# Future platforms for toxicity testing

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**Abstract:** The toxicology community is in the midst of a paradigm shift, moving from high-dose testing in animals with extrapolation to expected responses in humans to an *in vitro* approach based on a mechanistic understanding of key toxicity pathways. Tools such as cell-based assays that interrogate specific toxicity pathways, multi-omics technologies, bioinformatics, and computational systems modelling enable deeper understanding of human biology at the molecular level. Integration of these tools provides a powerful new approach to toxicity testing. The tools and technologies used in toxicity testing platforms are continuously emerging, evolving, and being applied in novel ways. The goal of this article is to: 1) describe recently emerged technologies; 2) highlight advances in various existing platforms; 3) provide relevant examples of how these platform components are currently being applied in toxicity testing; 4) discuss advantages and limitations of each platform and identify gaps where further developments are required.

**Keywords:** multi-omics; metabolomics; proteomics; *in vitro* toxicity testing; systems toxicology.

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## 1 Introduction: scientific toolbox for 21st century toxicity testing

Toxicity testing of industrial chemicals, pesticides, pharmaceuticals, consumer and personal care products, environmental substances and foods typically involves exposing animals to high doses of toxicants, observing adverse effects and extrapolating to expected human responses at lower doses (Krewski et al., 2010). These approaches are expensive (Bottini and Hartung, 2009), time-consuming (Hartung, 2009), use large numbers of animals (Taylor et al., 2008) and provide results that are only marginally relevant to human toxicology (Greaves et al., 2004). The low-throughput of current toxicity testing approaches has led to a backlog of more than 80,000 chemicals to which humans are potentially exposed, with largely unknown toxicity profiles (Kavlock and Dix, 2010). The pharmaceutical industry spends billions each year on research and development of new medicines. Approximately 30% of drugs tested in clinical trials fail owing to unanticipated and unacceptable toxicology profiles, and toxicity often cannot be fully assessed until late in the developmental stage (Kola and Landis, 2004). The prediction of drug toxicity early in the drug development process remains one of the greatest limitations to the successful development of safe pharmaceuticals, and new approaches are urgently needed to improve the efficiency of predictive toxicology (Anson et al., 2011).

With progress in molecular, cellular and computational biology, new tools are available for studying the responses of cells, tissues, organs and the whole organism to drugs, chemicals and other environmental stressors at the molecular level. Advances in the life sciences are driving development of new technologies that are enabling researchers to study complex biological responses to chemical exposures in humans. The toxicology community is in the midst of a toxicity testing paradigm shift, moving from traditional high dose testing in animals to an *in vitro* approach based on a deep mechanistic understanding of key toxicologically-relevant cell-signalling pathways (toxicity pathways). Major components of this new approach include the use of cell-based assays (of human origin) to evaluate perturbations in toxicity pathways coupled with omics measurements and computational modelling in an integrated systems biology approach to address dose-response and *in vitro*-to-*in vivo* extrapolation (IVIVE) (National Research Council, 2007; Krewski et al., 2010). By implementing these new testing strategies one can accelerate the ability to:

1 test chemical substances using a rational, risk-based approach to chemical prioritisation

2 identify suitable drug candidates earlier in the developmental process

3 provide test results that are more predictive of human toxicity than current methods (Schmidt, 2009).

In addition, the new approach may significantly reduce costs and time required to conduct chemical safety assessments and could markedly diminish and potentially eliminate animal testing.

Future platforms for toxicity testing will combine these new tools and technologies to provide novel approaches for evaluating adverse effects of chemicals. Future platforms may include primary cell cultures, human stem cells, three-dimensional (3D) culturing systems or organs-on-chips, cell-based assays that enable interrogation of specific toxicity pathways, quantitative high-throughput screening (HTS), cell-based imaging

technologies, multi-omics technologies, bioinformatics and visualisation tools, and computational systems modelling. These tools have the potential to facilitate development of predictive toxicology based on models built with existing *in vivo* data (animal and human), as well as new and existing *in vitro* and *in silico* data. Integration of these tools provides a powerful new approach to toxicity testing. It is now possible to map and annotate toxicity pathways, conduct systems analysis of pathway function and link pathway perturbations to cell and tissue responses, thereby enabling both dose-response modelling and IVIVE (McMullen et al., 2014; Molinelli et al., 2013).

Given the rapid advances in biology and biotechnology, the tools and technologies that might be used in future platforms of toxicity testing are continuously emerging, evolving and being applied in novel ways. Although many of the tools and technologies listed above were reviewed in an outstanding article only a few years ago (van Vliet, 2011), the field is evolving rapidly and recent significant developments across these platforms warrant another appraisal. The platform components described here are not an exhaustive list of all of the tools and technologies that scientists are using in their studies; rather, this article attempts to

1 describe emergent technologies

2 highlight significant contemporary technological advances in various existing platforms

3 provide relevant examples of how these platform components are currently being applied to toxicity testing

4 discuss advantages and limitations of each platform and identify gaps where further development is required.

## 2 In vitro model systems

As the toxicology community shifts from a reliance on animal testing *in vivo* to *in vitro* approaches, a key question arises: how well do these *in vitro* models represent *in vivo* systems?

### *2.1 Cells and cell culture*

The process of growing and maintaining human cells in media in flat dishes [i.e., two-dimensional (2D) cell culture] has been standard for more than 50 years. Cell culturing techniques have been improved through the use of specialised media, co-cultures and cell lines including immortalised cell lines and primary human cells (Bhogal et al., 2005). However, cells grown on 2D cell culture constructs may exhibit altered morphology and have limited cell-cell and cell-matrix interactions; thus, these systems generally provide a poor representation of the complex physiology and anatomy of 3D tissues (Yamada and Cukierman, 2007). Moreover, the selection of cell type and quality of cells used as the basis of an *in vitro* model system is of paramount importance; the cells need to serve as reliable representatives of intact human cells and human cellular systems and exhibit properties that enable testing and evaluating the compound of interest. Many existing *in vitro* assays do not meet these criteria as they rely on

immortalised cell lines or isolated primary cells. Immortalised cell lines provide a simple model for more complex biological systems. Immortalised cells offer several advantages as they

1 can be grown indefinitely in culture

2 easily cloned

3 are cost-effective.

However, as immortalised cells are genetically altered, they may exhibit responses to chemicals that are significantly different or perhaps clinically irrelevant from unaltered cells.

Primary cells are isolated from human or animal tissues. Primary cell models have been developed for most human organs, including liver, kidneys, central nervous system and skin (Li et al., 2004). The major disadvantage of primary human cells is their limited availability owing to the requirement of a human or animal tissue donors, but other limitations include their variable quality, the high variation observed between donors, their phenotypic instability, and the potential loss of functionality when cultured (Combes, 2004).

Stem cells are a promising alternative source of human cells for development of predictive *in vitro* models for toxicity testing. Embryonic stem cells (ES cells) are derived from the inner cell mass of a blastocyst, an early-stage embryo. Human embryonic stem cells (hESC) are pluripotent – that is, they may

1 differentiate into any of the more than 200 cell types in the human body

2 propagate almost endlessly

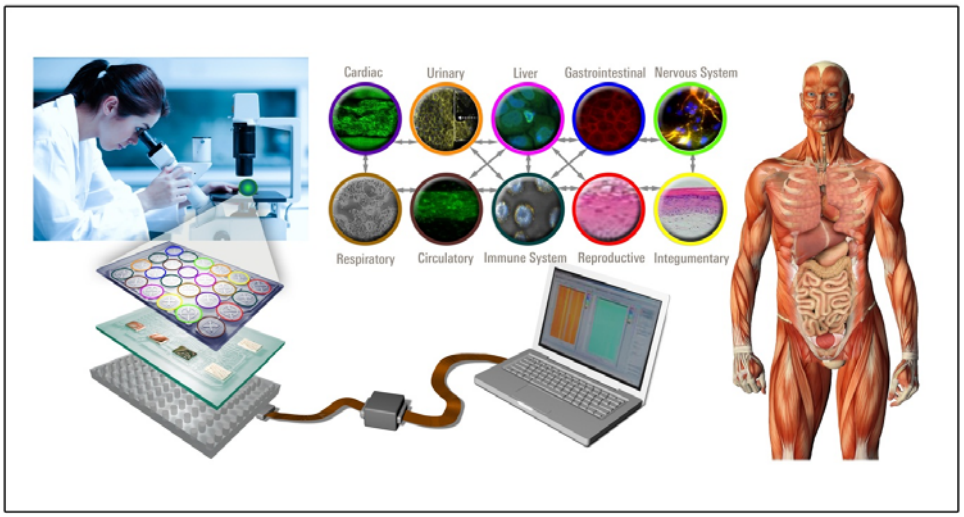
3 be amenable to genetic modifications.

Induced pluripotent stem cells (iPSC) are those that are derived from differentially-terminated adult cells and treated with a cocktail of transcription factors to 'induce' them to return to their undifferentiated pluripotent state. These cells do not require the destruction of an embryo, enabling access to stem cell technology that avoids the legal and ethical constraints of hESC. Their plasticity enables the generation of an array of cell types and proliferation indicates that researchers have easy access to large numbers of cells. iPSC can be derived from any individual's cells, opening the possibilities of creating cell lines that capture human genetic diversity for use in broad screening applications, creating disease-specific cell lines that may be used to study disease progression and pathology, and creating cells lines from individuals that may then be genetically altered for therapeutic applications and re-introduced to the donor, thus minimising the probability of donor rejection.

Within the USA, there is a great deal of interest in the use of stem cells for drug discovery and toxicology testing purposes from both public and private sectors. The National Institutes of Health (NIH) invested $650 M in stem cell research in 2012, a significant funding increase compared to previous years. Other federal agencies, including the Environmental Protection Agency (EPA) and the Department of Defense (i.e., DTRA, DARPA) are also funding stem cell research. In 2012, DARPA, NIH, and FDA established the Microphysiological Systems Program (or 'Organs-on-Chips'), a coordinated $145 M effort to create and integrate systems that utilise human primary or stem cell sources that represent ten major organ systems (Figure 1) (Sutherland et al.,

2013). Seven states in the USA have established stem cell research institutions and programs that support stem cell research initiatives; in 2013 the California Institute of Regenerative Medicine (CIRM) awarded Cellular Dynamics International (CDI) a $16 M contract to create three iPS cell lines for each of 3,000 healthy and diseased donors. Large pharmaceutical companies are also interested in the use of stem cells for drug discovery applications that include target identification, disease modelling and cell replacement therapies. Functional iPS cardiomyocytes were used to study long QT syndrome while other iPS cells were generated from patients with Huntington's disease, Parkinson's disease, autism, and others (Itzhaki et al., 2011; Rubin and Haston, 2011). Fourteen of the top 20 pharmaceutical companies (70%) are already engaged in stem cell research initiatives (Wobus and Löser, 2011). Roche invested $20 M in a deal with Harvard University to use cell lines and protocols to screen for drugs to treat cardiovascular and other diseases. GlaxoSmithKline has signed a similar deal worth $25 M (Hook, 2012).

**Figure 1** Human on a chip




Notes: chip Conceptualisation of a 'human-on-a-chip' system under development at MIT in collaboration with the Draper Laboratory and Zyoxel. The goal of the project is to create a versatile *in vitro* microphysiological system that incorporates and integrates ten individual engineered human organ system modules in an interacting circuit that mimics human physiological systems. The system will facilitate the assessment of candidate drugs, vaccines, biomarkers, and other chemicals to predict bioavailability, efficacy, and toxicity of therapeutic agents prior to clinical trials. Image used with permission from Draper Laboratory.

A number of groups reported on the use of stem cells in toxicology for applications such as hepatotoxicity, cardiotoxicity, neurotoxicity, and reproductive toxicology (Anson et al., 2011; Chapin and Stedman, 2009; Davila et al., 2004; West et al., 2010). Although applications based on stem cells are evolving rapidly, their value in predictive toxicology assays has not yet been established. One important challenge is directing stem cell differentiation *in vitro* to generate pure populations of specific cell types that are fully functional. Although iPS hepatocytes exhibit liver-specific morphology and certain

functional characteristics, these cells do not yet fully reproduce all the functions of *in vivo* hepatocytes. Considerable effort is focused to achieve this, and even now these cells play an increasingly important role in toxicity testing. Assuming that human stem cells will ultimately yield more accurate predictions of human toxicology than current animal tests, these cells may find their greatest utility when combined with multi-omics and other high-content approaches to fully evaluate and understand how toxicants perturb normal cell signalling pathways.

### *2.2 3D cell culture, tissue engineering, and organs-on-chips*

Despite the promise of stem cells for use as the basis of future *in vitro* model systems, at the moment, primary cell cultures employing 3D culturing systems are the most suitable *in vitro* models to represent *in vivo* conditions. 3D culture systems range from relatively simple aggregates of cell cultures embedded in a porous extracellular matrix to complex devices that integrate microfluidics and microelectronic technologies with human cells such as organs-on-chips. Growth of 3D cellular structures was facilitated using a variety of approaches such as multicellular spheroids, gel matrices scaffolds, and hanging drop plates as reviewed recently by Page et al. (2013), Haycock (2011), and Rimann and Graf-Hausner (2012). These constructs enable far more cell-cell interactions and enhanced intercellular signalling than 2D culture systems thereby providing a better representation of *in vivo* environments compared to 2D cultures. Several 3D primary cell models have shown promising results for identifying specific toxicity pathways and predicting *in vivo* toxicity (Leite et al., 2011; Pfuhler et al., 2014). The use of 3D cell cultures is markedly increasing in research areas including drug discovery, cancer biology, regenerative medicine and basic life sciences. Consequently, many 3D culture systems are now commercially available (Rimann and Graf-Hausner, 2012). Despite the significant advantages that 3D cultures confer over 2D systems, challenges still remain. Currently, 3D systems are not ideally suited or readily integrated into high-throughput applications although improvements in this area represent an active area of investigation (Deiss et al., 2013). Most of these models still do not adequately represent the biological characteristics and functions of tissues.

Tissues are ensembles of cells from the same origin that carry out a specific function. Tissues represent an intermediate level of organisation between cells and organs. Over the past decade, investigators developed and used tissue-engineered constructs as *in vitro* models for a wide array of applications including toxicity and drug safety testing, testing of stents and other devices, tumour biology, wound healing, organ replacement and regenerative medicine (Cosgrove et al., 2006).

Tissue engineering has made tremendous advancement in the field of tissue-engineered preclinical models. Such models now exist for many whole tissues including skin, muscle, cartilage, blood vessels, bone, bladder, liver, cornea, reproductive tissues, adipose, small intestine, neural tissues, kidney and cardiopulmonary systems (Gibbons et al., 2013).

The next wave is to create 3D models with living cells and tissues that accurately simulate structure and function of human organs, such as lung, liver and heart. This field is an active area of research with groups reporting a range of organ-specific features for multiple organs, including liver, lung, kidneys, intestine, bone, and others (Huh et al., 2011; Kamei et al., 2013). As described previously, significant investments are fuelling development of functionally-relevant 3D cellular microsystems that accurately reflect the

complexity of the tissue of origin, including genomic diversity, disease complexity and pharmacological responses. Studies are underway to produce representative human organ systems and to integrate those systems into a single chip-based system that will provide research tools for drug efficacy and toxicology screening of new molecular entities (Figure 1). Recent progress reports for all of the NIH-funded projects under the 'Organs-on-Chips' program can be found at: http://www.ncats.nih.gov/research/reengineering/tissue-chip/tissue-chip.html.

Looking beyond the 'Organs-on-Chips' efforts, it seems almost like science fiction to think about creating functional, implantable organs using 3D printing (i.e., bioprinting), a technique analogous to ink jet printing where instead of different coloured inks, the printer applies different materials and cells. However, scientists have been experimenting with 3D printing of human organs since the 1990s. The Wake Forest Baptist Medical Center's Institute for Regenerative Medicine (http://www.wakehealth.edu/WFIRM) is leading a project to develop a 'body on a chip' (Esch et al., 2011). Similar to the 'Organs-on-Chips' described above, the goal is to create a single integrated chip with miniaturised organ systems all connected into a single representative 'human system', which then serves as an *in vitro* platform for toxicity testing, disease modelling and other applications. For 'body-on-chip' efforts, organs will be miniaturised and printed onto the chip using Wake Forest Baptist's unique 3D printer.

## 3 In vitro assays and HTS

Realising the full potential of the *in vitro* model systems described above will require *in vitro* assays that can be used to identify perturbations in toxicity pathways and molecular mechanisms linked to disease. Toxicity testing based on evaluation of pathway perturbations will require suites of *in vitro* tests that can identify the range of significant perturbations of human pathways that might occur as a result of chemical exposure, and provide the right level of detail and an ability to provide appropriate read-outs across different response levels (Andersen, 2010; Kavlock and Dix, 2010; Hartung and McBride, 2011).

Efforts have focused to develop pathway-based assays using well-known toxicity pathways. The cellular stress pathway ensemble such as apoptosis, antioxidant responses, cytotoxicity, DNA damage responses, endoplasmic reticulum stress responses, heat shock, inflammatory responses, or mitochondrial damage consists of a limited and manageable number of pathways that are activated and respond in similar ways (Simmons et al., 2009). These pathways have been well-characterised at the molecular level. Using a suite of cell-based assays that cover various components of the pathways, compounds that activate the pathways based on certain key events can be studied and the mechanism-of-action (MoA) of such compounds may be elucidated. Further, since the pathways are activated and respond in similar ways, it may be possible to infer common mechanisms across multiple related pathways. Human nuclear receptor pathways such as estrogen, androgen, thyroid, aryl hydrocarbon, glucocorticoid, peroxisome proliferator, and others have been well-studied. These pathways play key roles in endocrine and metabolism pathways. In a similar way, suites of pathway-based assays directed towards components of each of these pathways may greatly accelerate toxicity testing studies.

HTS employs liquid robotics handling systems and computerised data processing to minimise manual inputs, reduce errors, and enable vastly improved speed, reliability and reproducibility. HTS is already used for toxicity testing and applied using different approaches. HTS can be used to screen a single compound against a large number of assays to identify toxicity pathways or to test a large number of compounds using a single assay analogous to candidate target selection in the pharmaceutical sector. A very impressive demonstration of the utility of HTS to toxicity testing comes from Tox-21 Consortium (Tox21C). The Tox21C, a collaborative research effort between the EPA, NIH and FDA (http://tox21.org/), was established to develop and demonstrate HTS assays to assess biological activity and identify MoA of thousands of chemicals in order to provide a scientifically rigorous, data-driven process for chemical prioritisation and risk assessment (Kavlock et al., 2009). The ultimate goal of this collaboration is to establish *in vitro* 'signatures' of *in vivo* rodent and human toxicity by comparing data generated in HTS assays with the rich historical database generated by the National Toxicology Program (NTP) at the National Institute of Environmental Health Sciences (NIEHS) using traditional *in vivo* and *in vitro* toxicologic assays (Shukla et al., 2010). Tox21C has established a testing library of 11,000 environmental and pharmaceutical chemicals, an assay library comprising more than 80 biochemical and *in vitro* cell-based assays, and a dedicated robotics system, informatics databases and algorithms to analyse, visualise and model the data, along with targeted testing paradigms to examine predictive and *in vivo* relevance of the models created (Attene-Ramos et al., 2013).

The EPAs ToxCast program is evaluating HTS for chemical safety assessments. Although EPA is also a partner in Tox21C, the ToxCast program's primary goal is to develop HTS *in vitro* assays that provide predictive signatures of toxicity and to use those signatures to prioritise the large backlog of untested chemicals for additional testing (Dix et al., 2007; Martin et al., 2010; Judson et al., 2010). Both the Tox21C and ToxCast research programs have been conducted in phases. Table 1 summarises the progress of these programs to date. The associated references contain significant additional details, including lists of compounds tested, specifics on the assays used and preliminary data analysis and interpretation. All of the data from these efforts is now publically accessible via various databases, including the NTPs Chemical Effects in Biological Systems (CEBS), the US EPAs Aggregated Computational Toxicology Resource (ACToR), PubChem, and the NCATS Tox21C Chemical Browser, to encourage independent evaluations of Tox21C findings. Other programs (now all concluded) also examined the utility of HTS *in vitro* assays to identify chemical hazards and predict *in vivo* responses. These included Japan's High Volume Production Program Challenge, and in Europe, the AXLR8 Consortium, the Sens-it-iv Consortium, ACuteTox, and carcinoGENOMICS.

**Table 1** Summary of HTS program phases

| *Program* | *Phase 1* | *Phase 2* | *References* |
|---|---|---|---|
| Tox21C | 2,800 compounds, 200 assays (2008) | 10,000 compounds, 80 assays (2012) | 7, 54, 79 |
| ToxCast | 309 chemicals, 600 assays (2009) | 1,800 chemicals, 800 assays (2012) | 26, 48, 64 |

Despite the impressive advances in HTS screening technology, *in vitro* models and assay development, thus far these research efforts have yielded mixed results. Retrospective analysis shows that most HTS assays are based on the use of fluorescent probes or

reporter gene constructs. While these methods are universal and easy to apply, use of these molecular entities may influence or bias the physiological environment within cells (Möller and Slack, 2010). Thomas et al. (2012) performed a comprehensive analysis of the ToxCast phase 1 data to evaluate the predictive performance of *in vitro* assays. Data showed that *in vitro* assays used in ToxCast phase 1 screening are poorly predictive of *in vivo* responses. The review also suggests that one reason for observed poor predictivity is that the panel of assays used are not yet adequate for their intended purpose, and Thomas et al. (2012) also suggest that perhaps the best use of HTS of *in vitro* assays may not be in screening for *in vivo* hazard prediction for subsequent chemical prioritisation, but rather as a means to survey and understand MoA. The Tox21C program was also recently reviewed, and a number of significant challenges identified. These include

1 limitations imposed on both assays and assay selection by the use of 1,536-well plate format

2 issues associated with cell sourcing (e.g., limited availability of primary cells, few primary cells with reporter gene assays, special requirements of certain cells)

3 currently no method for including metabolic activation in the HTS screens because liver S9 mix is toxic to cells when used beyond a few hours and the current HTS assay protocols do not include aspiration steps

4 need for development of additional assays suitable for HTS format

5 limited biological output where each assay yields 1–2 signals (Tice et al., 2013).

In addition, the toxicology community is considering how to advance results from *in vitro* HTS assays for validation and acceptance by regulatory agencies. The current paradigm for validating new or revised tests for potential acceptance by regulatory agencies, which includes a formal process to evaluate reliability, relevance, and fitness for purpose of each assay, is slow and expensive – too slow to validate the many new HTS assays already in use in the research setting (Judson et al., 2013). New validation approaches were proposed and employed to validate new innovative assays more efficiently (Wind and Stokes, 2010).

Conducting risk assessments from *in vitro* assays results requires a comprehensive understanding of how chemicals perturb normal cell-signalling pathways, and how those pathway responses relate to chemical exposure. Assay data generated from suitable *in vitro* model systems need to be placed into meaningful biological context, which requires a systems-biology level of knowledge of the mechanism(s) of action that chemicals employ, coupled with dose-response. Understanding MoA requires that one possesses a comprehensive map of toxicity pathways, and appropriate tools to detect, measure, and quantify perturbations to those pathways.

## 4 Cell-based imaging technologies and high-content screening

Modern molecular imaging technologies enable non-invasive visualisation of cellular and molecular processes occurring in living cells or animals. Molecular imaging in cell-based systems typically involves dosing cells with a compound of interest and analysing cellular structures and molecular components. Molecular imaging may be used for

1 measuring physical parameters such as cell size, surface area, cell morphology, and changes in concentrations of cellular products

2 disease detection, drug development, and *in vivo* monitoring of therapeutic effects

3 providing cellular, molecular and mechanistic information and could help to identify particular targets or pathways.

The next generation of imaging tools includes innovative microscopy methods, ultrasound, Computed Tomography (CT), Magnetic Resonance Imaging (MRI), and Positron Emission Tomography (PET) (Kherlopian et al., 2008). Most of these tools have not yet found utility in toxicity testing. To date the most widely-used technologies include optical imaging techniques based on fluorescence or bioluminescence and high-content imaging based on automated multicolour fluorescent microscopy. Developments in fluorescence microscopy are enabling new possibilities in high-resolution and molecular imaging both in *in vitro* and *in vivo*. Recent technical advances, as well as developments in the rapidly expanding spectrum of biological probes and fluorophores were reviewed by Wessels et al. (2010).

The combination of automated HTS cell-based assays with automated high resolution microscopy is referred to as 'high-content screening' (HCS) or sometimes as high content analysis. HCS has been enabled by technical advances in both microscopy hardware and software. Hardware improvements include auto-focusing and automated sample positioning. Software advances include integrated software platforms that comprise automated image extraction and data management, sophisticated statistical analysis, improved image analysis algorithms and network access to additional informational databases (Zanella et al., 2010).

In contrast to the assays similar to those used in Tox21C or ToxCast that average a single biological readout over thousands of cells, HCS acquires information from individual cells using multiple readouts simultaneously. HCS data extracted from cellular images include fluorescence intensity changes, fluorescence distributions, morphology and cell movement (Zanella et al., 2010). This information, when combined with other quantitative and qualitative imaging outputs including cell number, size and concentrations, provides a variety of parametric datasets that can be tailored to suit individual research needs. As HCS instrumentation has improved, it is finding increased utility in toxicity testing. Pharmaceutical companies are using HCS as a tool in lead candidate selection for target validation and hit qualification (Möller and Slack, 2010). HCS can identify cytotoxic compounds early in the process. In lead compound optimisation, HCS might help unravel the complexities of protein cascades to identify specific protein targets or other complexes, and facilitate toxicity and MoA studies. Abraham et al. (2008) developed an HCS assay in a HepG2 cell line that supports lead compound optimisation through predictive toxicology. HCS applications were noted for apoptosis, oxidative stress, human genotoxicity and neurotoxicity (Zanella et al., 2010).

Additional research and development will continue to drive new HCS applications. Currently, the major limitations of HCS are the overall quality of the cells (many assays still use immortalised cell lines) or in the labelling approaches used (as previously discussed, fluorescent probes may alter the physiological environment of the cells). The relatively high costs of fully automated imaging systems continue to place the HCS technology out of the reach of the mainstream and are not yet suitable for real-time live cell imaging. The use of stem cells continues to hold promise for use in HCS and 3D cell

culture systems based on primary human cell co-cultures were adapted for HCS approaches (Evensen et al., 2010).

## 5 Omics approaches

The advent of 'omics' technologies such as genomics, transcriptomics, proteomics and metabolomics are challenging our fundamental understanding of human biology. In just the past few years, a number of previously unassailable beliefs such as

1 DNA encodes mRNA and mRNA codes for proteins

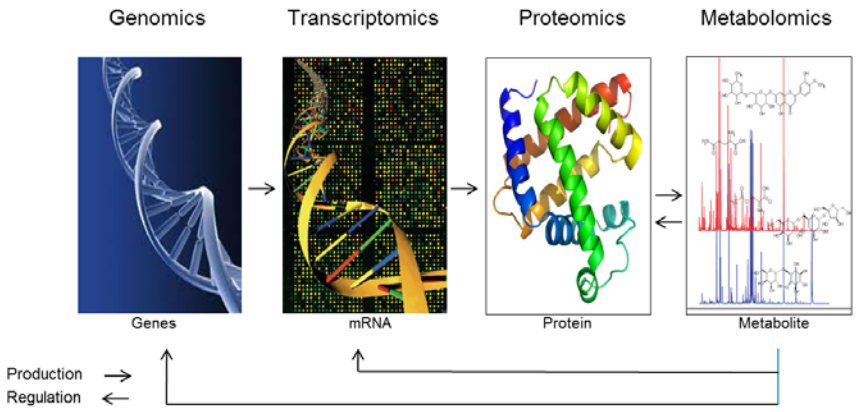
2 genome is static throughout an organism's lifetime

3 genome is identical in all cell types

4 all of the necessary information for cellular function is contained within the gene sequence (Figure 2).

have been called in to question by studies using omics approaches, with similar-scale revelations occurring in our understanding of the biology of mRNA, proteins and metabolites (Franklin and Vondriska, 2011).

**Figure 2** Central dogma




Notes: The simple picture of biological information flow (DNA is transcribed to RNA, mRNA is translated to proteins, and the flow of information is unidirectional) is constantly challenged by omics discoveries that reveal significant information flow between biomolecules. For example, transcriptional regulation occurs by epigenetic changes (direct structural modifications to DNA), by protein-DNA and RNA-DNA interactions, and by small functional RNA molecules (siRNA, miRNA). The products of translation are subject to post-transcriptional, translational, and post-translational modifications.

Systems toxicology – the integration of traditional toxicology approaches with high-throughput, high-content 'omics' technologies, cell-based assays, bioinformatics and computational tools – has the potential to facilitate development of predictive toxicology (Hege-Harrill and Rusyn, 2008). Traditional toxicology evaluates end points such as death, disease or observable changes in the organism or cells of the organism,

while ‘omics’ measurements are made across multiple levels of biological organisation and provide information that may be used to understand cellular processes as an integrated system rather than as a collection of disparate measurements. Although single ‘omic’ approaches might be utilised to correlate a static molecular profile (e.g., metabolites) with physiological endpoints, it is increasingly clear that no single ‘omic’ approach, by itself, is sufficient to characterise the complexity of biological systems (Zhang et al., 2010; Schäfer et al., 2012). Although all proteins are based on mRNA precursors, the expression level of a given gene that codes for production of a protein does not correspond to the amount of protein produced, as the expression level alone does not account for post-translational modifications or other ways in which proteins are regulated. Investigators (Waters and Fostel, 2004; Lohr et al., 2012) are increasingly using integrated, multi-omic approaches to link exposures and outcome-specific patterns obtained from ‘omics’ profiles to

1 identify toxicity pathways and MoA

2 study the underlying cause of disease and specific chemical or drug targets

3 identify signatures (biomarkers) of toxicity.

Recently Snyder and colleagues (Chen et al., 2012) conducted extensive multi-omics (genomic, transcriptomic, metabolomic, and proteomic) profiling of a generally healthy person over 14 months and used that information to create an integrative personal omics profile (iPOP) – something akin to a personalised medicine profile. In an earlier study, Heijne et al. (2005) employed high-throughput genomics, proteomics and metabolomics measurements to examine molecular and biochemical pathways that control homeostasis. Several of the most common ‘omics’ are described in greater detail below.

### *5.1 Genomics*

The completion in 2003 of the human genome sequencing project catalysed the application of genomics to understanding the effects of drugs, industrial chemicals and other environmental stressors on biological systems (Collins et al., 2003). Genomics is a scientific discipline that studies genome structure and function. Genome sequencing provides the specific order and identity of DNA nucleotide bases. Sequence information might be used to identify functional regions of the genome such as protein-coding genes, regulatory sequences, non-coding regions, and genomes may be compared to look for structural variations within DNA including single nucleotide polymorphisms (SNP), insertions, deletions, duplications, and copy number variations for differences between genomes. The most common structural variation is the SNP which are mutations in single nucleotides found throughout the genome that have a phenotypic consequence that are often associated with a disease. Consequently, considerable effort has been focused to identify these SNP ‘biomarkers’.

Genomics technologies have developed and evolved at an amazing pace in recent years transforming our ability to catalogue and study the information stored in genomes. Conventional sequencing methods used for the past several decades to determine the order of these bases one by one have all but been replaced by next-generation sequencing (NGS) approaches that enable extensive parallel sequencing of billions of DNA molecules simultaneously. NGS has substantially reduced the time and costs of sequencing and dramatically increased sequence output. Advances in NGS have enabled

a host of new applications including the 1000 Genomes Project (a population-based whole genome sequencing effort to identify common genetic variants), the Cancer Genome Atlas (TCGA), an effort to accelerate our understanding of the molecular basis of cancer, and many other large-scale research efforts (Green and Guyer, 2011). Ultrafast DNA sequencing represents the third generation in DNA sequencing and many strategies are under development. These include sequencing-by-hybridisation, nanopore sequencing, and sequencing-by-synthesis. Third generation strategies and platforms were reviewed and compared but all of these approaches provide improvements over current methods including higher-throughput, faster turn-around times, longer read lengths, and reduced costs (Pareek et al., 2011). The biggest challenge for investigators using NGS approaches is managing the data that is generated. Data analysis tools are not yet able to effectively process the sheer volume of data that comes from sequencing although a lot of research effort is being directed towards improving these tools. NGS, coupled to other technologies such as DNA microarrays have enabled significant advances. Genome-wide association studies (GWAS) are one of the most commonly used approaches to compare genomes. A typical GWAS experiment might involve comparisons of large numbers of genomes from well-phenotyped individuals to look for structural variants including SNP. GWAS studies are focused largely on finding small differences between genomes. These discoveries direct research towards targeted therapeutics for diseases, and have given rise to entirely new disciplines such as epigenetics (looking for ways in which the DNA itself rather than the nucleotide bases or the sequence gets modified) which in turn affects gene expression and gene regulation.

Hybrid technologies, like chromatin immune-precipitation coupled to DNA microarray (ChIP-chip) or sequencing (ChIP-seq) have been used to probe the genome-wide location and function of DNA binding proteins (Schäfer et al., 2012). These technologies also facilitate studies of DNA-protein interactions to unravel how various transcription factors and other proteins interact with DNA to regulate gene expression. RNA sequencing (RNA-seq) enables sequencing of RNA transcripts, a technique that vastly expands upon, and compliments, microarray-based gene expression studies.

## *5.2 Transcriptomics*

Many genomics-based studies for toxicology use microarray technology to establish human genome-wide gene expression profiles by measuring all of the approximately 100,000 mRNA molecules or 'transcripts' produced in a cell or a population of cells. This technique, referred to as transcriptomics, captures the characteristic and specific patterns of gene expression (i.e., 'signatures') that result from exposures to a given toxicant under a given set of experimental conditions for thousands of genes simultaneously and provides quantitative measurements of the dynamic expression of mRNA molecules in contrast to the static measure of DNA provided by gene sequencing. Cellular response to toxicant exposures for the entire gene compliment of the human genome (about 21,000 genes) may be probed in a single microarray experiment. Gene expression profiling enables identification of specific genes that are differentially expressed as a result of changes in environmental conditions. Linking these gene changes to a chemically-induced phenotype (i.e., 'phenotypic anchoring') facilitates predictive toxicity and elucidation of MoA (Cui and Paules, 2010). Gene expression profiles between arrays

might be compared to evaluate the effects of different compounds, doses and exposure times across species, or between/within populations. Genes with common expression profiles may be identified using statistical methods (e.g., clustering techniques), leading to potential insights regarding common pathways, or MoA, assuming the clustered genes are functionally related (Afshari et al., 2011). Gene function and gene relationships within networks may be established and verified using gene knockout or silencing techniques. Gene expression signatures also enable toxicants to be grouped or classified into different toxicity classes, usually based on potency or MoA, and facilitate prediction of toxicity of chemically-related compounds.

Gene expression profiles might guide identification of biomarkers of toxicity even at low exposure doses when no phenotypic changes were observed. Heinloth et al. (2004) demonstrated how the analysis of gene expression profiles from liver samples obtained from rats exposed to sub-toxic doses of acetaminophen indicated subtle cellular injury that was not detectable by histopathology or clinical chemistry methods. Such biomarkers of toxicity might identify potentially toxic drug candidates even when there are no indicators of toxicity in preclinical studies (McBurney et al., 2009, 2012). These biomarkers might serve as the basis for suites of *in vitro* assays to assist in

1 compound screening
2 to group chemicals by toxicity class or MoA
3 to monitor drug therapies for safety and efficacy
4 to monitor for exposures to environmental toxicants, even at sub-critical exposure levels.

Suites of assays based on gene signature biomarkers have already been developed – one example is a set of gene signatures that distinguish genotoxic carcinogens from non-genotoxic carcinogens (Ellinger-Ziegelbauer et al., 2009). As a cautionary note, however, two studies by Kirkland et al. (2005, 2006) showed that *in vitro*-only genotoxicity tests, even combined as a battery of tests, yielded a high false-positive rate, and that if these test results were the sole source of decision making, many common products would have been erroneously rejected for commerce. These results underscore the need to fully utilise all available biological information like that obtained from proteomics and metabolomics and to combine that information with computational modelling.

The recent technical advances of NGS enabled advances in transcriptomics. RNA transcripts are sequenced in a cell (RNA-seq) and used to study RNA expression patterns, point mutations, alternative gene spliced transcripts, post-transcriptional changes, gene fusion, SNP and other mutations and changes in gene expression. RNA-seq is increasingly being used to discover and study different types of RNA including miRNA, siRNA, lincRNA, and tRNA. Other important transcriptomic technologies include RT-PCR, which provide higher sensitivity and accuracy than microarrays.

### *5.3 Proteomics*

The comprehensive study of the entire complement of proteins and their modifications (i.e., the proteome) of an organism to understand cellular processes is known as proteomics (Miller et al., 2014). Proteomics includes

1 global identification of all proteins in a sample (protein profiling) using discovery or 'shotgun' proteomics

2 quantitative measurement of protein expression (i.e., abundance)

3 study of protein structures, including protein variations and modifications

4 interactions of proteins and other molecules.

The human proteome, estimated to comprise between 250,000 and one million proteins (along with their post-transcriptional, translational, and post-translational modifications), is highly dynamic varying not only over time but from cell to cell as well. Proteins exist in concentrations that may span nine orders of magnitude, making low abundance proteins extremely difficult to detect and characterise. Thus, proteomics measurements are far more complex and challenging compared to the relatively straightforward and somewhat static human genome and smaller, more tractable human transcriptome.

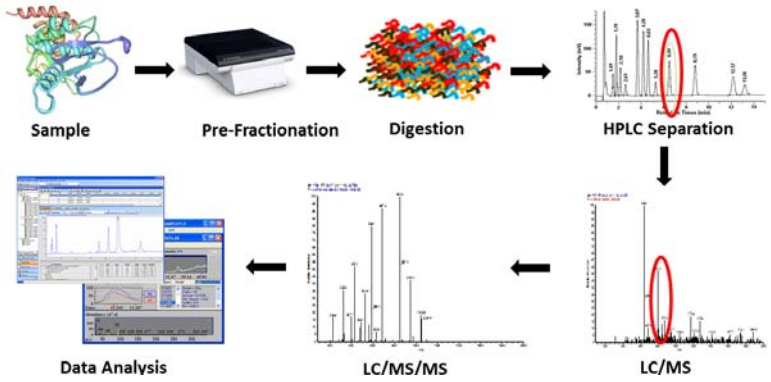
Advances in mass spectrometry (MS) in just the past few years now enable routine identification and quantification of thousands of protein components in samples. Indeed, most proteomics studies are now performed using liquid chromatography/mass spectrometry (LC/MS) because of sensitivity, selectivity, accuracy, speed and throughput (Chen and Pramanik, 2009). A typical MS-based proteomics workflow is shown in Figure 3. For most proteomics experiments, the first step involves extracting protein(s) of interest from cells, tissues or other complex sample matrices. Sample preparation steps, which are determined by the overall objectives of the experiment, may include cell lysis, protein separation using gel electrophoresis, dialysis, and concentration. Because analysis of intact proteins by MS is extremely difficult, extracted and purified proteins are enzymatically digested into smaller constituent peptide fragments. Samples containing multiple proteins generate many thousands or hundreds of thousands of peptide fragments; therefore samples are subjected to fractionation or enrichment to further reduce complexity of analysis. The two most commonly employed approaches are:

1 2D gel electrophoresis (2DGE), in which proteins are separated according to their isoelectric points and molecular mass, followed by mass spectrometric (MS) identification

2 gel-free liquid-phase separation methods such as size-exclusion, affinity, and ion-exchange chromatography with automated tandem mass spectrometry (LC-MS/MS) (Miller et al., 2014).

Although the relative merits of these two approaches have been debated, each has its advantages and limitations and both separation strategies are widely used (Manadas et al., 2010). Fractions are then further separated and identified using LC and subsequently, peptides within fractions are ionised and passed to the mass spectrometer. The mass analyser filters the ions and records their mass-to-charge (m/z) ratio along with their relative abundance as peaks that populate a mass spectrum. Ions comprising specific peaks (precursor ions) are selected and further analysed by tandem LC (MS/MS) to generate characteristic fragment ions. The combinations of precursor m/z and their associated fragment ions are then compared to sequences of known peptide fragments and identified. Fragments are further assembled to enable identification of the protein sequence.

**Figure 3** Proteomics workflow




Notes: A typical mass-spectrometry-based proteomics workflow, applicable to both discovery and targeted proteomics. Proteins samples are prepared in a series of steps that are determined by the overall objectives of the experiment and may include cell lysis, pre-fractionation, or other separation, purification and concentration techniques. Proteins are enzymatically digested into their smaller constituent peptide fragments; peptide fractions are then further separated and identified using HPLC. HPLC peptide fractions (a single fraction is indicated in the red circle) are ionized and passed to the mass spectrometer. The mass analyser filters the ions and records their m/z ratio along with their relative abundance as peaks that populate a mass spectrum. Ions comprising specific peaks (precursor ions, indicated by the red circle in the LC/MS spectrum) are selected and further analysed by tandem LC (MS/MS) to generate characteristic fragment ions. The combinations of precursor m/z and their associated fragment ions are then compared to sequences of known peptide fragments and identified. Fragments are then quantified and may be further assembled to enable identification of the protein sequence.

To date, most proteomics research has been conducted in an untargeted or discovery mode. This approach was used primarily to identify all proteins in a given sample (protein profiling), and more recently employed for differential quantification of the identified proteins (Tuli and Ressom, 2009). While this approach enabled significant advances to whole proteome identification and mapping, it suffers from significant shortcomings:

1 the analysis of a complete proteome remains challenging, expensive and time-consuming and only a few labs have become truly expert in this approach

2 due to the way in which precursor ions are selected, results often cannot be reproduced, even within the same lab using the same sample

3 the approach does not enable identification of low-abundance proteins

4 in any experiment designed to address a specific scientific question, a large numbers of 'irrelevant' proteins are identified, while some number of relevant proteins are missed (Domon and Aebersold, 2010).

The emerging strategy of targeted proteomics enables researchers to detect, identify and quantify specific aspects of the proteome. In a targeted approach, the proteins of interest are known in advance and the MS is programmed to select only those certain signature

peptides using a technique known as selected reaction monitoring (SRM), sometimes referred to as multiple reaction monitoring (MRM). This approach enables greater sensitivity over discovery-based approaches, and enables detection of low-abundance proteins. It also provides vastly improved reproducibility such that multiple labs generate identical results (Marx, 2013).

Within toxicology, proteomics research efforts (toxicoproteomics) have been largely directed towards identification of biomarkers with prognostic or diagnostic value, reflecting the fact that discovery (untargeted) proteomics has been the dominant strategy for the past decade. Biomolecules serve as early indicators of disease and used to monitor disease progression, pharmacologic therapeutic responses, and adverse responses to toxicants. Biomarker discovery and identification has largely focused on liver and kidney as a consequence of studies driven by the pharmaceutical sector, although disease-specific markers were also identified (van Vliet, 2011; Altelaar et al., 2013). Progress in biomarker discovery, identification and validation for toxicology has been slow. The slow progress does not reflect a lack of suitable biomarkers; but rather, inherent challenges of using an untargeted approach to discovery. Targeted proteomics enable rapid advances within *in vitro* toxicology, for both biomarker discovery as well as for expanding and developing our understanding of pathway-based molecular mechanisms of toxicity. Identification and quantitation of proteins in a sample may reveal that a signalling pathway is active; conversely, knowledge of signalling pathways might be used to map and model human responses to chemical exposures or to pharmaceuticals (Collings and Vaidya, 2008).

### *5.4 Metabolomics*

Metabolites are small molecules, such as amino acids, lipids, organic acids and sugars that are intermediate or end products of metabolism. Unlike genes and proteins that are altered and subject to regulatory processes, metabolites

1 are downstream products of gene expression (and also the end product of a toxic insult)

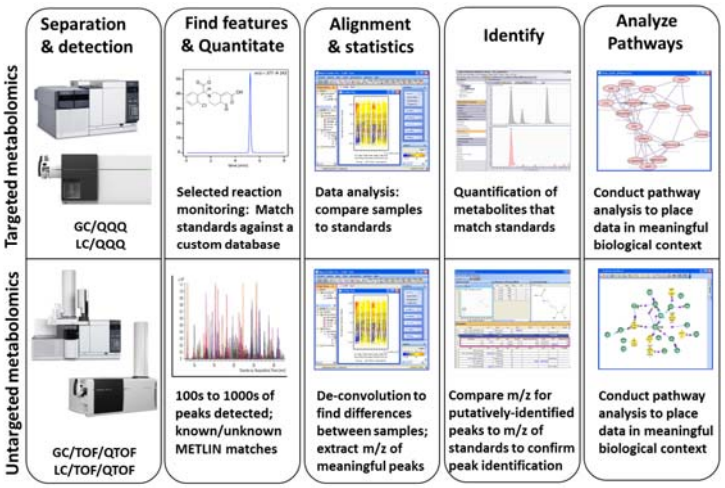
2 directly reflect biochemical end products that are closer to the phenotype (van Ravenzwaay et al., 2007).

Metabolomics is the study of metabolites and is used to identify all of the metabolites present in a given cell or organism at a specific time (global metabolite profiling) or to characterise specific metabolites with respect to concentration or other parameters. In 2007, scientists completed the first draft of the human metabolome, cataloging approximately 2,500 metabolites, 1,200 drugs and 3,500 food components; this information is available in the Human Metabolome Database (http://www.hmdb.ca), although it is still incomplete (Wishart et al., 2007).

Modern metabolomics research had its origins in nuclear magnetic resonance (NMR) spectroscopy but over the past two decowing due to high sensitivity, specificity, and ability of MS to detect and identify large numbers of metabolites. Gas chromatography/mass spectrometry (GC/MS) was used to study complex samples and later expanded into LC/MS, driven by the advent of affordable, accurate mass, time-of-flight (TOF) instruments. The advantages and limitations of each technology have been the subject of numerous reviews (Bouhifd et al., 2013; Dunn and Ellis, 2005).

Metabolomics experiments are conducted using either targeted or untargeted strategies (Figure 4). Targeted metabolomics is a method used to determine the relative abundances and concentrations of a specific set of pre-selected metabolites, usually related to a specific metabolic pathway. While the method is quantitative and enables direct comparisons of metabolites between samples, it also requires that the exact structures of the metabolites under study are known and usually requires use of analytical standards. Therefore, targeted metabolite studies are limited to those metabolites catalogued in searchable mass spectra libraries such as available metabolomics databases, along with bioinformatics tools to facilitate data analysis and interpretation (Baker, 2011; Go, 2010). Untargeted (discovery) metabolomics methods are used to establish the metabolite profile of a given sample. Discovery metabolomics experiments involve examining an untargeted and unbiased suite of metabolites, finding the ones with statistically significant variations in abundance within a set of experimental versus control samples, and determining their chemical structure. An interpretation step allows the investigator to connect the metabolite with the biological process or condition.

**Figure 4** Workflow diagrams and discussion of experimental steps




Notes: Mass-spectrometry-based metabolomics workflows, for targeted (upper) and untargeted (lower) applications. Targeted metabolomics is a method to determine the relative abundances and concentrations of a specific set of pre-selected metabolites, usually related to a specific metabolic pathway. Targeted applications typically employ triple quadruple LC/MS or GC/MS because the QQQ provides reliable, sensitive and reproducible quantitative analysis. The method requires that the exact structure of metabolites are known; therefore the instrument is first optimised against standard compounds in selected reaction monitoring. Sample metabolites are compared to standards and exact matches quantified. Untargeted metabolomics approaches usually employ TOF or QTOF mass analysers, as the instrument enables high resolution and accurate mass measurements for identification and characterisation, particularly with unknown compounds. Untargeted (discovery) metabolomics experiments involve examining an untargeted and unbiased suite of metabolites, finding the ones with statistically significant variations in abundance within a set of experimental versus control samples, and determining their chemical structure. An interpretation step allows the researcher to connect the metabolite with the biological process or condition.

Metabolomics has been expanding rapidly and applications are now routine in the areas of system biology, drug discovery, pharmaceutical research, early disease detection, toxicology, newborn screening, food safety and nutrition science. Metabolomics is finding broad acceptance and ready adoption in toxicology. Even as early as 2000, metabolomics was explored as a technique for rapid *in vivo* screening. The Consortium for Metabonomic Toxicology (COMET) performed NMR-based studies to predict liver and kidney toxicity using serum and urine samples from rodents; that data is still used today (Lindon et al., 2005). The same approach was extended more broadly and now *in vivo* metabolomics are routinely used in drug development to screen for potential toxic effects of drug candidates, as well as for MoA studies (van Ravenzwaay et al., 2012). Metabolomics is also being applied to *in vitro* toxicology. Ramirez et al. (2013) provided a long list of suggested *in vitro* metabolomics applications for toxicology and connected these suggestions to their actual implementation through active research efforts. Just a few of the application areas identified are:

1 development of prediction models, where metabolite profiles obtained from training compounds of known toxicities could be compared to unknown compounds to predict their potential toxicity

2 rank/prioritise compounds and sort or classify molecules with respect to their MoA or predicted toxicities

3 use pathway-based knowledge to pinpoint potential drug/compound molecular targets and predict their MoA and map and model pathways of toxicity

4 biomarker discovery.

## 6 Data integration, analysis and interpretation: bioinformatics and visualisation tools

Experimental omics approaches are high-throughput, data-driven, top-down approaches that generate large amounts of data (Zhang et al., 2010). Combining data from different platforms and assays across multiple experiments into a coherent approach that appropriately weighs and evaluates different data sources will be a challenging task and represents the next generation of pathway identification tools.

The two main challenges for integrated systems toxicology are

1 limitations of bioinformatics and visualisation tools to enable researchers to analyse and interpret their data within a meaningful biological context

2 the overall processing, storage, and curation of data into databases such that data can be easily accessed, retrieved, shared, and archived.

Bioinformatics tools will need to be built on novel, flexible architectures, to provide a broad foundation for joint analysis and visualisation of orthogonal data.

Several key processes critical to pathway-based orthogonal analysis such as

1 shuttling of different kinds of data between different software applications

2 facilitating new custom visualisations

3 enabling statistical analyses involving pathway databases

4 providing workflow and help facilities in order to ensure that the software is accessible to users with different levels of experience, must be considered.

Integrative software and open-source data repositories provide the opportunity to share, reduce, and analyse data from multiple sources. One example is GeneSpring, a bioinformatics platform developed collaboratively by Agilent Technologies and Strand Biosciences, representing cutting edge in

1 integrated 'omics' bioinformatics and visualisation

2 providing comprehensive analytical and visualisation tools for datasets obtained from NGS such as transcriptomcs, genomics, metabolomics and proteomics using NGS sequencing, microarray, MS and NMR platforms.

Heterogeneous data, such as gene expression, miRNA, exon splicing, genomic copy number, genotyping, proteomic, and metabolomic abundance can be combined into one project, allowing investigators to analyse and view results from different experiments in a single user interface. A comprehensive suite of statistical tests is provided to enable robust differential analysis on a variety of experimental designs, and clustering and classification algorithms facilitates pattern discovery. Intuitive graphical displays that employ a variety of plots, graphs and diagrams help users conceptualise and interpret the information in their data, and other interactive visualisation tools make it easy to import/export graphical images and to compare results from different experiments. The GeneSpring Pathway Architect module enables scientists to view and analyse curated pathway content, by leveraging WikiPathways, a publically-available resource for building, annotating and querying biological pathways. GeneSpring also incorporated Gene Ontology (GO) analysis, Gene Set Enrichment Analysis (GSEA), Gene Set Analysis (GSA) and network analysis tools. GeneSpring can be used in an individual desktop environment, or in a scalable, client-server collaborative environment.

Given the complexity and sheer volume of data generated in 'omics' studies, there is an emerging need for comprehensive, publically-accessible databases. Databases such as CEBS, ACToR, PubChem, GO, Gene Map Annotator and Pathway Profiler, Science Signaling Connections Map, BioCarta, Reactome and KEGG are useful in this regard. Of the more than 1,000 biologically-relevant databases are already publically available, several hundred are specifically relevant to toxicology but many of those contain data that is not necessarily in a format that is directly useable (Judson, 2010). EPA's ACToR is an example of a knowledgebase that brings together diverse types of information into a system where interrelationships of individual database elements including traditional toxicology, chemical structure information, high throughput screening data, molecular pathway analysis, chemical data repositories, peer reviewed published literature, and internal Agency databases can be explored and utilised (Judson et al., 2008). The ACToR database links information from more than 400 source databases and datasets on chemical identity. All published data associated with the ToxCast, ToxRefDB (a mineable, searchable database of pesticide toxicity data) and Tox21C programs are consolidated within ACToR and the knowledgebase is publicly accessible. Given the existing utility and advanced stage of development of ACToR, it might serve as the foundation upon which to build out a complete knowledgebase for all 21st century toxicology testing data and metadata.

## 7 Computational toxicology

Computational toxicology is the integration of mathematical and computer models created using modern high-powered computational capabilities with toxicology and molecular biology to map, model and understand the biological circuitry of toxicity pathways to predict the toxicity of environmental chemicals and pharmaceuticals and their dose-response relationships (Kavlock and Dix, 2010; Krewski et al., 2011). Computational toxicology approaches range from simple models that use purely statistical methods to look for correlations between *in vitro* assays or calculated chemical descriptors and *in vivo* endpoints, all the way through to complex models that couple information from comprehensive high-content datasets with knowledge of network biology to identify toxicity pathways and evaluate pathway perturbations (Judson, 2010).

Countless numbers of models have been developed for computational toxicology. A few of the most commonly-employed types are described here. Structure-activity relationships (SARs) are used to relate the structure of a chemical (molecule) and its biological activity (or toxicity). Quantitative structure-activity relationships (QSARs) provide a statistical relationship between the physicochemical properties of a chemical and its effects (toxicity and fate). Machine learning techniques 'train' computer systems to identify biologically-active compounds from those that are inert. Molecular modelling tools had their origins in computer-aided drug design (CADD). A classic example would be to use crystal structures of proteins to model a receptor site and then use that model to conduct *in silico* simulations of new structures to evaluate how well new structures might 'fit' the receptor (Hartung and Hoffman, 2009). Molecular modelling tools provides an approach for estimating chemical activity when relevant data are not available to simulate critical processes in the specific mechanisms that lead to toxicity, and to model toxicity pathways (Kavlock et al., 2008). Physiologically-based pharmacokinetic (PBPK) models are by far the most often-used approach to model ADME (absorption, distribution, metabolism, and elimination) toxicokinetics. PBPK models are used for predictions of toxicologically-relevant internal or target dose from environmental and pharmacologic chemical exposures, and for IVIVE. Traditionally, these models have been used for performing extrapolations between different routes of exposure and between different species (Caldwell et al., 2012). Finally, computational models of cellular response networks are increasingly being used to predict dose-response behaviour. Omics approaches enabled a detailed characterisation of molecular signatures; however, a mechanistic understanding of the underlying biological processes requires an even more focused quantitative analysis of specific pathways and networks, with computational systems biology pathway models expected to play a key role in the process of studying the dynamic behaviour of toxicity pathways across dose and time domains. Some specific examples of this type of model were described by Bhattacharya et al. (2011) and found here http://www.thehamner.org/education-and-training/drm_workshop.html.

The field of computational toxicology is advancing just as rapidly as all of the other scientific tools in toolbox for 21st century toxicity testing. Key developments include:

1 data from high-throughput, HCS assays to help to facilitate the identification of toxicity pathways

2 development of new computational modelling tools that enable integration across multiple levels of biological organisation and provide information that might be used to understand cellular processes

3 construction and curation of large-scale data repositories like those mentioned above (Kavlock et al., 2008).

## 8 Making it happen: implementing 21st century approaches to toxicity through partnership and collaboration

The landmark National Research Council (NRC) report 'Toxicity Testing in the 21st Century: A Vision and a Strategy', chartered a transformative change in toxicity testing, calling for the use of high-throughput cell-based assays (of human origin) to evaluate perturbations in key toxicologically-relevant cell-signalling pathways, coupled with 'omics' measurements and computational modelling in an integrated system biology approach to address dose-response and IVIVE (NRC, 2007; Krewski et al., 2010). There are many projects underway now to develop new test methods and every day, new researchers are drawn to these kinds of studies in an effort to understand the relationships between human health and human biology. On a larger scale, at least three different approaches to *implementation of the vision* articulated in the report are currently underway: the US EPA ToxCast program together with the associated multi-agency Tox21C initiative, the human toxome approach, and the case study approach. Each of these approaches has strengths and contributes to the overall goal of modernising toxicity testing.

The ToxCast/Tox21C programs seek to develop ways to predict potential toxicity and establish scientifically rigorous, data-driven, cost-effective processes for chemical prioritisation and risk assessment. As previously described, substantial progress has been made in the first two phases of these programs, although significant challenges remain. In addition to prioritising compounds for testing, this program has already combined analysis of dose-response evaluation of the *in vitro* screens with high-throughput dosimetry to develop high-throughput risk assessment tools (Judson et al., 2011; Wetmore et al., 2012). The output of these assessments is derivation of biological pathway altering concentrations (BPAC). The 'Human Toxome' project, a consortium of researchers led by Dr. Thomas Hartung at Johns Hopkins University, along with scientists at The Hamner Institutes, Agilent Technologies, Georgetown University, Brown University and the EPA ToxCast program received an NIH grant to begin mapping the totality of human toxicity pathways (i.e., the human toxome). The group has begun by mapping estrogenic pathways in human breast cancer cells using a combination of transcriptomics and metabolomics (http://altweb.jhsph.edu/news/current/caatnihgrant.html). In the case studies approach, well-studied compounds that are known to affect specific pathways are being evaluated using a suite of *in vitro* model systems and specific technology platforms to assess dose-response and perform IVIVE (Judson et al., 2011). The Hamner Institutes, along with a variety of partners from the private sector spanning multiple sectors (e.g., chemical, personal care, ag/bio, technology providers) established the Toxicity Pathway and Network Biology Program. Under this

program, researchers at the Hamner have deliberately selected a small number of well-studied 'prototype' pathways and are using well-characterised compounds to map and model those pathways (Andersen et al., 2011). The program intends to use these case studies to demonstrate how the application of new understanding of toxicity pathways might be applied for human safety assessments through use of computational systems biology dose response models of pathway circuitry (Boekelheide and Anderson, 2010). The case study approach has several advantages but the most significant is that this approach is most likely to accelerate the adoption of new toxicity testing approaches and enable us to quickly 'learn as we go' and adjust/adapt our efforts in response to what is learnt.

## 9 Conclusions

When fully implemented, the new approaches to toxicity testing might significantly reduce the cost and time required to conduct chemical safety assessments and markedly reduce and potentially eliminate high-dose animal testing. An integrated systems toxicology approach offers large scale potential benefits to human health, translational medicine, energy and the environment. The toxicology research community working to advance these efforts is establishing the foundation for a full-scale effort to implement the NRC vision of toxicity testing.

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