Chapter XXIX: Biodosimetry

High Throughput Biodosimetry Methods

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Introduction

*Why **Bio**dosimetry*

Following a large scale radiological event, it will become important to screen tens or hundreds of thousands of individuals for radiation exposure, both to identify those that would benefit from medical treatment and also to alleviate the concerns of the "worried well". With almost any type of accidental exposure, physical dosimetry will be incomplete or more likely absent, thus requiring *a posteriori* dosimetry. Biodosimetry provides a unique tool in that it allows using the exposed individual's tissues or biofluids to serve as a dosimeter providing an *individualized* dose estimate.

Advantages over physical dosimetry

The main advantage of biodosimetry is that it is **retroactive**, there is no need for the individual to be exposed to carry any physical dosimeter on them – the individual's body serves as the dosimeter. Furthermore a biological assay folds into itself the radiosensitivity (or radioresistance) of the individual, thus rather than reporting a physical dose, a biodosimeter can be tuned to identify individuals requiring treatment, which is, obviously much more relevant.

Why High throughput

The need for *high throughput* biodosimetry is well illustrated by the 1987 radiation incident in Goiânia, Brazil [2], a city with about the same population as Manhattan. In the first few days after the incident became known, about 130,000 people (~10% of the population) came for screening, of whom 20 required treatment. Mass radiological triage will thus 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 be equally crucial in what will be a highly resource-limited scenario, as well as potentially to reduce the number of individuals unnecessarily fleeing a small event.

Necessity of automation

Using automated assays has two distinct advantages over a manually applied assay:

Throughput – Through automation, even complex assays can be easily parallelized, increasing throughput from tens to thousands of samples per day, easily achieving the large sample throughputs required for population triage in a way that cannot be done when relying on the skilled labor of a clinical lab.

Precision – When performing any assay manually, small variations (e.g. in culture conditions and scoring criteria) between experimenters and between labs can result in large variations between the biomarker values reported for duplicate samples. By automating the entire procedure most of the variability introduced by human handling is eliminated. Thus, it is much easier to guarantee that multiple replicates processed in parallel or at different sites will yield the same results.

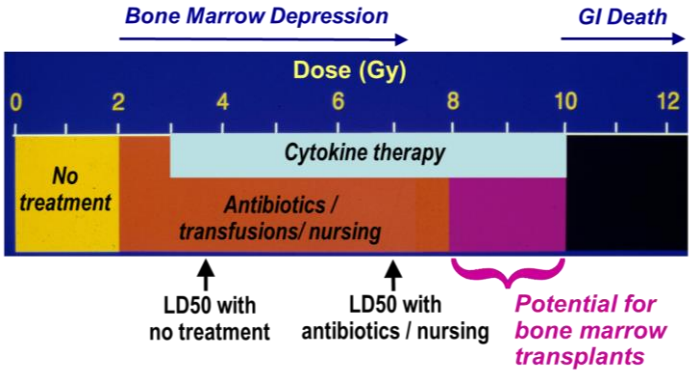

Figure 1: Relevant dose ranges for biodosimetry, reproduced from [1]

Relevant doses

In terms of the need for medical intervention, the best estimate for the LD50 at 60 days in humans is in the 3 to 4.5 Gy range [3], but this value can be roughly doubled by the use of antibiotics, platelet and cytokine treatment [3], so it is crucial that individuals who actually received whole-body doses above, say, 2 Gy are identified. It would be undesirable to give these treatments to "all comers" irrespective of radiation exposure, not least because there is some evidence of long-term toxicity with cytokine treatments [4, 5]. Some individuals exposed in the 2 to 5 Gy dose range will be identifiable through early nausea, vomiting, and acute fatigue, but by no means all. 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 [6, 7], was initially almost entirely asymptomatic and yet developed acute bone marrow failure [8]. Thus accurate biodosimetry is crucial in this dose range.

At higher doses, there is only a quite narrow dose window (currently approximately 7-10 Gy [1]) in which bone-marrow transplantation is a useful option [<7 Gy, survival rates are good solely with medication, >10 Gy patients will generally have lethal gastrointestinal (GI) damage]. Thus it is critical to ascertain whether a patient's dose is within this dose window, such that a bone-marrow transplant would be a useful option.

Cytogenetic Biodosimetry

Established cytogenetic assays of human lymphocytes are recommended by the International Atomic Energy Agency (IAEA)[9] for response to radiation emergencies. These assays probe DNA damage and its processing via a variety of endpoints, as listed below, with many novel assays under development.

The Dicentric Assay

The Dicentric Chromosome Assay (DCA) is currently the gold standard for clinical radiation biodosimetry:

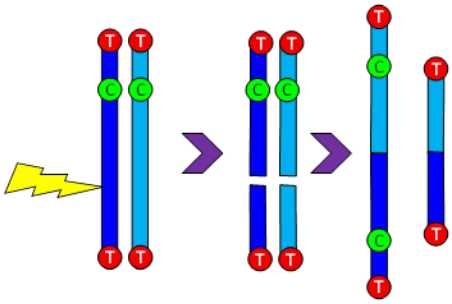

Figure 2: Schematic representation of the formation of a dicentric chromosome. Centromeres are marked with a "C", Telomeres are marked with a "T". Our scoring distinguishes between TCT, for a normal chromosome, and TCCT for a dicentric.

Dicentric chromosome formation is the result of induced breaks in two chromosomes which, after mis-rejoining, result in a single chromosome entity with two intact centromeres – the dicentric. This is illustrated in Fig. 2. Because ionizing radiation is a very efficient inducer of DNA double-strand breaks (DSBs) and dicentric formation generally requires induction of DSBs in two chromosomes, dicentrics are a highly specific marker for radiation exposure, particularly in the context of the radiation doses (≥1 Gy) relevant to medical management. The very low baseline frequency, of approximately 1-2 dicentric chromosomes per 1,000 metaphase cells, is largely independent of age and sex and not strongly influenced by life style, including smoking. For comparison, a radiation dose of 1 Gy typically results in roughly 50 to 70 dicentrics per 1,000 metaphase cells.

The DCA has been successfully employed for radiation biodosimetry in many radiation accidents where the number of people assessed is comparatively small. However, as implemented in a standard cytogenetic laboratory, the DCA is too labor intensive to be widely applied when the need is to assess large numbers (thousands or more) of individuals. Various strategies have been developed in this context such as establishing networks of biodosimetry labs, web based scoring, and new scoring strategies. At present, however, even large cytogenetic laboratory networks based on manual DCA assays can only analyze some hundreds of samples per day. Various approaches have also been reported in terms of automating the DCA assay. These automation approaches are promising, though high throughput (e.g. >1,000 samples per day) has not yet been achieved.

The DCA protocol in macroculture was standardized by IAEA and details are given in Annex I of [9]. However this assay is not automation friendly. Both in that it requires large cultures and in that it requires manual scoring.

A more automation friendly protocol [10] is given below:

- 20 µl of whole blood is cultured in 1 ml/well multiwell plates with 180 µl of complete medium PB-MAX (Life Technologies) in a humidified atmosphere at 37°C with 5% CO_2 for 47 hours.
- Colcemid (0.1 µg/ml final concentration) is added and the samples incubated for an additional 3 h.
- A hypotonic shock is performed at 37°C during 10 min (0.075 M KCl).
- The samples are washed in fixative (methanol: acetic acid; 3:1) three times.
- 100 µl of fixed cell suspension is transferred into 96-well glass-bottom plates with 400 µl of 50% acetic acid and centrifuged for 3 min at 400 g.
- The liquid is aspirated from the wells and the plate left at room temperature for 10 min.
- Samples are denatured for 1 min in 200 µl of 0.07 N NaOH/70% methanol and washed with 200 µl of methanol.
- Hybridization is performed with 2 nM centromere PNA probe (Panagene, Thousand Oaks, Ca, USA) in 0.001 N NaOH/2XSSC (pH 7.0-7.5) at 55°C for 10 min.
- Samples are washed with 200 µl of 0.001 N NaOH/2XSSC and counterstained with DAPI (Life Technologies).

The Cytokinesis Blocked Micronucleus assay

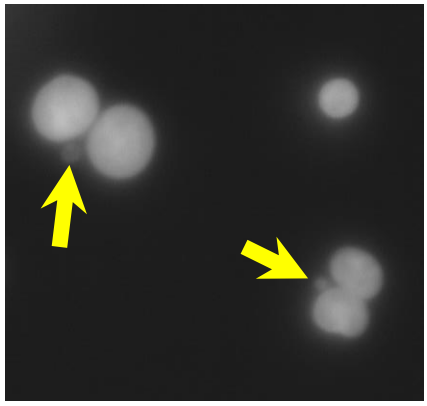

Figure 3: Example of binucleated cells with micronuclei, marked by arrows.

The Cytokinesis Blocked Micornucleus assay (CBMN) quantifies radiation-induced chromosome damage expressed as post-mitotic micronuclei, in once-divided cells. Micronuclei are small, generally round objects in the cytoplasm of the cells outside of the main nucleus. They represent chromosome fragments or whole chromosomes that are not incorporated into the daughter cell after nuclear division. By adding a cytokinesis block it is easy to identify the subset of lymphocytes that have divided exactly once, thus eliminating confounding parameters associated with nondividing cells or cells that have divided more than once. The CBMN test is easy and reproducible and has become one of the standard tests for genotoxicity assessment. Its use in biomonitoring studies has greatly increased in the last 15 years, and recent international efforts such as the HUMN (human micronucleus) project (http://www.humn.org) have greatly contributed to improving the reliability of this assay, providing technical guidelines and analyzing major sources of variability.

As with the DCA, IAEA provides a standardized protocol for performing this assay in macro-culture, see Annex IV of [9].

An automation-friendly micro-culture protocol is given in [11].

The γ-H2AX assay

The □-H2AX assay is a direct measure of DNA double strand breaks (DSB), and it has a highly linear relationship with dose. It quantifies, through immunostaining, the phosphorylated H2AX histone, which localizes to DSBs. The main advantage of the γ-H2AX assay over other assays is that it does not require culturing the cells, and thus can provide a same day answer rather than requiring a 2-3 day wait. However the persistence of the γ-H2AX signal is directly related to DNA repair times and thus this assay is only useful if blood is obtained within 24-36h following irradiations. An exception to this is in chronic irradiation situations [12].

When manually implemented, the yield of phosphorylated H2AX is quantified by counting foci at high magnification. Several automation systems based on counting foci have been described in the literature but they require acquisition of Z-stacks and high resolution imaging. Alternatively, equivalent results can be obtained at the relevant dose range, by using quantitative fluorescence (either using microscopy or flow cytometry) to evaluate the total fluorescence per nucleus (Fig. 4).

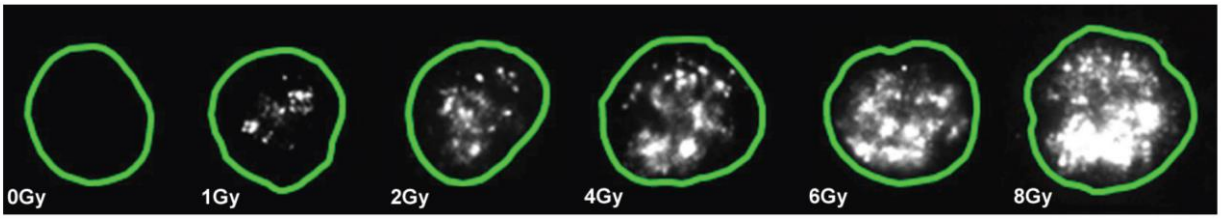

Figure 4: While γ-H2AX foci can be counted at doses up to about 2 Gy, at higher doses the foci merge and can no longer be counted. At these higher doses, a quantitative fluorescence method works better.

Other chromosome assays

The mBand assay is a well-established technique for scoring intra chromosomal rearrangements [13]. It consists of "painting" a single chromosome using region-specific partial chromosome paints. This results in a banded image of a single chromosome, where each band is defined by a combination of 1, 2 or 3 fluorofores. Within Chromosome 5 for example, 11 bands are typically seen (Fig. 5). By analyzing the sequence of chromosome bands, intra-chromosome aberrations, which are characteristic of high LET radiations (e.g. neutrons) can be detected.

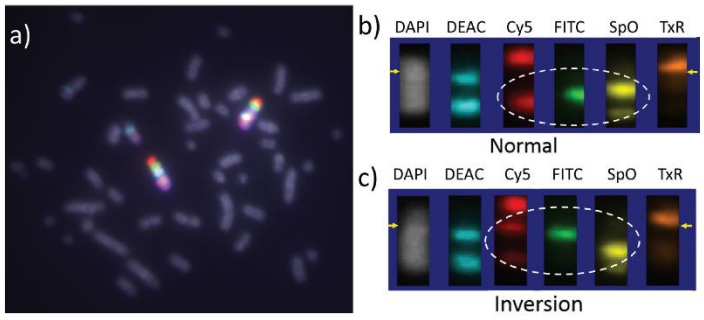

Figure 5: image of chromosome banding, following high linear energy transfer (LET) irradiation, the order of the bands within a chromosome change, due to intra-chromosomal rearrangements.

This is a highly labor intensive assay (both sample handling and image analysis) utilizing expensive reagents and therefore it is not really appropriate for automation.

Manual vs. automatic processing

In stark contrast to the tens of thousands of samples per day that would be required to be analyzed even following a small radiological event, a typical cytogenetic laboratory has a throughput of less than a hundred of samples per day. Even large national and international networks of labs are only capable of scoring a few hundreds of samples per day. The benefits from the introduction of high-throughput formats for cytogenetic assays are obvious: manipulating multiple samples simultaneously raises the throughput of the sample preparation as well as decreases the cost of the reagents required for analysis.

While several labs have developed custom robotic systems for automating cytogenetic preparations, the rapid increase in commercial High Throughput/High Content Screening (HTS/HCS) platforms situated both in academic and industry settings suggests that it would be possible to drastically increase biodosimetric capacity by utilizing these, already existing, systems.

Gene expression for biodosimetry

Newer radiation biodosimetry techniques are also being developed, based on the 'omics technologies of transcriptomics, proteomics, and metabolomics. RNA expression-signature based assays are the most developed of these approaches.

Much of the cellular-level response to ionizing radiation and other stresses is mediated through changes in gene expression. Broad transcriptional reprogramming can reflect changes in cellular activity after radiation exposure, with a reduction in specialized functions in favor of cell- or tissue-protective functions, such as cell cycle arrest, apoptosis, DNA repair, and damage signaling pathways. Radiation exposure does not simply switch cells from one transcriptional state to another, however, and complex temporal expression patterns result from successive waves of signaling. In practical terms, this means that the time after exposure must be considered in assay development.

Development of gene expression approaches to biodosimetry has focused mainly on whole blood or lymphocytes, as these are among the most transcriptionally responsive cells following radiation exposure, as well as being minimally invasive to biopsy. Such efforts have generally used whole blood or total white cells in order to minimize the processing needed for a high throughput or point-of-care (POC) assay. Research is also ongoing to support the development of measurement platforms appropriate to the needs of mass radiological triage, since the discovery platforms being used for signature development are expensive, time-consuming, and require a high level of technical expertise.

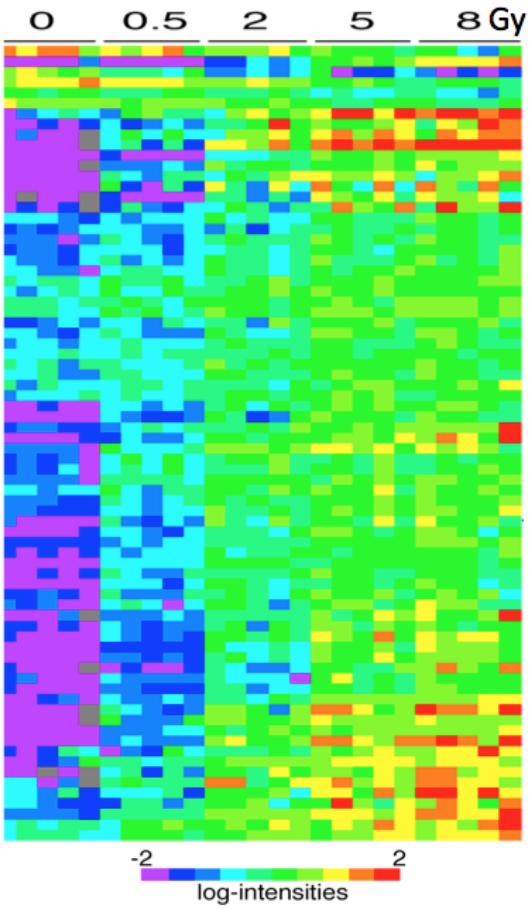

Figure 6: Heat map showing relative expression of 74 genes 24h after exposure of human peripheral blood to different doses of x-rays. Cooler colors (purple, blue) indicate lower levels of expression, while hot colors (yellow, red) indicate high levels of expression. Adapted from [15].

Signature Development

Most transcriptomic biodosimetry studies have focused on development of signatures for acute, whole-body radiation exposure within the first week after irradiation. Multiple models have been used in the development of gene expression biodosimetry. These include *ex vivo* exposure of human peripheral blood from healthy donors [14-20] (Fig. 6), animal models (mostly mice [21-29], but also rats [30] and non-human primates [31]), and human patients undergoing total body irradiation

(TBI) [21, 22, 24, 32, 33]. No individual model is sufficient for biodosimetry development. Gene expression responses to radiation are known to differ between rodents and humans, and between *in vivo* and *ex vivo* exposures. TBI patients generally have cancer, and receive multiple treatments in addition to the radiation under study. The doses and times post-exposure available for study are also limited by the treatment regimen. Nonetheless, many responses are broadly conserved between the models. Signatures that incorporate response information from multiple models are generally held to be the most robust.

MicroRNAs, the small regulatory transcripts that can downregulate large sets of genes, are also radiation responsive and have been suggested as radiation biodosimeters using either whole blood [27, 34, 35] or serum [36, 37]. Because of their small size and the protection afforded by bound proteins, miRNA are generally much more stable than mRNAs, an attractive feature for biodosimetry. They can also be isolated from urine or saliva, presenting the possibility of a completely non-invasive assay.

Nuances of Exposure

While most biodosimetry studies have focused on total body acute gamma- or x-ray exposures, characteristics of an exposure in addition to dose can modify the health impact. 'Omic approaches in general hold great promise to distinguish such modifying details of an individual exposure. Exposure factors that have been explored in the context of gene expression biodosimetry include partial body exposure, dose rate, and radiation quality.

Studies in mice have indicated that partial body exposures produce signatures distinct from those of TBI, and that TBI signatures do not predict the irradiation status of partial body exposed mice [23]. Studies in non-human primates found that gene expression signatures correlated better with the extent of radiological injury than with the administered dose [31]. Together these findings support the further development of gene expression signatures to distinguish non-uniform exposures.

Reduced dose rate studies indicate that while some genes respond similarly to acute and protracted exposures, for many genes dose protraction reduces the response in both mouse [29] and human [38] models. When exposure dose rate changes over time, such as from fallout resulting in internally deposited radionuclides, the patterns of gene expression in relation to dose can be complex [26, 27]. Current findings support the feasibility of developing gene expression signatures that can distinguish protracted from acute exposures.

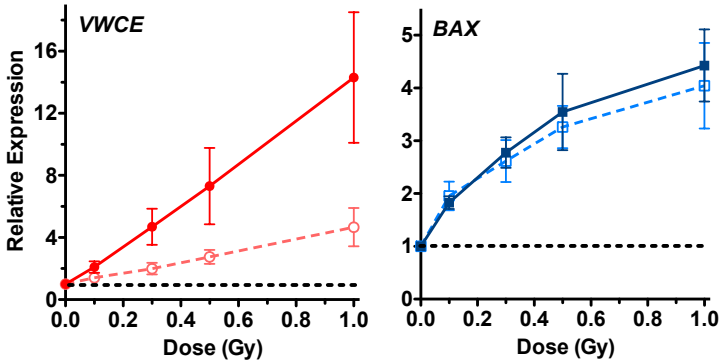

Figure 7: Example of genes with different relative dose-response relationships to neutrons (solid symbols and lines) and photons (open symbols, dashed lines). VWCE is more responsive to neutrons per unit dose, while BAX shows the same response to both radiation qualities. The black dashed lines indicate the level of expression in un-irradiated controls.

Depending on the material used to construct a dirty bomb, alpha,

beta, or gamma exposures could result. In the case of an improvised nuclear device (IND), a significant neutron component to the prompt radiation is likely, and isotopes in the fallout plume can also contribute alpha-, beta-, or gamma-radiation. High LET irradiation, such as neutrons (Fig. 7), causes more severe biological damage and has a greater health impact per unit dose, making this another exposure characteristic important to understand for triage and treatment purposes. The impact on radiation biodosimetry of alpha emitters ^{211}At [39, 40] and ^{241}Am [41, 42] and the beta-emitter ^{90}Sr [27] and IND-spectrum neutrons [43] have been investigated. All show some broad similarities with the response to gamma radiation, but unique characteristics of the responses suggest that gene expression signatures may be able to provide radiation quality information in addition to dose.

Dose Prediction Algorithms

An array of algorithms have been applied for radiation dose prediction from gene expression signatures, most involving some form of regression analysis followed by either leave-one-out or n-fold cross validation [16, 21-25, 31]. Signatures developed using random forests [44], forward variable selection [18], support vector machines, 3 nearest neighbors [16, 20, 29] and nearest centroid [15, 16] have also been reported with sample classification accuracies from 70-100% depending on the dose range and other variables included in the analysis. Extensive confounder studies have not yet been undertaken, and may be best addressed once there is some consensus on signatures. Gene expression signatures for radiation biodosimetry have been reported that are robust against sex and smoking [16], administration of countermeasures such as Granulocyte colony stimulating Factor (G-CSF) [25, 45], and inflammatory processes, as modeled by LPS [25, 45].

No consensus has been reached in the field in terms of either algorithm or signature to be used, and although a clear best approach has yet to emerge, the fact that multiple algorithms select many of the same biodosimetric genes and produce similar results supports the further development of gene expression approaches for radiation biodosimetry. Standardization of approaches may not begin in earnest until specific approaches gain or near regulatory approval.

Development of Assay Platforms

Development of gene expression measurement platforms for radiation biodosimetry is also ongoing. These efforts have mostly been addressed to the development of self-contained microfluidic approaches (Fig. 8) for point-of-care (POC) implementation and initial triage assessment of exposure above or below 2 Gy. Microfluidic assays provide a number of features potentially useful for large-scale triage. The requirement for very small amounts of blood as input means these assays can be developed for fingerstick volumes, rather than requiring phlebotomy, which itself presents a critical bottleneck for all blood-based assays. Integration of an automated finger lancet would further enhance throughput, as the test could essentially be self-administered. Small reaction volumes mean less reagent costs, and faster reaction times, with some assays aiming at results in less than an hour.

While it may be possible to adapt microfluidic platforms for medical management needs, to assess actual dose within ±0.5 Gy, the field has focused mostly on the idea of a networked approach that would make use of facilities already running clinical quantitative real-time PCR (qRT-PCR) diagnostics. In this scenario, stabilized blood samples would be shipped to existing facilities, which would switch their focus to biodosimetric analysis during a radiological emergency. Sample shipping would add to the turnaround time, but with a large network, high throughput should be feasible. Protocols exist for high-throughput robotic RNA purification and qRT-PCR, and can be optimized for radiological medical management. National or international networks have not yet been established, however, and will require standardization and testing.

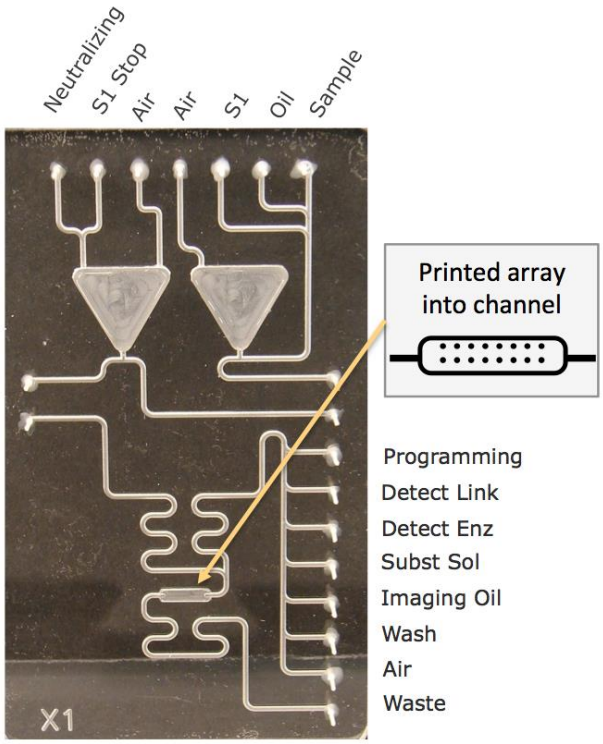

Figure 8: Prototype microfluidic cartridge for self-contained gene expression assay showing reaction ports and integrated detection array.

Metabolomic signatures for biodosimetry

What is Metabolomics

Metabolomics is the characterization and quantification of metabolites (<1 kDa), the chemical products of metabolic pathways, which are present in cells and tissues and can be deposited in biofluids. The composition and concentration of these metabolites vary in the body in response to injury, stress, and environmental stimuli. Early metabolomic analyses utilized nuclear magnetic resonance (NMR) approaches that provided structural information on metabolites and limited destruction of material, lacking however the appropriate sensitivity for a thorough investigation of responses to injury. Other technologies such as liquid chromatography (LC) coupled to mass spectrometry (MS) and/or gas chromatography (GC) MS provided solutions to biomarker identification due to high sensitivity and selectivity.

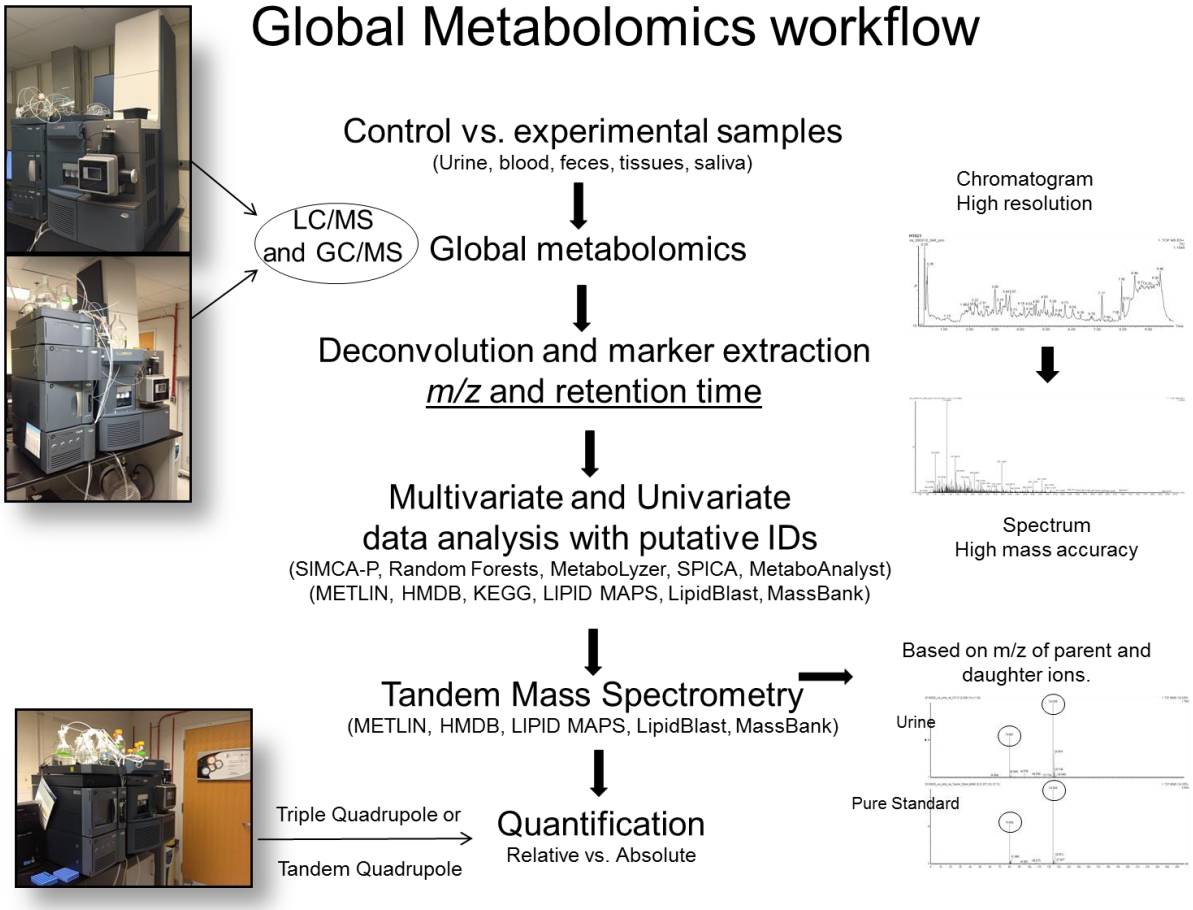

Figure 9: Global metabolomic analyses involves the deconvolution of complex chromatographic data followed by statistical approaches and quantification of candidate markers.

The use of global profiling technologies (Fig. 9) has contributed substantially to the understanding of the radiation cellular stress response and has contributed to the elucidation of many of the complex biological networks associated with gene expression and signal

transduction. On a similar level, global understanding of how ionizing radiation exposure affects small molecule concentrations (such as metabolites) would be expected to lead to the identification of metabolites that can be used to monitor for exposure and extent of injury. Metabolomics is a rapidly advancing field that aims to identify and quantify the concentration changes of all metabolites (i.e., the metabolome) in a given biofluid or model system. In addition, lipidomics, the full assessment of changes in lipids, can be considered a component of metabolomics, however with the variability and number of possible lipids some consider it an -omics field itself. Finally, targeted metabolomics (Fig. 10) can be utilized to quantify a select number of potential biomarkers, either as single biomarkers or in the form of a panel.

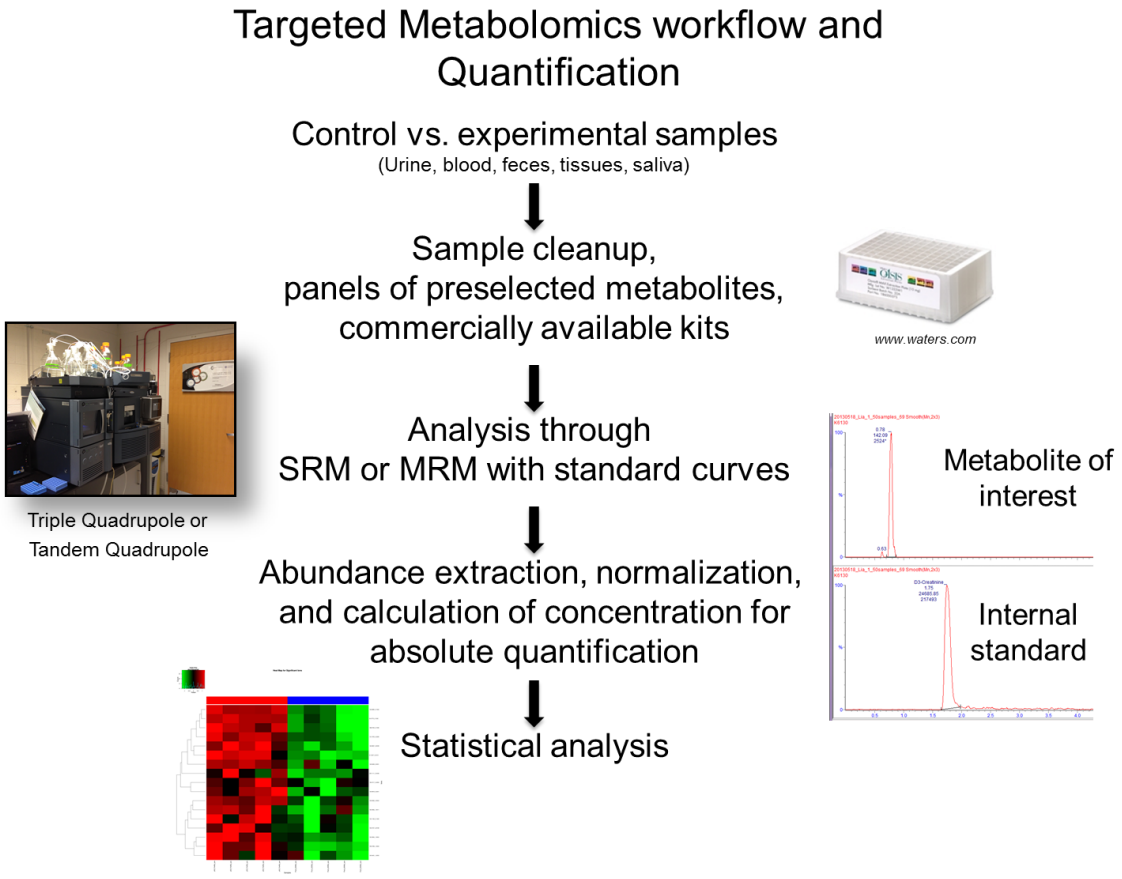

Figure 10: Targeted approaches are more focuses on a select subset of markers with concurrent quantification in biological samples.

Radiation metabolomics

For the purposes of biodosimetry, easily accessible biofluids (urine, blood, saliva, feces) have been the primary focus with minimally invasive methods of acquisition in the field. To date, the primary research has been conducted on urine and blood (serum, plasma), with saliva and fecal material showing great promise in this evolving field. Potential biomarkers have been identified in mice, rats, minipigs, non-human primates, and humans, with cross species validation of markers, allowing for the confidence in the use of smaller animal models.

Investigation of sex differences delivered different levels of potential biomarkers that should be taken under consideration during the construction of a panel of metabolites.

Several radiation exposure scenarios have been investigated that have contributed to specific radiation metabolomic signatures. Dose, dose rate, whole vs. partial body exposure, internal vs. external contamination and exposure, radiation quality, and genotypic contribution are some of the conditions that have been dissected to date. Metabolic pathways that have shown the most significant perturbations include fatty acid β-oxidation, amino acids, omega-3 and-6 pathways, glycolysis/ gluconeogenesis, oxidative phosphorylation, nicotinate and nicotinamide metabolism, purine and pyrimidine metabolism, tricarboxylic acid (TCA) cycle, riboflavin, and taurine and hypotaurine metabolism.

Of great importance is determining the specificity of the radiation signature. Efforts have been initiated with regards to endotoxin infections, trauma, and sepsis that are some of the confounding conditions that will be encountered in a real life situation. Future directions should also focus on combined injuries and further elucidation of tissue specific circulating biomarkers. To date, the only identified reliable biomarker associated with gastrointestinal injury is citrulline.

The increased potential value of radiation metabolomics has been highlighted in the scientific literature, e.g. [46-48], as a new approach to reliably and rapidly identify radiation exposed victims. The ability to accurately quantify the markers also allows for the potential utilization of this method for biodosimetry, with downstream categorization of victims into those that require immediate medical intervention and those who do not. Therefore, generation of a panel of metabolites that can accurately predict the dose exposure is the focus of radiation biodosimetry. This panel will include markers with high specificity as to differentiate those with immediate needs for medical intervention.

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