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Beyond the vertebrate: Integrating advanced in vitro, in silico, and human-based models to implement the 3Rs in biomedical research and education

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Abstract
The growing use of experimental animals in biomedical research and education has intensified ethical, scientific, and economic concerns. Millions of animals are used in laboratories worldwide each year, and animal experimentation raises unavoidable moral questions while also requiring skilled personnel, specialised housing, and substantial financial investment. The Three Rs framework — Replacement, Reduction, and Refinement, first articulated by Russell and Burch (1959) — provides a structured strategy for minimising animal use without compromising scientific quality. This review surveys the principal categories of non-animal and reduced-animal alternatives currently available, including in vitro cell- and tissue-based systems, in silico and computational models, organ-on-a-chip microphysiological systems, donated human tissue, human patient simulators, non-invasive human imaging techniques, and invertebrate model organisms. For each category we summarise the underlying technology, its demonstrated applications, and its comparative performance against traditional animal tests where data are available. We also examine the practical, regulatory, and scientific limitations that currently constrain wider adoption of these alternatives. We conclude that no single alternative method can yet fully replace animal models across all research contexts, but that the coordinated use of multiple complementary approaches can substantially reduce reliance on animal experimentation while improving the human relevance of research and educational outcomes.

Keywords: laboratory animals, animal ethics, 3Rs, alternative methods, model organisms, organ-on-a-chip

Introduction

Animal models have long been used in experimental research to advance human knowledge and address biological and biomedical questions. However, growing concern for animal welfare and increasing recognition of animal rights have brought greater scrutiny to the ethical dimensions of this practice. Although animals have featured in human inquiry for millennia, the systematic development of animal models expanded chiefly during the 18th and 19th centuries, when scientists such as Jean Baptiste van Helmont, Francesco Redi, John Needham, Lazzaro Spallanzani, Antoine Lavoisier, and Louis Pasteur conducted animal experiments to investigate the origin of life (Oparin, 1957) [20]. Animal models have since contributed to major advances across many biological fields (Institute of Medicine and National Research Council, 1988, 1991; Lieschke and Currie, 2007) [14, 15, 17], and animals continue to play a significant role in behavioural and biomedical research: many medical advances that improve human health stem from carefully designed and strictly regulated animal studies.

Animals have contributed to the development of new drugs, vaccines, surgical techniques, and anaesthesia protocols. Despite ongoing concern about the reliability of extrapolating clinical relevance from animal data (Greek and Menache, 2013) [11], a wide range of species continues to be used in biomedical research, including insects (Drosophila), nematodes (*Caenorhabditis elegans*), fish (*Danio rerio*), amphibians (*Xenopus*), and mammals such as mice, rats, dogs, cats, pigs, and non-human primates, largely on the basis of their phylogenetic proximity to humans (Institute of Medicine and National Research Council, 1991) [15].

Animals are used in research for several interconnected reasons. They provide models for understanding how diseases develop and spread — for example, primate studies of viral infections analogous to human AIDS. They support the development of new treatments, as illustrated by mid-20th-century canine studies that informed heart-valve replacement and vascular graft procedures. Controlled animal studies allow researchers to standardize variables such as temperature, diet, and medication exposure, improving the precision of biological inferences. Findings from such studies can also feed back into veterinary and animal welfare practice, and animal research often lays the groundwork for later human clinical studies.

At the same time, animal experimentation carries substantial costs beyond the primary ethical objection that sentient animals should not be made to suffer for human benefit (Rollin, 2003) [21]. These include the need for highly trained personnel, labour-intensive protocols, and the high recurring costs of animal breeding, housing, and regulatory compliance (Balls, 1994) [3]. Replacing animal testing where scientifically feasible does not imply accepting greater risk to patients or slowing medical progress; rather, substituting validated non-animal methods can enhance both the scientific quality and the ethical standing of research (Directive 2010[6]/63/EU). This review summarizes the principal non-animal and reduced-animal alternatives now available, evaluates their comparative strengths and limitations, and considers the practical barriers to their wider adoption.

Animal Models Commonly Used in Biomedical Research

Table 1 summarises the animal and non-mammalian model organisms most frequently used to study fundamental aspects of disease biology and drug discovery, alongside key features relevant to their experimental use.

Table 1: Common model organisms used to study fundamental aspects of disease and drug discovery.

Common Name	Scientific Name	Body Size	Genome Size	Whole Genome Sequenced	Homology to Humans*	Generation Time / Key Features	Typical Use
Baker's yeast	*Saccharomyces cerevisiae*	~5–10 µm	~12 Mb (~6,000 genes)	Yes (1996)	~23%	2 hours; single-celled, genetically tractable	Cell cycle, gene expression, toxicology
Roundworm	*Caenorhabditis elegans*	~1 mm	~100 Mb (~20,000 genes)	Yes (1998)	~40%	3 days; transparent body, simple anatomy	Aging, apoptosis, neurobiology
Fruit fly	*Drosophila melanogaster*	~3 mm	~140 Mb (~14,000 genes)	Yes (2000)	~60%	10 days; short lifespan, well-mapped genome	Genetics, behavior, neurodegeneration
Zebrafish	*Danio rerio*	~3–4 cm	~1.4 Gb (~26,000 genes)	Yes (2013)	~70%	3 months; transparent embryos, rapid development	Developmental biology, neurobiology, drug screening
Mouse	*Mus musculus*	~8–10 cm	~2.7 Gb (~23,000 genes)	Yes (2002)	~85%	3 months; vertebrate model, rapid development	Drug screening, toxicological testing

Genome sequencing milestones: S. cerevisiae (Goffeau et al., 1996) [10]; C. elegans (C. elegans Sequencing Consortium, 1998); D. melanogaster (Adams et al., 2000); D. rerio (Howe et al., 2013) [1, 4, 12]; M. musculus (Mouse Genome Sequencing Consortium, 2002) [18].

When feasible, toxicology testing is increasingly performed using biochemical or cell-based (*in vitro*) systems rather than live animals. Researchers have, for example, developed *in vitro* methods to identify severe eye irritants and substances likely to cause allergic contact dermatitis. Developing reliable in vitro tests for endpoints such as carcinogenicity or teratogenicity remains more difficult, however, owing to the biological complexity of these processes. Laboratory animals therefore remain extensively used across biomedicine, keeping questions about their welfare, and about the scientific and economic costs of their use, firmly on the research agenda.

The Three Rs Framework

Russell and Burch's The Principles of Humane Experimental Technique (1959) [22] introduced the 3Rs — Replacement, Reduction, and Refinement — as a structured strategy for minimising animal use without compromising the quality of scientific work. The framework remains the internationally recognised ethical standard for animal use in science and underpins current legislation such as the European Union's Directive 2010[6]/63/EU, which requires that an animal procedure not be performed if another scientifically satisfactory method is reasonably and practicably available.

- **Replacement:** substituting traditional animal models with non-animal systems (computational models, biochemical or cell-based systems) or replacing a more sentient species with a less sentient one (for example, a mouse with a nematode).
- **Reduction:** decreasing the number of animals used to the minimum necessary to achieve valid, statistically robust results.
- **Refinement:** minimising pain and distress in animals that are still used, and improving their overall well-being through better housing, handling, and enrichment.

Several factors continue to slow the adoption of alternative methods despite the 3Rs' formal legal and ethical standing. Institutional conservatism favours established protocols because they are easier to compare against historical animal data; regulatory review can default to a checklist approach that does not always reflect current scientific capability; and even validated alternatives face substantial bureaucratic hurdles before they are formally accepted by regulators. Organisations such as Cruelty Free International conduct in-house research and support the development of new or modified bioassays intended to replace animal experiments or reduce the number of animals used in existing *in vivo* assays, and engage directly with regulators to encourage acceptance of validated alternatives.

Alternative and Complementary Approaches

1. *In Vitro* Testing

In vitro tests allow researchers to predict, with increasing accuracy, how drugs, chemicals, cosmetics, and other consumer products will affect humans. The Wyss Institute at Harvard University has developed "organs-on-chips" containing human cells cultured to mimic the structure and function of human organs and organ systems, enabling drug and toxicity testing and disease research without relying on animal models. These systems can replicate aspects of disease progression, drug response, and human physiology with greater relevance than many traditional animal experiments, and several organ-chip products are now commercially available to other research groups.

- Human-blood-cell-based pyrogen tests now allow researchers to detect drug contaminants that would previously have been assessed by injecting or infusing test substances into restrained animals.
- Devices such as the VITROCELL exposure systems (developed by a German manufacturer) expose human lung cells *in vitro* to inhaled substances, replacing methods that historically confined rodents to restraint tubes for prolonged inhalation exposure.

2. Computer Models (In Silico)

Advances in computing power have made it increasingly possible to model organ systems computationally. Computer models of the heart, lungs, kidneys, skin, digestive tract, and musculoskeletal system already exist and can be used to run virtual experiments based on existing physiological and pharmacological data. Data-mining and quantitative structure–activity relationship (QSAR) approaches, of the kind incorporated into several OECD test guidelines for chemical hazard assessment, allow researchers to predict the likely toxicity of a novel substance based on data from structurally similar compounds already characterised.

3. Organ-on-a-Chip Systems

Three-dimensional tissue culture, commonly known as organ-on-a-chip technology, is one of the most notable developments in the search for *in vitro* human microphysiological systems capable of replicating organ-level, and in some cases organism-level, function. These microfluidic devices contain hollow channels lined with living human cells and tissues, cultured under dynamic fluid flow, and vary considerably in scale and design (Ingber,

2022) [13]. Some devices reproduce organ-level structural features such as tissue–tissue interfaces and physiologically relevant mechanical cues — for example, breathing motion or peristalsis-like movement — that are important for accurately modelling organ physiology and disease states (Esch *et al*., 2014) [7]. Fluidically coupling two or more organ chips allows researchers to build "body-on-a-chip" multi-organ systems that approximate whole-body physiology, including drug distribution and disposition. Advances in stem cell technology, including induced pluripotent stem (iPS) cells and patient-derived organoids, now also allow patient-specific cells to be incorporated into organ chips, opening the door to individualised preclinical models.

4. Human Tissue and Cell-Based Models

Human volunteers can donate healthy and diseased tissue for the direct study of human biology and disease, offering a more physiologically relevant alternative to animal-based studies. Donated tissue can be sourced from surgical transplants, elective procedures, and biopsies. Reconstituted human skin and other tissue-equivalent models, for instance, have been developed to replace the historically used Draize irritation tests conducted on rabbits.

5. Human Patient Simulators

Human patient simulators (HPS) represent one of the newer approaches in health-professions education. An HPS is a lifelike manikin designed to respond physiologically in a manner comparable to a real patient, made possible by increasingly sophisticated embedded software. This technology allows both normal and abnormal physiological responses — such as an asthma attack — to be simulated for instructional and training purposes, and in some systems can also replicate therapeutic interventions and adverse drug responses (Alinier *et al*., 2004) [2].

6. Non-Invasive Human Imaging and Microdosing

Brain imaging technologies that allow non-invasive visualisation of internal brain structure and function can be used to monitor the progression and treatment of neurological disease, and to compare patients against healthy volunteers in order to better understand disease mechanisms. A complementary technique, microdosing, allows researchers to measure how very small, radio-labelled doses of an experimental drug behave in the human body — typically assessed in blood samples using a highly sensitive accelerator mass spectrometer — without exposing volunteers to pharmacologically active doses.

7. Invertebrate Models

Invertebrate animals have been used as medicinal agents for around 4,000 years and as formal research and teaching models since the late 1800s (Wilson-Sanders, 2011) [26]. Interest in invertebrate models has grown substantially over recent decades as the scientific community has responded to public concern over the use of vertebrate animals in research, and invertebrates are increasingly recognised as valid models for a wide range of diseases and conditions. Their use has contributed to advances across nearly every field of biology and medicine, including the study of ageing and embryonic development. The invertebrate species used as models range from terrestrial nematodes and insects to freshwater and marine organisms such as planarians, crustaceans, and molluscs (Wilson-Sanders, 2011) [26]. The fruit fly Drosophila melanogaster and the nematode Caenorhabditis elegans remain the most widely used invertebrate models; C. elegans in particular is valued for its short generation time and transparent body, which greatly simplify experimental observation (Strange, 2006; Gilbert, 2006; Karathia *et al*., 2011) [9, 16, 24].

Comparative Performance of Alternative Methods

Where validation data exist, several non-animal methods have demonstrated predictive performance comparable to, or exceeding, the traditional animal tests they are intended to replace. Cruelty Free International (n.d.) reports that combined chemistry- and cell-based alternative approaches to skin-sensitisation testing can achieve considerably higher concordance with human outcomes than the guinea-pig assays they replace, and that reconstituted human skin models substantially outperform the rabbit-based Draize skin-irritation test. Cell-based embryotoxicity assays are likewise reported to improve detection of strongly toxic developmental hazards relative to standard rodent teratogenicity testing, and analytical chemistry methods for shellfish toxin detection have replaced the mouse bioassay historically used for this purpose. These figures originate from advocacy and validation summaries rather than from a single peer-reviewed comparative study, and should be treated as indicative of a general trend toward improved predictivity rather than as precise, independently verified statistics; readers seeking exact concordance figures should consult the primary validation studies conducted by regulatory bodies such as the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM).

More broadly, the predictive validity of animal models themselves has been questioned in the systematic-review literature. Greek and Menache (2013) [11] argue that, even after accounting for methodological improvements such as protocol standardisation, animal models frequently fail to reliably predict human responses to drugs and disease — a finding that strengthens, on scientific as well as ethical grounds, the case for developing and validating human-relevant alternatives.

Benefits of Alternative Approaches

- **Ethical improvement:** reduced reliance on higher vertebrates such as primates and dogs.
- **Reusability:** many *in vitro* and in silico models can be reused across multiple studies and research groups, independent of time and location.
- **Cost-effectiveness over time:** although initial implementation costs can be high, alternative methods may ultimately reduce the recurring costs of animal acquisition, transport, and housing (Van der Valk *et al*., 1999) [25].
- **Educational flexibility:** alternative models allow students to repeat procedures and self-assess until learning objectives are met, without repeated animal use.
- **Visualisation:** video, 3D, and computational tools can demonstrate physiological phenomena that are difficult or impossible to observe directly in live animal models.
- **Alignment with the 3Rs:** direct support for the Replacement, Reduction, and Refinement of animal use.

- **Speed:** many alternative models have short life cycles or rapid assay turnaround, accelerating the pace of research.
- **Relevance:** some models offer closer genetic or physiological correspondence to the specific human process under study than a traditional animal model would.

Key Considerations in Selecting Alternative Models

- Relevance to human physiology for the specific research question being addressed.
- Degree of genetic and molecular similarity to humans.
- Availability of validated genetic, molecular, and analytical tools for the chosen model.
- Ethical acceptability relative to the traditional animal method being replaced.
- Practical considerations of cost, infrastructure, and ease of handling.

Limitations and Challenges

Despite their promise, current alternative methods have important limitations that should temper expectations of a rapid, wholesale replacement of animal models. *In vitro* and organ-on-a-chip systems, however sophisticated, typically model isolated organs or tissues and cannot yet fully reproduce whole-organism phenomena such as systemic immune responses, neuroendocrine regulation, or complex behaviour. Computational models remain constrained by the quality and completeness of the biological data used to build and validate them, and can propagate errors or biases present in their training data. Organ-chip and iPS-cell-based platforms remain relatively costly and technically demanding to produce at scale, and standardised validation protocols for regulatory acceptance are still being developed for many endpoints. Invertebrate models, while genetically tractable and ethically less contentious, diverge from human physiology in ways that limit their applicability to certain disease areas, particularly those involving complex organ systems absent in the model species.

Regulatory acceptance also lags behind technical development in many jurisdictions: a validated alternative method is not automatically accepted for regulatory submissions until it has passed formal, often lengthy, validation and endorsement processes (for example, through EURL ECVAM or OECD test-guideline adoption). As a result, animal testing frequently persists not because it is scientifically superior for a given question, but because it remains the default comparator against which regulators and researchers judge new evidence. Addressing this gap will require sustained investment in cross-validation studies, harmonised international standards, and continued engagement between scientists, regulators, and educators.

Conclusion

No single alternative method currently available can fully replace animal models across the entire range of biomedical research and educational applications. However, the methods reviewed here — *in vitro* and organ-on-a-chip systems, computational and data-driven models, donated human tissue, human patient simulators, non-invasive imaging, and invertebrate model organisms — are individually capable of replacing, reducing, or refining animal use in specific, well-defined contexts, and their capabilities continue to expand rapidly. Applied in concert, and guided by the 3Rs framework, these approaches can meaningfully reduce the scale of animal experimentation required in science while improving the human relevance of research findings and preserving the quality and safety of downstream medical and educational outcomes. Continued investment in the validation, standardisation, and regulatory acceptance of these methods — alongside sustained institutional support for researchers adopting them — will be essential to realising this potential.

Conflict of Interest

The authors declare no conflict of interest.

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