voltage (compensation electric field) levels are adjusted to select the particular ion species allowed to pass through the ion filter. Once the selected ion species passes the ion filter electrodes, it is detected as an ion current, on collision with the detector electrodes. Depending on the electric field conditions applied to the DMS, ion species are selected and permitted to pass through the ion filter region to be collected at a detector, which consists of a simple charge collector electrode. Unwanted (i.e., uncompensated) ions are scattered towards the ion filter electrodes, are neutralized, and are swept out by the carrier gas. The filtering mechanism is governed by the interaction between the ion and the net applied field, which alternates between high and low electric field strengths.

The particular compensation voltage required to select an ion species to pass through the filter is governed by its differential mobility ($\alpha$). The mobility of an ion in air is field-dependent and can change significantly as the field strength increases. The compensation voltage required to allow a particular ion to pass through the filter exploits this field/mobility relationship. The electric field conditions required to permit a particular ion to penetrate through the filter to the
detector are specific to each ion species. As illustrated in the two Figures, by noting the applied field conditions, or voltages, and the current level at the detector electrode, various ions species can be identified.

[0011] Because DMS functions as a filter, the longer the field conditions are set at one particular value, the more ions are collected at the detector. This improves the signal-to-noise ratio, thus enabling increased sensitivity. Since mass production techniques and batch fabrication methods can be employed in producing this miniaturization, significantly less expensive devices can be manufactured.

[0012] DMS has several advantages over conventional ion mobility spectrometers, which are much larger, more expensive, and operate with short pulses of ions. In DMS analysis, the ions are introduced continuously into the ion filter with nearly 100% throughput of the “tuned” ions reaching the detector. This allows the DMS to have an extremely high sensitivity even though it is significantly smaller. This approach also avoids the complexity of generating short, spatially well-confined, ion pulses required in the conventional IMS. In fact, the DMS approach actually benefits from miniaturization, since the electric fields required to filter the ions are on the order of 10,000 V/cm. By reducing the gap dimensions to the order of 500 microns, the voltages required for ion filtering are easily achievable.

[0013] Sionex Inc. has developed a development platform (SDP-I, shown in the following Figure).

[0014] The SDP-I platform allows use of the SDP-I device in parallel with the mass spectrometer to ascertain sensitivity and selectivity limits for the metabolite set previously generated.
Synthetic laboratory reagents are used. The SDP-I is optimized for metabolite set identification and detection using parameters such as flow rate, dopant choice, and concentration. Dopants are used to change mobilities and therefore spread out the peaks of different metabolites (and interferants), should the peaks be close together. An example is given here from the use of DMS in explosives detection.

Potential interferants potentially leading to false positives and false negatives are also identified. Examples of possible interferants include metabolites from tobacco, such as cotinine, trans-3'-hydroxycotinine, nicotine-N'-oxide, cotinine, N-oxide, and nornicotine, and cocaine and its metabolites benzoylecgonine and ecgonine methyl ester. Synthetic laboratory reagents are used in an exemplary embodiment.
The SDP-I parameters are optimized for metabolomic set identification and detection, in the presence of potential interferants. The signal optimization parameters can be, for example, flow rate, dopant choice, and concentration.

An appropriate front-end is selected for coupling with the microDMx to the detect metabolomics set. Suitable alternatives include: direct (no front-end microDMx), gas chromatography (GC, including a standard laboratory GC), and use of a membrane, prior to microDMx.

In one embodiment the microDMx™ sensor is adapted for biomarker testing and a compact Nano-electrospray coupled microDMx (ESI-DMS) system is used for detection of metabolic radiation biomarkers. It includes an electrospray ion source for ionization of liquid samples combined with direct detection of DMS spectra. Integration of a nanospray ion source directly with the microDMx sensor allows DMS to be used for very fast screening of low volatility species in fluids and digests. This system provides rapid analysis (seconds) and is simple to use. DMS sensitivity and selectivity should provide accurate detection and identification of radiation-exposure biomarkers. Performance of this system is enhanced by the use of drift gas modifiers tailored to the analyte. The system is being optimized to provide mass spectrometric investigation of the five preliminary radiation-exposure biomarkers for analytical performance testing of this prototype.

Once a sampling system and a front end are selected, detection of the identified metabolites is demonstrated with reference to sensitivity and selectivity characteristics, as above. The ion chemistries and flows of the current best DMx parameter set can be modelled, to further optimize the breadboard design.

In an alternative embodiment, "conventional" technology can be used in combination with the microDMX, for example an ultra-miniature mass spectrometer, again with or without a GC front end. For example, the Ferran Symphony micropole mass spectrometer
(Boumsellek, S. & Ferran, RJ. (2001) Trade-offs in miniature quadrupole designs. J. Am. Soc. Mass Spectrom., 12, 633-640) is suitable. The Ferran Symphony has four advantages over conventional mass spectrometers: 1) extremely small size, 2) low cost, 3) high sensitivity (up to 10 ppb), and 4) the ability to operate at 10-2 Torr pressures.

[00123] Designed for detecting residual gases in situ, this instrument can be configured with a mass range of 4-300 m/z, meaning that it could be used to detect ionized molecules and their daughter ions with molecular weights below 300, which is a characteristic possessed by most, if not all, of our likely metabolomic biodosimetry set.

[00124] The microDMx chip for the breadboard device is built by Sionex, according to the specifications defined above. Where a GC front end is desired, the SLS miniature GCM 5000 gas chromatograph can be used, shown here, which is about the size of a credit card.

[00125] All fluidic and electronic module components on this miniature GC are on an integrated multi layer printed circuit board. The breadboard device can be tested for adherence to specifications (sensitivity and specificity), using the laboratory reagent approach outlined supra.

[00126] The sensitivity and specificity of the breadboard device is tested on human samples, specifically urine, sputum, capillary blood, and sweat. Testing is done, for example, with samples obtained from multiple volunteer patients.
Example 12: Sensitivity and Specificity Testing

[00127] Suitable patients have been identified at the brachytherapy spa associated with Charles University, Jachymov, Czech Republic (Navratil, L., Hlavatý, V. & Dylevsky, I. (1996) Our experience in brachytherapy of certain noncancerous diseases. Sb Lek., 97, 103-113). Here, patients are treated with whole body irradiation of 1.8 to 3.5 Gy (over ~6 h), for a variety of rheumatic diseases, from an extended 226 Ra source, in what is known as a "Jachymov box." Blood, urine, sweat swabs and saliva will be obtained before, immediately after treatment (i.e. 6 h after the start of treatment), and 24 hours after the treatment. In selected case, samples at 1 week post irradiation will be obtained. These samples will then be analyzed for the radiation metabolomic signature defined earlier. As before, the breadboard parameters can be optimized to maximize sensitivity and specificity. Based on data from 60 individuals, a decision will be made regarding which subset of the four matrices (blood, urine, sweat, and saliva) should be built into the specifications for the prototype.

[00128] The device will then be tested on samples from TBI (total body irradiation) pediatric patients from Memorial Sloan Kettering Cancer Center (MSKCC). Similarities and differences can be investigated between the results from Prague, which were for a 4-6 hour radiation exposure, and those for the Pittsburgh TBI patients, where the exposure was only a few minutes. For the Pittsburgh TBI patients, a protocol is already in place and patient blood has already been utilized to monitor for gene expression changes by TBI (Amundson, S.A., Grace, M.B., McLeland, C.B., Epperly, M.W., Yeager, A., Greenberger, J.S. & Fornace, Jr, AJ. (2004) Human in vivo radiation-induced biomarkers: gene expression changes in radiotherapy patients. Cancer Res, 64, 6368-6371). As already described, the patients receive two doses of 1.5 Gy, 6 hours apart per day. Samples will be collected prior to irradiation, 6 h after the first dose fraction, and at 24 h, i.e. after two fractions.

[00129] Size and power requirements are also important factors in determining additional configurational details. The Sionex Inc.chip has already been designed into a handheld, battery powered device such as the prototype shown in the next figure.
This device uses membrane sampling, 63Ni ionization, and has a lower power requirement (4W), allowing for optional battery operation. Suitable software is also incorporated into the device.

Various embodiments of the device can be tested with the Prague whole-body brachytherapy patients, the Pittsburg TBI patients, and the Memorial TBI children. Preferably, in these studies the operators will be blinded between irradiated individuals and controls. Testing will involve, for example, studying 120 TBI adults in Prague, with a corresponding number of unirradiated controls, and 120 adults and 24 children in the US, again with approximately equal numbers of controls. These large scale studies will provide the opportunity to identify possible effects of age, gender, and smoking status.

Example 13: Set of Candidate Metabolites That Distinguish Between High and Low Dose Radiation Exposures in Mice

Through the analysis of 614 mouse urine samples, a number of candidate metabolite biomarkers of radiation exposure have been identified. Two basic types of multivariate data analysis were performed. Unsupervised analysis using MarkerLynx software used principal components analysis (PCA) separations of data into groups. Supervised analysis was then performed using the SIMCA-P platform to build a robust model using partial least squares discriminant analysis (PLS-DA). Ions that differ significantly between groups of urines appear as outliers in these analyses. For instance, in comparisons between mice irradiated with 0.1 and 6 Gy, sets of ions showing either increased or decreased urinary excretion after exposure to 6 Gy ionizing radiation have been identified. Currently, tentative structural assignments of these ions have been made, which include the sulfate conjugate of either o-, m- or p-cresol, and several other sulfate conjugates. The identification of these ions will form the basis for our future studies of dose- and radiation-specificity, and will guide the early stages of adapting the Sionex microDMx™ platform into a working prototype for metabolomic radiation biodosimetry.
Example 14: Marker for Monitoring Non-Specific Cage Stress in Metabolomic Experiments

[00133] As with all biodosimetry approaches, it will be important to discern radiation-specific metabolomic changes from confounding variables both experimentally and in the field.

[00134] A number of candidate metabolite biomarkers of radiation exposure have been identified using our metabolomic profiling approach and the Waters UPLC-MS(TOF) (ultra-performance liquid chromatograph + time-of-flight mass spectrometry) machine. Both unsupervised and supervised methods of multivariate data analysis were performed to identify ions that differed significantly between groups of urines from mice treated with different doses of ionizing radiation. Sets of ions showing either increased or decreased urinary excretion after exposure to 6 Gy ionizing radiation have been identified. Currently, a set of ions with tentative structural assignments has been identified, including the sulfate conjugate of either o-, m- or p-cresol, and several other sulfate conjugates. The identification of these ions allows studies of dose- and radiation-specificity.

[00135] Non-specific stresses not related to the radiation treatment of interest may also alter the excretion of metabolites in urine. "Stress molecules" have been identified that were excreted by sham-irradiated animals. By analysis of ion mass, elemental composition analysis, putative compound analysis and other confirmatory testing, one such "stress molecule" is stachydrine (proline betaine), and the excretion of stachydrine is used as a monitor for nonspecific cage stress associated with the metabolic cages required for these experiments. This has allowed to optimization of the acclimatization of mice to the experimental conditions. This approach eliminates one potential source of noise in the experiments, and enables radiation studies with increased confidence in the resulting metabolomic profiles. This marker of non-specific cage stress is also valuable to other researchers developing metabolic signatures of disease or other stresses.

Example 15: Metabolite Profiling

[00136] With bioinformatic approaches, radiation stress signatures are being developed using functional genomic and proteomic approaches. Expression profiling studies both in irradiated cells, and in patients undergoing radiotherapy, point to the overproduction of various proinflammatory cytokines and growth factors. Through the inflammatory cascade, these in turn cause the production of an array of intermediary metabolites, for example Krebs’ cycle intermediates and prostaglandin derivatives. Initial experiments indicated that certain urinary metabolites were elevated in male C57BL/6 mice after γ-irradiation in the range 10 cGy to 6 Gy. A total of 150 urine samples were collected from 5 groups of 3 mice (10, 10, 40, 150 and 600 cGy). Animals varied in age from 8.5 to 15 weeks at first radiation exposure. There were no control/sham groups studied and urine samples were collected from the same mice prior to irradiation. However, elevations in urinary output of urea, uracil, lactate, succinate, citrate, leucine, and isoleucine, in relation to radiation exposure,
demonstrated proof of principle. These preliminary investigations were conducted by metabolite profiling using solid phase-extracted acid hydrolyzed urines that were then chemically treated to yield trimethylsilyl derivatives suitable for gas chromatography-mass spectrometric (GCMS) analysis.

**Example 16: Irradiation of Mice for Urine Metabolomics**

Three independent experiments were made using C57BL/6 mice. In Experiment 1, 6 week old male C57BL/6 mice were treated as follows: Group A (n=4): 3 mice irradiated at 10 cGy + 1 sham control. Group B (n=4): 3 mice irradiated at 6 Gy + 1 sham control. Because it is not possible to keep mice in metabolic cages (which are required for urine collection) beyond 24 h duration, Groups A and B were placed into four metabolic cages on alternate days. By irradiating groups on consecutive days, it was possible to collect day 1, 3, 5, 7, 9, 11, 13, 15, and 17 urines for all eight mice using just four metabolic cages. In addition, animals were placed in metabolic cages for several days prior to irradiation to condition them to their environment. Urines were collected on four separate days prior to irradiation. In total 112 urine samples were collected in this experiment. In Experiment 2, 5 week old male C57BL/6 mice were treated as follows: Group A (n=7): 3 mice irradiated at 10 cGy + 4 sham controls. Group B (n=7): 4 mice irradiated at 10 cGy + 3 sham controls. In this experiment a total of 175 urines were collected. In Experiment 3, sham controls from Experiment 2, which were reasoned to be better acclimatized to the metabolic cages were used, together with additional older C57BL/6 mice, which it was thought would acclimatize better than younger mice. The mice were treated as follows: Group A (n=8): 4 mice irradiated at 3 Gy + 4 sham controls, then four weeks later irradiated with 6 Gy (shams remained sham controls). Group B (n=8): 4 mice irradiated with 3 Gy + 4 sham controls (shams remained sham controls). The total number of urine samples collected in this experiment was 327. In all cases, urine volumes and daily body weights of the mice were recorded. Overall, 614 daily mouse urine samples, including pre- and post- irradiation plus sham controls, have been collected.

**Example 17: Analysis of Urine by UPLC-MS(ToF)**

The chromatogram obtained from UPLC-MS(ToF) analysis of each urine sample contains data from between 4,000 and 6,000 ions, the majority of which represent individual urinary constituents, and the remainder result from unintentional fragmentation of parent ions in the source of the mass spectrometer. The mass spectrometer can be set to generate and analyze either positively or negatively charged ions. Typically, experiments are carried out in -ve ion mode. Each sample therefore has an associated data set of, for example, 5,000 ions, each of which has a known accurate mass (to four decimal places), intensity, and retention time on the UPLC column. This means that ~15,000 data are typically collected for each sample. Therefore, including some additional preliminary experiments, our current dataset for metabolomic analysis, including both +ve and -ve ions, is approximately 764 X 5,000 X 3 X 2, equivalent to in excess of 20 million data. Because each
ion has a measured retention time and a determined accurate mass, these data can be used to identify
the chemical nature of any biomarkers that are associated with radiation.

**Example 18: Metabolomic Analysis and Data Mining**

Two basic types of multivariate data analysis have been performed on various subsets of this database. The first employs unsupervised analysis using the MarkerLynx, software that is provided with the UPLCMS(ToF) instrument. Here the analysis is blind to sample categorization, in this case irradiated vs. sham. Using principal components analysis (PCA), it was attempted to separate data into groups, for example, for each day of urine collection. More advanced analysis was then done using the SIMCA-P platform to perform supervised analysis of groups, where the software is now told which samples come from untreated and treated animals. The software attempts to build a robust model using partial least squares-discriminant analysis (PLS-DA). The PCA and PLS-DA analyses both yield so-called scores plots (separation of samples) and loadings plots (depiction of the ion/retention time data that gave rise to the sample separations). Most ion/retention time pairs do not differ significantly between groups and therefore appear as part of a dense cloud around the null point of the scores plot of component 1 vs. component 2. However, when ions do differ significantly between groups of urines, they appear as outliers from this cloud, their displacement representing the magnitude of their contribution to the sample group differences. A typical example is shown in the following Figure.
The scores plot shows the separation of three urines from mice (Bl) exposed to 6 Gy radiation from three urines of mice (Al) exposed to 10 cGy radiation (used in this example as controls). The loadings plot identifies a number of ions that contribute to this difference. The outlying ions on the left side correspond to compounds whose urinary excretion is elevated by 6 Gy radiation exposure (positive biomarkers). The outlying ions on the right side represent compounds whose urinary excretion is depressed by 6 Gy radiation, relative to 10 cGy exposure (negative biomarkers). Ions with the same letter represent a group of isomeric compounds, e.g. F1, F2, and f, the last being a negative biomarker and the first two, positive biomarkers. This may represent a switch in metabolism triggered by radiation.

To date, tentative structural assignments of most of these biomarkers have been made on the basis of their accurate masses, which, in turn, generate empirical formulae. For example, ion C has a mass [M-H]- of 187.0084, which corresponds to an empirical formula of C7H7O4S1 with an acceptable error of 10.2 ppm. It would appear that the parent compound is the sulfate conjugate of either α-, m- or p-cresol (2-, 3- or 4-methylphenylsulfate). p-Cresol sulfate is a well
established constituent of human urine, possible derived from tyrosine sulfate, that is also found in urine of patients with multiple sclerosis and referred to as "myelin basic protein-like material" (MBPLM). It may thus represent aromatic amino acid catabolism. The other positive biomarkers found in this experiment (6 Gy vs. 10 cGy, day 1) are also all sulfate conjugates. This basic pattern of the excretion of sulfate conjugates of aromatic amino acid catabolism products was found consistently across the sample sets from 1 to 5 days post irradiation with 6 Gy. The chemical nature of each putative biomarker must next be identified through the purchase or chemical synthesis of authentic metabolites. The robustness of the biomarkers can then be tested in the database, in relation to specificity and sensitivity, with respect to radiation dose and time course. Positive identification of this set of potential biomarkers is can be readily made using this approach.

Example 19: Characterization of Radiation Stress-Signaling Pathways With a Mouse Model Approach

[00142] To have confidence in a biodosimetric test, it is necessary to develop insight into the biological mechanisms that gave rise to the test result. In addition, insights into the molecular responses driving metabolomic changes may promote better understanding of the biological consequences of radiation exposure, severity of injury, and routes for development of possible countermeasures. The use of a genetic approach with select mouse models focuses on signaling pathways contributing to radiosensitivity in key target organs, namely lymphoid/hematopoietic and gastrointestinal (GI). The current approach will also allow for more detailed in vivo dose response and timecourse studies in healthy wildtype mice, and will supplement more limited studies in human patients.

[00143] As already discussed, radiation injury triggers an inflammatory-like response which can affect immune cell function and viability, and can trigger even long-term effects in this regard, such as reported many years later in A-bomb survivors. Both p53-null and Gadd45a-null mice show perturbations of immune cell function and signaling. While Gadd45a can be regulated by p53, its role in immune cell function appears to be mediated primarily by the p38 pathway. This highlights the central role for p38 in inflammation and its importance as a therapeutic target. While p38 has a central role in many genotoxic and oncogenic stress signaling pathways, it is typically not rapidly responsive to ionizing radiation in many cell types other than lymphoid and myeloid lineages, where early studies indicated that ionizing radiation triggered transcriptional responses by stress MAP kinases including p38.

[00144] To directly measure p38 activation, immunoblotting was used to detect the activated form and have found rapid activation in spleen and thymus. A representative result is shown in the figure to the right for thymus from wt mice. Transient activation has also been seen in fibroblasts. Since the major isoform, p38 alpha, is required for viability, a knock-in mutant for this
gene has been engineered where activation is blocked by replacement of threonine 180 and tyrosine 182 with amino acids that cannot be phosphorylated (designated p38DN). This mutant of p38 is known to function as a dominant negative to block activation of wt p38 isoforms and thus will mimic the effect of anti-inflammatory therapeutics to this target. As shown in the last 3 lanes of the upper panel, our p38DN blocks activation of wt p38 expressed by the remaining wt allele. While activation of p38 (a key event in many inflammatory responses) is blocked, this model has shown a normal phenotype during more than a year of observation. In the human population, genetic variability, pre-existing conditions, and use of chemical modulators, such as anti-inflammatory agents, will need to be taken into account when developing reliable metabolomic markers for radiation exposures. The current approach with mouse models should provide a basis for deciphering signaling pathways that are affected by these variables.

Thymus

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embryo fibroblasts

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Upper panel: p38 activation (pp38) in mouse thymus after 3 Gy in wt and transgenic mice expressing a dominant negative p38α mutant. Lower panel: timecourse for p38 activation in wt mouse embryo fibroblasts.
In addition to metabolomic studies, strategic functional genomic studies can be performed. Initial studies have focused on responses to intermediate doses of radiation of 3 to 4 Gy in spleen. Microarray hybridizations were performed in both wild-type and selected mutant mouse lines.

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<td>71</td>
<td>IL-6 receptor and IL-1 receptor related proteins</td>
</tr>
<tr>
<td>Cell junction and cell membrane</td>
<td>27</td>
<td>gap junction proteins, annaxin A2</td>
</tr>
<tr>
<td>Secretory and plasma proteins</td>
<td>13</td>
<td>tissue plasminogen activator (TPA) inhibitors</td>
</tr>
<tr>
<td>Acute phase response related</td>
<td>18</td>
<td>TPA, cytochrome P450-related proteins</td>
</tr>
<tr>
<td>Tissue protease related</td>
<td>10</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>Eicosanoid biosynthesis</td>
<td>4</td>
<td>prostaglandin-related enzymes such as COX2</td>
</tr>
</tbody>
</table>

Representative radiation-responsive genes are shown for murine and human in vivo studies; this includes recent results with Agilent 22k mouse microarrays. The majority (>60%) of mouse genes showed attenuated responsiveness in all categories in p53-/- and p38+/ DN compared to wt. Results are pooled from spleen, thymus, and peripheral blood samples after doses of 1.5 to 5 Gy.

A full dose response can be determined from 0.5 to 13 Gy in wt mice at 4 and 24 h in spleen using full-genome 44k Agilent microarrays. The mutant lines studied were p53-null, p38+/DN, and Wipl-/- . The latter line is of particular interest in that Wipl inactivates p38 and its deletion results in heightened p38 signaling in vivo and increased sensitivity to DNA damaging agents. Emphasis has been placed on genes in the categories shown in the accompanying table (previous page) as those involved in injury responses that are most likely to impact on systemic metabolomic markers.

Responsiveness was substantially reduced for the majority of genes in all 7 categories in p53-null or p38+/DN mice (e.g., 73% of cytokine genes that were responsive in wild-type mice showed clear attenuation in p53-null mice, and all showed attenuation in p38+/DN). Results for cytokines and growth factors are shown graphically in the figure, illustrating the clear effect of deletion of p53. Genes showing heightened responsiveness in Wip-null mice are illustrated in the left panel, and are included for comparison in the other two panels. Our Wipl-null studies provide further support for the central role of p38 in radiation-induced cytokine signaling. Parallel raetabolomic studies are underway in wild-type mice, and will be extended to determine perturbations in splenic injury responses in p53-null and p38+/DN lines.

Example 20: Human Studies

Patient samples are being collected at the Pittsburgh University Medical Center, one of our sister CMCR sites, where protocols are in place to collect urine, blood, and sweat. In addition to organizational efforts prior to May of Year 1, urine has been collected from 3 patients (see Table below). Urine was collected prior to irradiation and 6 hr and 24 hr after the initial radiation.
dose. The samples were frozen and stored at -80°C before shipment to NCI for analysis on the UPLC-MS(TOF) machine.

<table>
<thead>
<tr>
<th>Patient identification</th>
<th>Irradiation Treatment†</th>
<th>Irradiation Dose</th>
<th>Urine Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCR-1</td>
<td>TBI</td>
<td>2 Gy</td>
<td>X</td>
</tr>
<tr>
<td>CMCR-2</td>
<td>TBI</td>
<td>1.5 Gy at 0 hr</td>
<td>X</td>
</tr>
<tr>
<td>CMCR-3</td>
<td>TLI</td>
<td>0.8 Gy at 0 hr</td>
<td>X</td>
</tr>
</tbody>
</table>

*TBI: total body irradiation, TLI: total lymphoid irradiation.

Example 21: Identification of Nonspecific (Non-Radiation) Stresses

[00149] An important issue in these studies will be to discern radiation-specific changes from confounding variables both experimentally and in the field. It was observed that sham animals often separated in the scores plots from cage controls, similar to the separation observed for irradiated animals. In other words, animals excreted "stress molecules" that were unrelated to radiation exposure, rather they appeared to be the result of housing the animals in metabolism cages. It was also observed that this phenomenon did not immediately resolve, but rather persisted over many days of urine collection. These observations caused us to modify our original protocol to allow longer conditioning of the mice to the metabolic cage environment prior to irradiation. One of the "stress molecules" produced in this situation has been identified as stachydrine (proline betaine). The pathway to identification of stachydrine illustrates the metabolomic methodology employed to identify biomarkers in this study. The stages are illustrated in the figure. Data from +ve ion total ion chromatograms (panel A) for groups of mice was subjected to multivariate data analysis as described. The loadings plot (data not shown) identified an ion of mass [M+I]+ = 144.101. Extraction of this ion from the total ion chromatogram is depicted in panel B, which shows a single peak. The ions that constitute this peak are shown in panel C, and this includes the ion 144.1022. Elemental composition analysis (panel D) revealed that this ion corresponds to C7H14NO2, with an error of 2.1 ppm. Deductive chemical reasoning then led to the putative chemical identity of this ion as stachydrine (panel E). This compound was purchased and subjected to LCMS/MS analysis, together with mouse urine. Panel F clearly shows that the substance in urine responsible for the ion of nominal mass 144 has the same retention time (0.77 min) and identical MS/MS fragmentation (102, 84, and 58 ions) as authentic stachydrine (0.78 min). It has therefore been established that "cage stress" results in the elevated excretion of stachydrine.
Whether or not stachydrine is produced de novo by the mouse or is in the diet remains to be established. It has been known for many years that stachydrine is a common amino acid metabolite in plants such as alfalfa. The figure demonstrates that the cage stress factor, as measured by urinary stachydrine excretion, is limited to the 6 days prior to irradiation.

It has therefore been possible, by careful planning and diligent observation, to limit the effects of cage stress, which could have seriously compromised our animal model for radiation and biomarker detection. In addition to cage conditioning prior to irradiation, it has been found that the use of flooring in standard animal facility cages that is equivalent to the flooring in our metabolic cages can facilitate conditioning.
Results with "cage stress" highlight the need to distinguish radiation injury responses from confounding variables and other stresses. As discussed in more detail earlier, evidence from RNA expression profiling indicates that various types of stress can be distinguished with an informatic approach. This study was carried out in a human lymphoid line after treatment with a variety of different types of DNA-damaging agents and non-genotoxic agents. Considering the relative high throughput of this cell line approach compared to in vivo studies, identical experiments were carried out and 204 aliquots of frozen cells and conditioned media sent for analysis on the UPLC-MS(ToF) machine at NCI. Also carried out were cytokine determinations in media from irradiated cells and elevated levels of proteins were found, such as 11-10 and MP-Ia. Studies with lipopolysaccharide (LPS), predator urine, and starvation (24 and 48 h) can also be made.

Example 22: Irradiation of Mice for Urine Metabolomics

Urine offers a variety of advantages including concentration of low-molecular weight metabolites, relatively low levels of protein and other high molecular weight species, and accessibility. An important priority is to demonstrate and refine the applicability of this biofluid for development of radiation metabolite markers. With "proof of principle" and applicability in one biofluid (urine), the rationale for development of radiation markers for other biofluids has been strengthened and should proceed efficiently. Based on our experience with expression profiling, robust ionizing radiation responses typically occur at higher physiologic doses and many show a dose response over a broad range. For this reason substantial focus has been on higher toxic doses of 6 to 8 Gy where clear changes could be seen compared to unirradiated mice. Current focus is over a broader dose range of 1 to 15 Gy. The LD50/30 for mice is typically 7 Gy. Hematopoietic/lymphoid failure and mortality typically occur at doses of 7 to 10 Gy, while GI failure with more rapid mortality occurs at higher doses. GI failure is pronounced at 15 Gy and can be modulated by various genetic and chemical approaches.

For these reasons responses in the 7 to 15 Gy range are examined to determine if marker signatures can be discerned for hematopoietic compared to GI toxicity. Another focus is on less toxic doses of 1 to 6 Gy. In extensive experiments done at 3 Gy significant responses at the metabolomic level have been observed. In the case of comparison to human IR exposures, a dose modification adjustment may be required since in vivo toxicity occurs at lower doses in humans (e.g., the LD50 for untreated human exposure is approximately 4.5 Gy).

One approach in mice employs metabolic cages for urine collections, metabolomic analysis by UPLC-MS(ToF), and additional chromatography methods, such as GC-MS
and LC-MS (triple quadrupole), for validation and quantization of select metabolites. Typical experiments use male C57BL/6 mice at 8 to 12 weeks of age. Both the Georgetown and NCI laboratories use 137Cs sources with procedures kept as similar as feasible. To minimize cage stress responses, mice are conditioned for several days in metabolic cages prior to irradiation, and are housed in the metabolic cages for 24 hour urine collection on alternate days. Urine samples are analyzed by UPLC-MS(TOF) after dilution in 50% acetonitrile in water and removal of any particulates. The molecular ions are resolved on a reversed-phase 50 x 2.1 ACQUITY 1.7 µm C18 column (Waters Corp, Milford, MA) using an ACQUITY UPLC system (Waters) with a gradient mobile phase comprised of 0.1% formic acid (solution A) and acetonitrile containing 0.1% formic acid (solution B). Each sample is resolved for 10 min at a flow rate of 0.5 mL-min-1. The gradient consists of 100% A for 0.5 min, 80% A/20% B for 3.5 min, 5% A/95% B for 4 min, 100% B for 1 min, and 100% A for 1 min. The column eluent is directly introduced into the mass spectrometer by electrospray.

[00156] Mass spectrometry is performed on a Q-TOF Premier (Waters) operating in negative-ion mode with a capillary voltage of 3000 and a sampling cone voltage of 65. The desolvation gas flow is set to 650 L-h-1 and temperature to 350 °C. The cone gas flow is set to 50 Lh-1, and the source temperature is 120 °C. Accurate mass is maintained by introduction by LockSpray interface of sulfadimethoxine (309.0658 [M-H]) at a concentration of 500 pg µL-1 in 50% acetonitrile and rate of 0.06 mlmin-1. Data are acquired in centroid mode from 50 to 800 m/z in MS scanning. Tandem MS collision energy is 5 to 35 V. Chemical formulae of urine metabolites of interest are derived from the accurate masses, and confirmed by authentic standards.

Example 23: Specificity of Radiation Injury Responses Compared to Other
Physiological Stresses

[00157] The specificity of stress metabolomic signatures must be tested. Radiation has well-documented inflammatory features and triggers many of the same cytokines seen in many inflammatory states. Stress responses triggered by infection, chronic inflammatory conditions or xenobiotics need to be considered. Furthermore, a radiologic or nuclear event would be expected to involve victims with mixed injuries as well as individuals with unrelated underlying pathologic processes, all of which could potentially complicate dosimetry based on biological responses.

Example 24: Cellular Models for Radiation Metabolomics

[00158] A cell-based radiation metabolomics approach with a focus on TK6, a spontaneously-immortalized human lymphoid line used in molecular toxicology. In contrast to animal models, a cell-based radiation metabolomics approach has much higher throughput and will allow us to assess metabolomic responses in a defined cell type. In addition, TK6 grows in suspension, making metabolite isolation easy and rapid, and has an engineered isogenic derivative, NH32, which lacks the tumor suppressor p53. The NH32 cell line maybe useful for future pathway dissection studies aimed at identifying the p53-dependent metabolic response after IR.

[00159] In one approach metabolomic analysis is performed on TK6 cells (and the culture media) that were exposed to 0.5, 1.0, or 4.0 Gy of ionizing radiation and then cultured for an additional 4 or 24 hours. Metabolites are analyzed by UPLCMS(ToF) as described earlier in either positive or negative ionization mode. As with the data collected from the mouse urines, the multivariate data analysis (MDA) program SIMCA-P+ is used to identify cellular metabolites that maybe indicative of stress associated with radiation exposure. A more sophisticated analysis procedure may be used, projection to latent structures (PLS) and orthogonal PLS (OPLS), that assigns all of the variation associated with radiation exposure to the first component, while the remaining components show unrelated variation. This analysis method proves to be an ideal approach for biomarker discovery. MDA using SIMCA-P+ remains an integral part of the metabolomics data mining for principal components analysis (PCA) and methods such as OPLS may be used as well as t-tests and z-score calculations for comparing normalized data. Adjusting protein concentration allows the normalization of samples across various timepoints and doses.
Fig. 3 Analysis by UPLC-MS(TOF) is well-suited for discriminating sham from irradiated TK6 cell-derived supernatants. A. A PCA scores plot of sham and 0.5, 1.0, and 4.0 Gy irradiated TK6 cells. B. A loadings S-plot demonstrating the metabolite differences between sham and 1.0 Gy irradiated TK6 cells. AMP and GSH are indicated as down-regulated metabolites in the 1.0 Gy irradiated samples.
For radiation metabolomics, scores plots of OPLS modeled four hour data show increased separation from sham-irradiated cells that was associated, most prominently, with the down-regulation of several metabolites.

As seen in the scores plot of Fig. 3A (above), clear separation exists between sham and irradiated cells (dotted line drawn arbitrarily between samples). This is a striking effect considered that unsupervised PCA of all metabolites showed a separation from unirradiated. Little variation exists between the sham-irradiated cells, while some weak separation between the groups of 0.5, 1, and 4 Gy-irradiated cells is visible (predominantly between the 1.0 and 4.0 Gy sample sets). In Fig. 3B, the corresponding S-plot (a combination of the loadings plot and the coefficients plot) of 1 Gy irradiated cells reveals several candidate metabolites, both up- and downregulated. The metabolites were further analyzed by quantifying peak areas in the raw UPLCMS(TOF) data for select metabolites such as glutathione (GSH). Some of the major metabolites significantly contributing (decreased in the irradiated samples) to group separation include GSH (308.091+) and AMP (348.0706+) as determined by empirical formula calculations and MSMS analysis of authentic compounds and cell samples. Reduced glutathione (GSH) is representative of the sham-irradiated cells as exposure to IR generates reactive oxygen species (ROS) that are rapidly consumed by scavengers such as GSH. Adenosine monophosphate (AMP) may be depleted in irradiated cells through the increase in unscheduled DNA repair mechanisms that are elicited following an IR event.

Other potentially informative metabolites include proline, oxoproline, spermine, and other nucleotide monophosphates. Concentrations of these and other metabolites are being determined through development of multiple reaction monitoring (MRM) methods using an ABI Triple-Quad mass spectrometer.

In order to distinguish the metabolic effects of IR from other stress agents, metabolic profiles of other toxicants are compared. In the case of heat shock, this stress could be clearly distinguished from both IR and untreated samples (not shown). Representative results are shown for the well known toxicants in Fig. 4 (above). While ions similar to those have been detected in irradiated cells, the overall contribution of specific ions to group separation was different. For example, when TK6 cells were treated with heavy metals such as cadmium, arsenic, or chromium, one of the major metabolites associated with chromium treatment was spermine (Fig. 4B), a polyamine often associated with cellular metabolism and nucleic acid stabilization.

Treatment with chromate is easily distinguishable from control and other stressors such as hydroxyurea (HU) and 4 Gy of IR (Fig. 4C). The clustering of HU near the IR samples suggests a similar metabolic profile and this may be through depletion of nucleotide pools as
is common following IR. In addition to these studies, the effects of other stressors such as the radiomimetic agent bleomycin, the Kreb's cycle inhibitor sodium fluoroacetate, as well as other ROS generators including hydrogen peroxide are also studied. The primary focus of these studies has been on the water soluble metabolites yet there remains an abundance of metabolites, such as lipids, that may be informative for defining the radiation metabolome. Specific extraction techniques are being developed to assess changes in the lipidome as a result of IR exposure. Our results to date indicate that, as is the case at the mRNA level, differences can be discerned at the metabolomic level for various types of stress agents.

**Example 25: Characterization of Radiation Stress-Signaling with Mouse Models**

Insight into the molecular responses driving metabolomic changes may promote better understanding of the biological consequences of radiation exposure, severity of injury, and prognosis of casualties. The use of a genetic approach with select mouse models in Aim 2 focuses on signaling pathways contributing to radiosensitivity in key target organs, namely lymphoid/hematopoietic and gastrointestinal. Key stress pathways, such as p53 and MAP kinase, are already known to regulate a variety genes involved in energy metabolism and other aspects of the metabolome. Currently, p53-/ mice are used to attenuate lymphoid/hematopoietic toxicity and are initiating studies with Asm-/ mice, which show attenuation of GI toxicity. Since radiation injury has inflammatory features, metabolomic responses may be affected by agents affecting inflammatory signaling. For example, nicotine can have immunosuppressive and anti-inflammatory effects, and the effect of this agent on IR responses will be determined. P38 MAP kinase is a major mediator of

![Image](https://via.placeholder.com/150)

**Fig. 4 Stress-specific metabolomic signatures.** A. A PLS-DA scores plot demonstrating the group separation between control and heavy metal (arsenic, cadmium, and chromate) treatments. B. The corresponding loadings plot (from plot A) depicting AMP and spermine association with the arsenic and chromate treated cells, respectively. C. A PLS-DA scores plot demonstrating the ability to distinguish various stressors including ionizing radiation, hydroxyurea, and chromate. D. The corresponding loadings plot (from plot C) demonstrating the specific association of various metabolites (spermine, AMP, and GSH are indicated) with different stresses.
inflammatory responses and an engineered mouse model has been developed where the wild type allele for the major isoform, p38α, was replaced (knocked in) with a dominant-negative mutant. Like p38α/− mice, the homozygote mutant is not viable, while the heterozygote, designated p38+/DN is phenotypically normal. However, p38-mediated stress responses are substantially dampened, so this model can be used as a general model for immune suppression or antiinflammatory therapeutics. It has been found that this is indeed the case after IR and a variety of transcripts involved in mediating inflammatory responses showed reduced responsiveness in p38+/DN mice. Since p53 plays a major role in IR transcriptional responses in vivo, these responses were compared to the p38+/DN line in Fig. 5 (above). The striking finding is that the majority of p53-inducible genes and a lesser proportion of p53-repressed genes show reduced responsiveness in p38+/DN mice, where the response was frequently intermediate between wt and p53−/−. An important caveat of this study is that anti-inflammatory agents may impact on IR markers at both the mRNA level and downstream at the metabolomic level.

![Fig. 5 Attenuation of many stress response genes in an anti-inflammatory mouse model. Log(Ratio) plot of microarray data in response in mouse spleen 4 h after 3 Gy. Genes were selected based on induction (A) or repression (B) in wt compared to p53−/− spleen with at least 1.5-fold response in wt and a reduced response in p53−/−. Individual blue symbols represent results of individual genes for wt which are rank ordered with greatest induction (A) or repression (B) on the left. The responses of the same genes are shown for p53−/− (yellow) or p38+/DN (purple). The ordinate axis represents the log of fold-change relative to unirradiated.](image-url)
Example 26: Nano-Electrospray-Coupled microDMx

[00166] (ESI-DMS): Integration of a nanospray ion source directly with the microDMx sensor allows DMS to be used for very fast screening of low volatility species in fluids and digests. This system provides fast analysis (seconds) and is simple to use. If it meets the performance criteria, this system is one of the most desirable designs for the final system, allowing rapid pre-screening of a large number of samples.

[00167] Sionex 63Ni ionization DMS-MS has been tested with a variety of chemical species and different DMS-MS interface designs. The example in Fig. 6 (above) shows the DMS signal as a function of DMS operating parameters (compensation and separation voltages) in a topographic plot, and the ability of DMS to perform mass spec CID analysis, and to separate a single fragment ion (C7H7+).

[00168] Chemical noise has been reduced by factors of 50 to 100 or more for a range of analytes. Typical results are shown in Fig. 7 for caffeine in a mixture of PEG (polyethylene glycol) and Na-PEG species, where ions of interfering species were completely suppressed.
The most recent advance, the Sionex microAnalyzer (miniature GC-DMS), combines a rapid micro-scale GC with microDMx technology. The microAnalyzer subsystem offers the sensitivity and selectivity associated with microDMx based products with the added benefits associated with pre-concentration and separation in a complete self-contained sub-system requiring no external gases. In many applications, the sub-system will be capable of detecting and identifying chemicals down to parts per trillion (ppt) levels in complex matrices. The complete system is enclosed in a 9x5x4 inch package, and is capable of analyzing complex mixtures, as illustrated in Fig. 8 for a BETX mixture, which contains five components in ppb level.
The compact Nano-electrospray-coupled microDMx (ESI-DMS) system includes an electrospray ion source for ionization of liquid samples combined with direct detection of DMS spectra. DMS sensitivity and selectivity provides detection and identification of radiation-exposure biomarkers (Fig. 9, above). Performance of this system is enhanced by the use of drift gas modifiers tailored to the analyte. Mass spectrometric investigation of the 5 preliminary radiation-exposure-biomarkers may be performed, and DMS-MS system analytical performance for these components may be evaluated.

Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.
WHAT IS CLAIMED IS:

1. A method for determining the radiation exposure of a subject, comprising:
   a) obtaining a sample from the subject;
   b) determining the metabolomic signature of the sample;
   c) comparing the metabolomic signature of the sample to at least one known radiation metabolomics signature; and
   d) quantifying the radiation exposure of the sample.

2. The method of claim 1, wherein the sample is comprised of blood.

3. The method of claim 1, wherein the sample is comprised of sweat.

4. The method of claim 1, wherein the sample is comprised of urine.

5. The method of claim 1, wherein the sample is comprised of sebum.

6. The method of claim 1, wherein the sample is comprised of blood plasma,

7. The method of claim 1, wherein the sample is comprised of saliva.

8. The method of claim 1, wherein the sample is comprised of cells.

9. The method of claim 1, wherein the sample is obtained non-invasively.

10. The method of claim 1, wherein the throughput of the system is greater than 50 samples per hour.

11. The method of claim 1, wherein the throughput of the system is greater than 1000 samples per hour.

12. The method of claim 1, wherein the throughput of the system is greater than 10,000 samples per hour.

13. The method of claim 1, wherein the throughput of the system is greater than 100,000 samples per hour.
14. The method of any of claims 1 through 13, wherein the subject is mammalian.

15. The method of any of claims 1 through 13, wherein the subject is a mouse.

16. The method of any of claims 1 through 13, wherein the subject is human.

17. The method of claim 1, wherein determining the metabolomic signature of the sample is comprised of chromatographic analysis.

18. The method of claim 1, wherein determining the metabolomic signature of the sample is comprised of mass spectrometry analysis.

19. The method of claim 1, wherein determining the metabolomic signature of the sample is comprised of radio-frequency differential ion mobility spectrometry analysis.

20. The method of claim 1, wherein determining the metabolomic signature of the sample is comprised of chromatography and mass spectrometry analysis.

21. A high throughput system for determining the radiation exposure of a sample, comprising:
   a) a device for determining the metabolomic signature of the sample; and
   b) a software package for comparing the metabolomic signature of the sample to at least one known radiation metabolomics signature.

22. The system of claim 21, wherein the sample is comprised of blood.

23. The system of claim 21, wherein the sample is comprised of sweat.

24. The system of claim 21, wherein the sample is comprised of urine.

25. The system of claim 21, wherein the sample is comprised of sebum.

26. The system of claim 21, wherein the sample is comprised of blood plasma.

27. The system of claim 21, wherein the sample is comprised of saliva.
28. The system of claim 21, wherein the sample is comprised of cells.

29. The system of claim 21, wherein the sample is obtained non-invasively.

30. The system of claim 21, wherein the throughput of the system is greater than 50 samples per hour.

31. The system of claim 21, wherein the throughput of the system is greater than 1000 samples per hour.

32. The system of claim 21, wherein the throughput of the system is greater than 10,000 samples per hour.

33. The system of claim 21, wherein the throughput of the system is greater than 100,000 samples per hour.

34. The system of any of claims 21 through 33, wherein the subject is mammalian.

35. The system of any of claims 21 through 33, wherein the subject is a mouse.

36. The system of any of claims 21 through 33, wherein the subject is human.

37. The system of claim 21, wherein the device for determining the metabolomic signature of the sample is comprised of chromatographic analysis.

38. The system of claim 21, wherein the device for determining the metabolomic signature of the sample is comprised of mass spectrometry analysis.

39. The system of claim 21, wherein the device for determining the metabolomic signature of the sample is comprised of radio-frequency differential ion mobility spectrometry analysis.

40. The system of claim 21, wherein the device for determining the metabolomic signature of the sample is comprised of chromatography and mass spectrometry analysis.