

## Toxicology

# Animal-Free Toxicology

## Sometimes, in Vitro is Better

The next time you use shampoo, air freshener, or moisturizing cream, consider this: How do you know it's safe? In all likelihood, whatever toxicologic screening its component ingredients were subjected to involved laboratory animals, the method of choice for decades and the industry's reigning "gold standard." Yet as Bob Dylan once put it, the times, they are a-changing. Animal-based testing is expensive and time-consuming, morally and ethically troubling, and most significantly, often a poor predictor of human toxicity. Animals aren't going anywhere just yet. But their numbers are dropping. Driven both by legislative mandate and scientific need, a new suite of in vitro and cell culture-based animal-free methods are gaining a foothold in toxicology labs. **By Jeffrey M. Perkel**

One key player in the modernization of toxicology screening is automation.

In 2010, as oil gushed from the broken wellhead beneath the stricken *Deepwater Horizon*, the **U.S. Environmental Protection Agency (EPA)** struggled to assess the safety of the chemical dispersants being used to treat that oil.

Eight commercial dispersants and 23 reference compounds were put through the analytical ringer, being probed for their cytotoxicity and activity on some 73 transcription factors, and—because one component of several of these dispersants was nonylphenol ethoxylate, a known “endocrine disruptor”—for their ability to activate estrogen and/or androgen-responsive pathways.

The take-home message from this analysis was that the compound then in use, Corexit 9500 appeared relatively safe, at least regarding endocrine activity.

But perhaps the bigger take-home message concerns how those data were collected. Rather than laboratory animals, the traditional go-to method of toxicity testing, the research team used a high throughput cell culture-based approach, finishing their analysis in about two weeks.

These days, an ever-increasing number of researchers, government agencies, and commercial entities have adopted a similar strategy. Partly, that's due to ethical considerations. But there are others as well.

For one thing, observes toxicologist Thomas Hartung, “We are not 70 kg rats.” But time, cost, and practicality also loom large. Some 55,000 chemicals or more were grandfathered in when the U.S. Congress first passed the Toxic Substances Control Act in 1976. About 100,000 chemicals are similarly situated in Europe. No toxicological data has ever been filed on most of those, a “knowledge gap” that represents the vast majority of compounds in use today.

Filling that gap, at least with animals, is both financially and practically impossible. According to Robert Kavlock, director of the U.S. EPA National Center for Computational Toxicology (NCCT), “the capacity of the U.S. industry to test is probably in the hundreds of chemicals a year at best.” And it can cost a company upwards of \$10 million and take five years to fully test just a single pesticide, at which point the EPA then needs

a million dollars or so to review those data and make a safety determination “If you extrapolate that to 80,000 chemicals, the math doesn't work very well,” Kavlock says.

But this huge backlog represents just one reason for pushing alternative methods. The European Union (EU) has for years been promoting a move away from animal-based testing in industries such as cosmetics, with a testing ban on cosmetic products and ingredients being implemented in stages since 2004. And where Europe goes, so goes the world, because U.S.-based companies that use animal testing, for instance, can no longer sell their cosmetics in Europe. But cosmetics testing represents only about 0.2 percent of the animals used for safety assessment testing in Europe, estimates Mathieu Vinken, of the Department of Toxicology at **Vrije Universiteit Brussel**. Another piece of European legislation, called REACH, covers the giant chemical backlog, suggesting that if animal-free alternative methods exist, they should be used there, as well.

“European legislation at the moment is the pacemaker worldwide on some of the demands in regulatory testing,” says Hartung, the Doerenkamp-Zbinden Professor and Chair for evidence-based toxicology at **Johns Hopkins University Bloomberg School of Public Health**.

The EU has, since 1986, invested some \$300 million on the development and validation of alternative approaches, Hartung estimates. A new initiative, called Safety Evaluation Ultimately Replacing Animal Testing (SEURAT) dedicates another 50 million euro specially to investing animal-free methods to long-term toxicity endpoints.

The United States has been slower to react, but a 2007 report by the National Research Council's Committee [continued>](#)

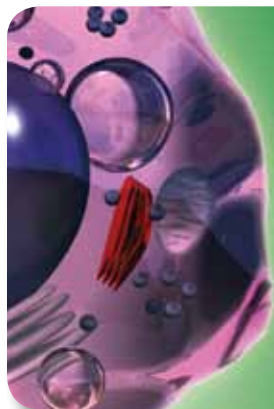
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## Toxicology



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on Toxicity Testing and Assessment of Environmental Agents, called *Toxicity Testing for the 21st Century: A Vision and a Strategy* (<http://scim.ag/zEJou2>), says Hartung, “was really a game-changer.” Laying out the issues with animal-based testing, the report “suggested to [researchers that they] embrace new technologies to overcome this problem.”

Today, from automated screening platforms to the incorporation of ‘omics technologies, the toxicology world is doing just that.

### TOXICITY FORECASTING

One key player in the modernization of toxicology screening is automation. For instance, the European Union Reference Laboratory for Alternative Methods to Animal Testing uses automated imaging on a **Cellomics** ArrayScan vTi platform and robotics in its work validating proposed animal-free testing methods. “Our aim is to challenge the assay with sets of reference chemicals/substances which have been well characterized in terms of their toxicity and preferably [with respect to] their mode-of-action,” says Maurice Whelan, head of the systems toxicology unit at the **European Commission Joint Research Centre**, which operates the lab.

Using such a system and 96-well plates, Whelan says his team has been able to reduce the time required to test a complete set of 90-plus reference compounds from 18 months (performed manually) to about eight weeks.

Automation plays an even bigger role at the EPA’s NCCT, which developed the Toxicity Forecaster (ToxCast) panel used in the oil dispersant study.

According to Kavlock, the NCCT, formed in 2005, was “basically given a blank wall” regarding the creation of a “more efficient, more effective, and maybe more intelligent” way to assess chemical hazards. The team’s strategy was to borrow the tools of the pharmaceuticals industry, pushing tens of thousands of compounds through automated, high throughput platforms.

In the first phase of ToxCast, the NCCT applied a battery of more than 500 tests to some 309 chemicals, using computational methods to develop what they call “predictive signatures” of some harmful biological action, which may then be used to infer the behavior of other chemicals. Three signatures have already been published, including one 36-assay signature of reproductive toxicity in rats.

Phase 2 of ToxCast, which currently is ongoing, extends this work to 1,061 chemicals (including pesticides, failed pharmaceuticals, industrial chemicals, and so on) and 700 tests.

According to Kavlock, those 700 assays represent some 10 platforms, including cell-free assays (such as receptor-binding assays); reporter gene assays; and co-culture assays in which activation of one-cell type produces a response in the second—plus one zebrafish-based test.

ToxCast’s first task, Kavlock says, is prioritizing chemicals for animal testing, specifically for endocrine toxicity. Currently, EPA is using the so-called Tier 1 Endocrine Disruptor Screening Battery of 11 animal and in vitro tests for that purpose. But each chemical costs between half-a-million and a million dollars to run, Kavlock says, and the full battery takes about a year. The full ToxCast panel costs about \$30,000 per compound, and EPA is testing another thousand compounds (in addition to the 1,000 Phase 2 compounds) on a subset of 80 or so endocrine-focused ToxCast tests at a cost of about \$4,000 apiece.

“We’ll have basically 2,000 chemicals we’ve tested through all of these different endocrine assays, and we’ll be able to rank order those and provide that information to the EPA program office to say, these are the ones you ought to ask industry to look at first, because they look like chemicals that we know can cause endocrine effects.”

### TOWARDS A HUMAN TOXOME

ToxCast is part of a broader federal program called Tox21, in which the EPA, the **National Institutes of Health (NIH)**, and the **U.S. Food and Drug Administration (FDA)** are collaborating to rapidly put some 10,000 compounds (including the 1,000 Phase 2 ToxCast compounds) through 30 assays in 1,536-well plates on a robotic platform at the NIH Chemical Genomics Center in Rockville, Maryland. The goal is to map the complete set of biochemical pathways implicated in toxicologic responses, so that more targeted assays of toxicity may be developed.

Hartung calls those “pathways of toxicity” the “toxome” and is using a \$6 million NIH Director’s Grant to help map them. His primary tools: transcriptomics and mass spectrometry-based metabolomics.

Hartung’s team will use **Agilent Technologies’** gene expression microarrays and liquid chromatography-mass spectrometry to map the gene expression and metabolome changes that result from exposure of two human breast cancer cell lines to some 53 pro-estrogenic agents, compounds like bisphenol A that activate estrogen-responsive pathways. They will use chemical inhibitors and RNAi to validate the identified pathways.

Hartung says he expects to find no more than perhaps a few hundred such pathways that, when mapped, could form the foundation for a set of simple cell and in vitro assays that almost any lab could run. “There’s only a certain number of critical infrastructures in the cell where you can harm the cell,” he explains, “and whatever we are using which is perturbing the physiology is somehow converging towards these critical infrastructures.”

Of course, his initial toxome efforts per se won’t be able to map all those mechanisms—it focuses only on two cell lines and “a handful” of toxicants, he says. But by moving onto other cell systems and inhibitor classes, Hartung can broaden his search. For instance, he was recently awarded another grant by the FDA to study mechanisms of developmental neurotoxicity. And he anticipates other researchers will join him, inputting data on still more pathways into their database through a kind of open wiki

system. "In a process which has some quality control as joint governance, but a process which is open, we hope more and more of these pathways will get into the system," he says.

### ANIMAL-FREE INDUSTRY

Industry, too, is moving away from animal testing. **Procter & Gamble (P&G)**, for instance, has been developing animal-free alternatives to toxicology testing for nearly 30 years, says Len Sauers, the company's vice president for global sustainability.

"The ethical and moral issue is a primary driver, but there are some real business drivers for wanting to get out of animal testing," he says. Over the years, he says, P&G's toxicologists—there currently are 150 on staff—have developed some 50 methods and published nearly 1,000 papers on the subject.

P&G employs a multi-tiered process for toxicology testing. The first step, says Sauers, is structure-activity relationship (SAR) analysis.

To feed those SAR studies, the company has compiled a database of "every toxicity study that's ever been run and is in the public literature," says George Daston, a Victor Mills Society Research Fellow at P&G, including published papers, public domain EPA submissions, and so on. "That literally results in hundreds of thousands if not one million or so line item pieces of information on the toxicity of materials," he says.

That database allows the company to make intelligent predictions about possible toxicities, and to test them directly. For instance, Daston says, perhaps some new ingredient has a structural fragment that previously has been associated with thyroid peroxidase inhibition. "We'll just set up an assay and evaluate the new chemical and see whether it does that."

Now, says Daston, the company has turned its sights on gene expression analysis, a technology P&G is "investing pretty heavily in." In one recent study, the company's researchers identified a 71-gene signature of uterine cell response by 17- $\alpha$ -ethynyl estradiol. "These results indicate that transcript profiling can serve as a viable tool to select reliable in vitro systems to evaluate potential estrogenic activities of target chemicals and to identify genes that are relevant for the estrogen response," the authors wrote.

### REDUCE, REFINES, REPLACE

For P&G, this emphasis of animal-free testing has reduced animal testing dramatically, says Sauers. "Ninety-nine percent of our assessments today are done without animal testing."

As the toxome comes into focus and the platforms become more widespread, the broader research community can likewise reduce their animal usage. Several such assays are commercially available, including the MucilAir assay from **Epithelix** (a cell culture system that includes the three cell types found in airway epithelium) and **Thermo Fisher Scientific's** ToxInsight Endocrine Profiler Panel (a fluorescence-based cell culture test for endocrine disruptors).

Yet no matter how sophisticated the system, cell culture and in vitro assays—not to mention computer models—are just no match for live animals whose many organ systems and cell types can react differently to chemical agents. For instance, researchers cannot reliably predict a priori a chemical's bioavailability and biodistribution as well as how it will be processed in the liver. "We're very good with in vitro methods at

### FEATURED PARTICIPANTS

**Agilent Technologies**  
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**Cellomics**  
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**Epithelix**  
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**European Commission  
Joint Research Centre**  
ec.europa.eu/dgs/jrc/  
index.cfm

**Johns Hopkins University  
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www.jhsph.edu

**National Institutes  
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www.nih.gov

**Procter and Gamble**  
www.pg.com

**Thermo Fisher Scientific**  
www.thermoscientific.com

**U.S. Environmental  
Protection Agency**  
www.epa.gov

**U.S. Food and Drug  
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www.fda.gov

**Vrije Universiteit Brussel**  
www.vub.ac.be/english/  
index.php

telling you, assuming a chemical reaches a cell, what happens," says Whelan. "We're not very good at saying how much of that chemical will be bioavailable in a certain tissue over time based on the exposure."

Some animal testing is thus inevitable, especially as pharmaceuticals are not covered by the European testing bans. But that doesn't mean there won't be improvements. For decades, the mantra in the world of laboratory animals has been the so-called 3Rs, which encourages researchers to *Reduce* the number of animals they use, *Refine* the assays to reduce distress, pain, and suffering, and ultimately, *Replace* animals with alternative methods.

Europe's cosmetics testing ban focuses just on one *R*, says Vinken: replacement. But a more realistic approach to limiting animal testing would focus on all three, he explains.

Take, for instance, the local lymph node assay (LLNA). The LLNA is a reduced and refined skin sensitization test that uses mice instead of guinea pigs and is based on the extent of stimulation of lymphocyte proliferation in regional lymph nodes draining the site of application of the test substance. The major refinement, Vinken says, is that the actual end stage of the sensitization process—erythema and edema—doesn't occur in the experimental animal, reducing its distress. That is not the case for conventional animal-based skin sensitization tests.

The U.S. regulatory body charged with validating animal-free alternatives, called ICCVAM, has to date approved 44 such methods. The equivalent European body has validated six more in such areas as eye irritation and reproductive toxicity. Yet one area for which no alternatives exist is long-term toxicity testing—mimicking, for instance, the allergic responses that might result from repeated, long-term exposure to a chemical.

Researchers are on the case, but the bottom line, says Kavlock, "is we're a long way away from animal-free toxicology."

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DOI: 10.1126/science.opms.p1200062