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REVIEW

High-throughput screening and small animal models, where are we?

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Current high-throughput screening methods for drug discovery rely on the existence of targets. Moreover, most of the hits generated during screenings turn out to be invalid after further testing in animal models. To by-pass these limitations, efforts are now being made to screen chemical libraries on whole animals. One of the most commonly used animal model in biology is the murine model *Mus musculus*. However, its cost limit its use in large-scale therapeutic screening. In contrast, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the fish *Danio rerio* are gaining momentum as screening tools. These organisms combine genetic amenability, low cost and culture conditions that are compatible with large-scale screens. Their main advantage is to allow high-throughput screening in a whole-animal context. Moreover, their use is not dependent on the prior identification of a target and permits the selection of compounds with an improved safety profile. This review surveys the versatility of these animal models for drug discovery and discuss the options available at this day.
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Keywords: *Caenorhabditis elegans*; *Drosophila melanogaster*; *Danio rerio*; high throughput screening; drug discovery; chemical genetics; disease; drug target

Abbreviations: BDSC, Bloomington Drosophila Stock Center; CGC, Caenorhabditis Genetics Center; COPAS, Complex Object Parametric Analyzer and Sorter; HTS, High Throughput Screening; RNAi, RNA-mediated interference; TILLING, Targeting Induced Local Lesion In Genome; ZFIN, Zebrafish Information Network; ZIRC, Zebrafish International Resource Center

Introduction

During the last two decades, drug research has been subject to major mutations. Entire genome sequencing, DNA microarrays, miniaturization, informatics and robotics have drastically changed the approach of this art. Pharmaceutical companies were the first to fully combine these emerging technologies and knowledge to establish robust methods for drug discovery (Figure 1.1). One is called High Throughput Screening (HTS) and consists in random screens of compounds to find hits showing an activity or an affinity on a selected target and/or in a model considered representative of a disease (Spring, 2005). This approach has contributed to identify therapeutic compounds, pathway, cell functions, chemical probes, with the ultimate goal of comprehensively delineating relationships between chemical structures and biological activities. Another one is called virtual screening and consists in *in silico* intelligent drug design against characterized targets (Bajorath, 2002).

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Despite numerous successes, the pharmaceutical industry is experiencing a slow-down in the development of new and innovative medical products, due to three major reasons. First, these methods are roughly reliant on the existence of identifiable and screenable targets (Lindsay, 2003). Despite a wealth of information about normal physiology and disease pathology, it is still difficult to predict which targets will effectively reverse a disease phenotype; this fact is particularly true for loss-of-function disease where the identification of pharmaceutically relevant targets is often difficult (Segalat, 2007b). Second, mechanisms involved in some diseases cannot be reproduced *in vitro*. Cells and tissues are physiologically connected and this interplay may be crucial in the evolution of some disorders. Third, most of the hits generated by traditional screening turn out to be invalid once tested in mouse, resulting in a waste of funds and efforts. Absorption, solubility, distribution, metabolic stability, toxicological problems in later animal studies present numerous difficulties which turn to a dead-end for most hits (Bleicher *et al*., 2003).

To by pass these limitations, efforts are now being made to screen chemical libraries on whole-animals (Figure 1.2). In medical sciences, the mouse *Mus musculus* is one of the most commonly used animal model due to its genetic, physiological and anatomical similarities to the human system (West

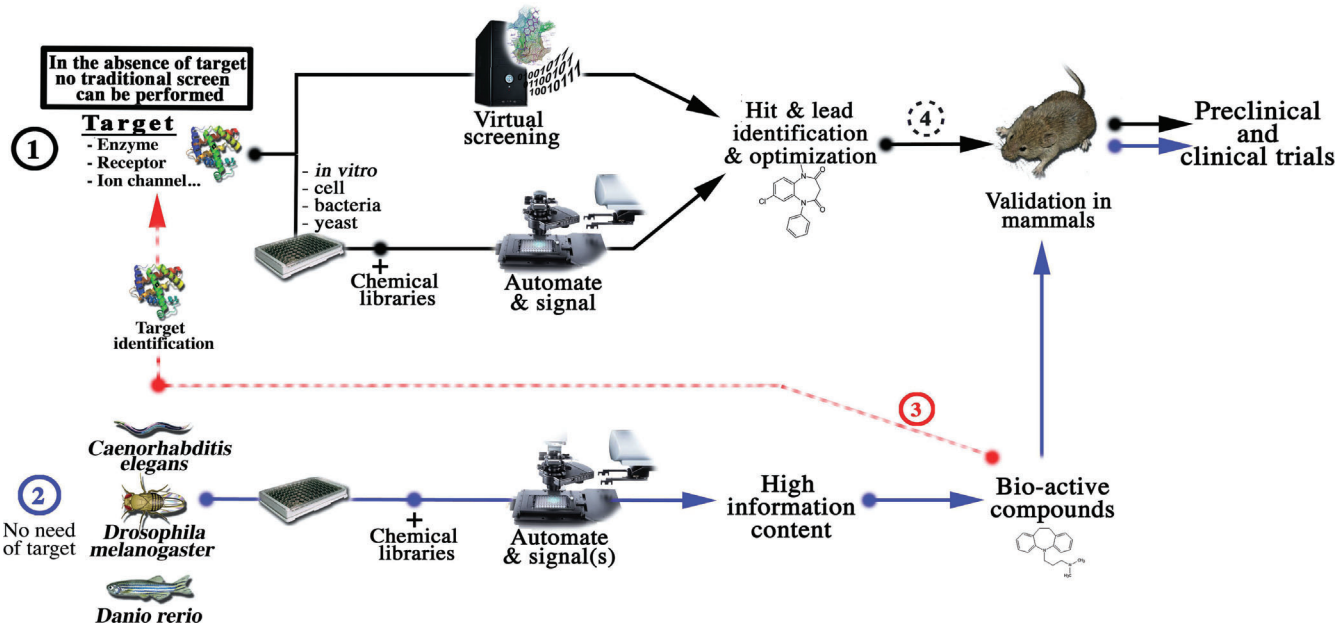

Figure 1 Whole-animal screening and drug discovery process. (1, black lines) show a schematic view of the different stages which came upon a drug discovery process based on traditional HTS. In the absence of target or in complex mechanism, screen can hardly set up. (2, blue line) An alternative may come from phenotypic chemical screens with small animal models like *C. elegans*, *D. melanogaster* and *D. rerio*. (3, red line) Identification of hits in these models may reveals new molecular mechanisms and targets. The target could be further used in traditional HTS. (4) *C. elegans*, *D. melanogaster* and *D. rerio* may also bridge the gap between traditional high-throughput screening and validation in mammalian models. HTS, High Throughput Screening.

et al., 2000). However, its cost limit its use in large-scale therapeutic screening. On the contrary, with the development of robotic and automated imaging, several tiny animal models, like *C. elegans*, *D. melanogaster* and *D. rerio*, are gaining momentum as screening tools for drug discovery (Lieschke and Currie, 2007; Segalat, 2007a). These organisms combine genetic amenability, low cost and culture conditions compatible with large-scale screening. Their main advantage is to allow high-throughput screening in a whole animal context. Moreover, their use is not dependent on the prior identification of target.

Finally, these new pharmaceutical screening tools will allow (i) the identification of new active compounds that may eventually be validated on mammals (Figure 1.2), (ii) the identification of unsuspected targets and/or molecular mechanisms which could be used in traditional HTS based on target binding or function (Figure 1.3), (iii) the generation of supplementary information which can turn useful to establish a database linking phenotypic activity to chemical structure.

This review discusses the versatility of these models for drug discovery, and try to make an overview of the options currently available at each step of the screening process.

Model attributes overview

C. elegans was introduced by Sydney Brenner in the 1960s as a model organism to study animal development and the nervous system. Since, this little worm has led to landmark discoveries on many molecular mechanisms such as cell death, ageing, development and neuronal function (Lendahl and Orrenius, 2002; Putcha and Johnson, 2004; Kenyon, 2005). It has also been used as a model for host-pathogen interactions and for neurotoxicological research (Moy *et al.*, 2006; Leung *et al.*, 2008; Peterson *et al.*, 2008). Its genome is fully sequenced and more than 50% of human genes have a *C. elegans* counterpart (Harris *et al.*, 2004). Several human diseases can be recapitulated in this model by knocking down a selected gene or by expressing a deleterious version of it. Unfortunately, the absence of tools to perform targeted mutations complexify this approach. However, many strains have already been generated by large-scale mutagenesis and are available at the Caenorhabditis Genetics Center (Table 1). Indeed, the conservation of such strains is easy and systematic as *C. elegans* can be stored indefinitely by cryo-conservation (Stiernagle, 2006). In parallel, the NemaGENETAG project has generated a large collection of 14 000 transposon-tagged mutants strains which can be obtained upon request (Bazopoulou and Tavernarakis, 2009). Additionally, double-stranded RNA-mediated interference (RNAi) is an alternative and useful method for gene disruption in *C. elegans* (Fire *et al.*, 1998). Moreover, several genetic tools have been developed to generate genetically engineered strains, like transgenesis or the MosTIC technology (Rieckher *et al.*, 2009; Robert *et al.*, 2009). Finally, the Wormbase website allows access to an exhaustive database containing comprehensive data on gene structures, mutants and RNAi phenotypes, microarray data, protein-protein interactions and more (Chen *et al.*, 2005).

Moreover, due to its little size, its short life cycle, its simple growth conditions and its low-cost, this animal has extensively been used in large-scale genetic screens (Sugimoto, 2004; Bazopoulou and Tavernarakis, 2009). The adult *C. elegans* reach ~1 mm in long and ~80 μm in diameter, whereas embryos are about ~50 μm long and ~30 μm in

Table 1 Overview of animal models characteristics for drug discovery

	C. elegans	*D. melanogaster*	*D. rerio*	*M. musculus*
Practical issues				
Generation time	3 to 5 days	10 to 14 days	3 to 4 months	3 to 4 weeks
Embryos size	50 μm	100 μm	1 mm	N/A
Adult size	1 mm	3 mm	6 cm	10 cm
Growth conditions	Solid or liquid medium	Solid medium	Liquid medium	Cage
Easiness to obtain individuals for testing	++++	++++	+++	+
Ressource				
Databases	Wormbase (Harris *et al.*, 2004; Chen *et al.*, 2005; Kaletta and Hengartner, 2006)	Flybase (Matthews *et al.*, 2005; Drysdale, 2008)	ZFIN (Sprague *et al.*, 2003; Henken *et al.*, 2004; Meli *et al.*, 2008)	N/A
Stock centre	CGC (Stiernagle, 2006)	BDSC (Matthews *et al.*, 2005)	ZIRC (Henken *et al.*, 2004)	N/A
Storage	++++ (frozen)	++	+++ (sperm frozen)	+
Strains available	++++	+++	+	++
Characterized inbred strains	+++	++	+	+++
Annual cost	++++	++++	+++	+
Similarity to human				
Number of gene (approximately)	19 000	13 000	25 000	25 000
Homology to human (genome)	>50%	>60%	>70%	>90%
Anatomical similarity	+	++	+++	++++
Pathological similarity	+	+	++	++++
Molecular and genetic similarity	++	++	+++	++++
Molecular tools				
Targeted gene KO/time	No	No	No	Yes months
Forward genetic tools	Yes (Jorgensen and Mango, 2002)	Yes (Venken and Bellen, 2005)	Yes (Solnica-Krezel *et al.*, 1994; Amsterdam, 2003)	No
Reverse genetic tools	Yes (Fire *et al.*, 1998; Bazopoulou and Tavernarakis, 2009)	Yes (Venken and Bellen, 2005; Matsushima *et al.*, 2007)	Yes (Nasevicius and Ekker, 2000; Wienholds *et al.*, 2002)	No (excepted targeted KO)
Generation of transgenic organism	Weeks (Rieckher *et al.*, 2009)	Weeks (Venken and Bellen, 2005)	Months (Davidson *et al.*, 2003; Esengil and Chen, 2008; Ogura *et al.*, 2009)	Months
Tissue-specific promoters available	++++	++++	++	+
Antibody reagents	+	+	+	+++
Issues for drug discovery				
Current use in drug discovery	+	+	++	++++
Available sorting equipment	Eggs to adults (Pulak, 2006; Rohde *et al.*, 2007)	Eggs to larvae (Pulak, 2006)	Eggs to embryos (Pulak, 2006)	N/A
Culture in microtiter plate	Eggs to adults	Eggs to larvae	Eggs to larvae	N/A
Equipment cost	++++	++++	+++	+
Screenning throughput	Medium to high	Low to medium	Low to medium	Very low
Examples of chemical screen	Gill *et al.*, 2003; Burns *et al.*, 2006; Kwok *et al.*, 2006; Breger *et al.*, 2007; Boyd *et al.*, 2009)	Stilwell *et al.*, 2006	Burns *et al.*, 2005; Murphey and Zon, 2006; Tran *et al.*, 2007; Hong, 2009	N/A
Review on drug discovery	Artal-Sanz *et al.*, 2006; Kaletta and Hengartner, 2006; Segalat, 2007a	Nichols, 2006; Whitworth *et al.*, 2006	Parng *et al.*, 2002; Lieschke and Currie, 2007; Rocke *et al.*, 2009	N/A

+,++,+++,++++, relative strength of the model in each category; CGC, Caenorhabditis Genetics Center; BDSC, Bloomington Drosophila Stock Center; N/A, not available; ZFIN, Zebrafish Information Network; ZIRC, Zebrafish International Resource Center.

height. This little size allow the use of a specific automated pippeting system at all developmental stages (see below). Animals can easily be cultivated in multi-well plates in agar or liquid medium with a diet of *Escherichia coli*. Its rapid life cycle is temperature-dependent (3.5 days at 20°C) which is convenient for experiment planification. Moreover, its optically transparent body allow to detect functional and morphological changes without having to kill or dissect the organism.

Despite these advantages for modelling molecular disorders and for large-scale screening, *C. elegans* has several drawbacks. The major one is that some diseases cannot be reproduced because the animal does not have the corresponding genes or organs. In this case, an alternative could be the fly *Drosophila melanogaster* or the fish *Danio rerio*, closer to mammals in the evolutionary tree.

The fruit fly *D. melanogaster* was introduced as an animal model in the beginning of the 20th century. *D. melanogaster* has typically been used over the years for genetics, development, signal transduction and cell biology studies, but has also recently been employed for pharmacological research purposes (Manev and Dimitrijevic, 2004; Arias, 2008). The entire *Drosophila* genome has been sequenced and annoted, and more than 60% of human genes have functional orthologs in *D. melanogaster* (Table 1) (Bernards and Hariharan, 2001; Celniker and Rubin, 2003; Bier, 2005). Over 75% of the human disease genes in the Online Mendelian Inheritance in Man database present strong protein sequence conservation with *D. melanogaster* genes (Reiter *et al.*, 2001). Then, this animal model has become a popular organism for studying human diseases (Botas, 2007; Doronkin and Reiter, 2008). *D. melanogaster* also benefit from a comprehensive range of methods for carrying out molecular genetic research such as mutagenesis, RNAi and transgenesis (Table 1) (Venken and Bellen, 2005; Matsushima *et al.*, 2007). Moreover, there are several efforts under way to mutate every predicted gene. Although *D. melanogaster* cannot be conserved frozen, it is easy to maintain, and thousands of strains are available from the Bloomington Stock Center (Matthews *et al.*, 2005). Additionally, an exhaustive database information relative to its genetics and its molecular biology is freely available (Drysdale, 2008). Indeed, the FlyBase database contains detailed phenotypic reports for over 2500 mutants and more are characterized every year.

Its size, although larger than *C. elegans*, allow manipulation of its eggs and its embryos (~100 μm) with a specific pipetting automate (see below). Although *D. melanogaster* has already proven its potential for large-scale genetic screening, this animal cannot be grown in liquid medium which limit its use in HTS (Nusslein-Volhard and Wieschaus, 1980; Nagy *et al.*, 2003). However, more and more chemical screens take advantage of it (Nichols, 2006; Whitworth *et al.*, 2006; Segalat, 2007a).

The high degree of functional conservation in cell-biological processes between mammals and invertebrates suggests that diseases resulting from disruption of conserved cellular processes can be recapitulated at a genetic and molecular level in flies and worms. However, one has to keep in mind that the anatomy and physiology of invertebrates are significantly different from those of humans and that, as a consequence, these animals can produce only a partial picture of the human symptoms. On the contrary, the zebrafish (*Danio rerio*) is a vertebrate model which is more similar to humans. During the last 20 years, this tropical aquarium fish was almost exclusively used to study organ development (Streisinger *et al.*, 1989; Grunwald and Eisen, 2002). Indeed, it was established as a model to bridge the gap between worm/fly and mouse/human for understanding embryonic development. Its interest for research increased substantially approximately 10 years ago, following the demonstration that it was amenable to large-scale forward genetic screens (Eisen, 1996). This resulted in the characterization of an exceptionally large number of genes involved in vertebrate pathways, and contributed to the establishment of the zebrafish as a relevant model for human diseases and pharmaceutical research (Driever *et al.*, 1996; Alestrom *et al.*, 2006). Moreover, the zebrafish genome has now been sequenced, and is part of a large database freely available at the Zebrafish Information Network (ZFIN) and at the FishMap website (Sprague *et al.*, 2003; Meli *et al.*, 2008). Additionally, ZFIN provides exhaustive information to serve the needs of the research community, such as methods, anatomical descriptions, developmental processes, mutants phenotype and more. Finally, working closely with ZFIN, the Zebrafish International Resource Center maintain numerous zebrafish strains and frozen sperm which can be obtained upon request (Henken *et al.*, 2004). Unfortunately, the number of available mutant strains with respect to the number of genes is still limited. The genetic resources for zebrafish continue to expand steadily with the existence of hundreds of genetic mutants from large-scale mutagenesis experiments, the availability of transgenic morpholino knock-down techniques and of the Targeting Induced Local Lesion In Genome and other genetic manipulations such as the sleeping beauty transposase system which allow gene insertion under the control of tissue- and/or time-specific promoters (Table 1) (Nasevicius and Ekker, 2000; Wienholds *et al.*, 2002; Davidson *et al.*, 2003; Esengil and Chen, 2008; Ogura *et al.*, 2009).

An attractive feature of zebrafish assays for pharmacology investigations is the optical transparency of its embryos and its larvae which allow *in vivo* observation of morphological defects. Moreover, in contrast to rodents, the zebrafish embryos develop externally from the mothers and most of the internal organs, including the cardiovascular system, gut, liver, kidney and muscle develop rapidly in the first 24–48 h (Westerfield, 1995). Then, a wide range of biological and disease process may be studied at an early developmental stages (Chico *et al.*, 2008). In addition, the zebrafish embryo is small (5 mm at 7 days post fertilization), inexpensive, hardy and easy to produce in large numbers. Then, screens may be conducted in microtiter plates (Barros *et al.*, 2008; Hong, 2009).

Screen for what?

HTS with whole animals can be subdivided in two distinct approaches (Figure 2). The first approach is comparable to the classical forward genetic screens which consist in random genetic modifications of wild-type animals by mutagenesis.

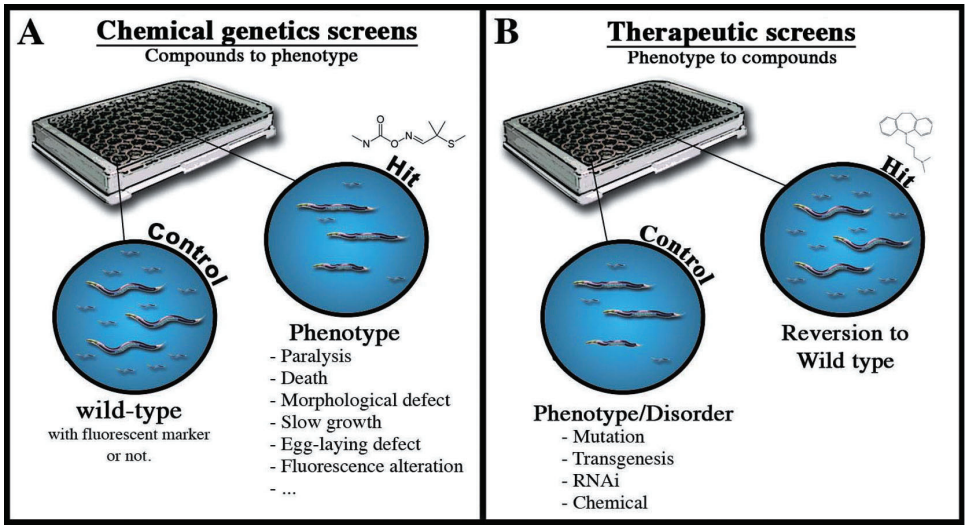

Figure 2 High Throughput Screening with whole animals can be separated in two distinct approaches. (A) Hits are selected for their ability to induce a phenotype in a wild-type strain (growth, behaviour, morphology defects or other detectable trait). (B) Hits are selected for their ability to reverse an abnormal phenotype to the wild-type phenotype. Once a bioactive compound is identified, the flexibility and the versatility of these small animals models may rapidly conduct to understand the mechanism of action of each lead at a low cost.

Similarly, chemical libraries may be tested on wild-type animals for their ability to produce a particular phenotype such as slow growth, lethality, uncoordinated movement, morphological defects and more (Figure 2A). In contrast to traditional biochemical assays that focus on specific molecular targets, a screen based on a phenotypic observation has the advantage of being independent of the specific molecular target involved. Then, depending on the end-point measure (readout), a large variety of bio-active molecules may be detected in the same screen. Additionally, experiments could further lead to the identification of unsuspected targets (see below) (Artal-Sanz *et al.*, 2006; Perrimon *et al.*, 2007). An example of this approach is well illustrated by the screen made by Kwok *et al.* which screened 14 100 small molecules for bioactivity in wild-type *C. elegans* and identified 308 compounds that induce a variety of phenotypes, including slow growth, uncoordinated movements and morphology defects (Kwok *et al.*, 2006). One of these compounds, named nemadipine-A, induces morphology and egg-laying defects. Through a genetic suppressor screen, Kwok *et al.* further identified *egl-19*, a calcium channel, as the sole candidate target of this compound in *C. elegans* (Burns *et al.*, 2006; all drug/molecular targets nomenclature follows Alexander *et al.*, 2008). Moreover, by showing that nemadipine-A can also antagonize vertebrate L-type calcium channels, they demonstrated the relevance of this approach for drug discovery.

The second approach consists in testing chemical libraries for their ability to reverse an abnormal phenotype to the wild-type phenotype (Figure 2B). HTS is performed on animals that reproduce a disorder or at least, some features of it. For this purpose, mutants strains which mimic human disease are already available or may be generated by mutation, transgenesis or in certain case by chemical treatments (Table 2). As an example, *C. elegans* and *D. melanogaster* models of Alzheimer's, Parkinson's and Huntington's disease are available and are being exploited (Link, 2006; Nichols, 2006; Kuwahara *et al.*, 2008). In the case of Alzheimer, loss-of-function mutations in *C. elegans* homologs of presenilin cause a defect in egg-laying (Smialowska and Baumeister, 2006). Then, the amount of eggs layed may be quantitated to monitor the therapeutical potential of chemical compounds. Comparably, amyloid deposits may be observed in transgenic *C. elegans* expressing the human β-amyloid peptide (Link, 2006). As this accumulation induces a paralysis phenotype, a screen based on a locomotion readout could be designed (see below). Similarly, because *C. elegans* is killed by many pathogens such as *Pseudomonas aeruginosa*, *Serretia marcescens*, *Salmonella enterica*, *Staphylococus aureus* and *Streptococcus pneumoniae*, a basic screen based on survival may lead to the identification of new anti-microbials agents (Tenor *et al.*, 2004; Sifri *et al.*, 2005; Moy *et al.*, 2006). Finally, in a more subtle approach, the help of fluorescent markers is an additional way of measuring a disorder parameter. Like *C. elegans*, many mutants and transgenic strains of *D. melanogaster* present relevant defects that may be used to design a screen (Table 2). Nevertheless, culture conditions of *D. melanogaster* limit its use to embryos in an HTS process.

Although invertebrate models present a high degree of functional conservation with human (Table 1), they often show a partial picture of human processes and, in certain cases, cannot reproduce the disorder as they do not have the corresponding gene and organ. To by-pass this drawback, the vertebrate fish *D. rerio* could be an alternative, in particular in immunity and cardiovascular disorders, but also in inflammation and in cancer (Table 2). For example, zebrafish develops malignant tumours in response to mutagens, carcinogens or by gene mis-expression (Beckwith *et al.*, 2000; Spitsbergen *et al.*, 2000; Mizgireuv and Revskoy, 2006). Inducing malignancy in transgenic fish carrying oncogenes with fluorescently tags, or fish with appropriate fluorescently marked cell types, results in fluorescent tumours, allowing recognition of tumour onset, location and the estimation of tumour bulk (Langenau *et al.*, 2003; 2005a,b). This feature, coupled with the optical transparency of its larvae, may be

Table 2 Example of disease models showing quantifiable defects

Disease or disorder	*Example of model*	*Quantifiable phenotype/defect*	*Reference*
Alzheimer's disease			
C. elegans	Transgenics: human beta-amyloid expression	Paralysis due to amyloid deposit	Link, 2006
	Mutants: *sel-12*, homologue of Human presenilin	Egg-laying defect	Smialowska and Baumeister, 2006
D. melanogaster	Transgenics: human beta-amyloid expression	Progressive locomotor defects, premature death	Nichols, 2006
D. rerio	Knock-down: Zebrafish *psen1* or *psen2* morpholinos injection	Developmental and morphological defects	Nornes *et al.*, 2009
	Knock-down: zebrafish *pen-2* morpholinos injection	Neuronal cells loss	Campbell *et al.*, 2006
Parkinson's disease			
C. elegans	Transgenics: human alpha-synuclein expression	Locomotor defects, dopaminergic cell loss	Kuwahara *et al.*, 2008
D. melanogaster	Transgenics: human alpha-synuclein expression	Locomotor defects	Nichols, 2006
D. rerio	Knock-down: zebrafish parkin morpholinos injection	Dopaminergic cell loss	Flinn *et al.*, 2009
	Chemical treatment: PD-inducing neurotoxins	Behavioural and locomotor defects	Bretaud *et al.*, 2004
Duchenne muscular dystrophy			
C. elegans	Mutants: *dys-1*, homologue of human dystrophin	Progressive muscle degeneration and paralysis	Gieseler *et al.*, 2000
D. melanogaster	RNAi: reduction of all dystrophin isoform expression	Progressive muscle degeneration and increased pupae lethality	van der Plas *et al.*, 2007
D. rerio	Knock-down: zebrafish dystrophin morpholinos injection	Locomotor and morphological defects	Guyon *et al.*, 2003
Spinal muscular atrophy			
C. elegans	Mutants: *SMN-1*, homologue of human SMN	Locomotor defects, pharyngeal pumping defects	Briese *et al.*, 2009
	RNAi: reduction of *SMN-1* expression	Egg-laying defect	Briese *et al.*, 2009
D. melanogaster	Mutants: SMN, homologue of human SMN	Larval lethality and developmental defects	Chang *et al.*, 2008
D. rerio	Knock-down: zebrafish SMN morpholinos injection	Embryonic lethality and developmental defects	Schmid and DiDonato, 2007
Other muscular disorders			
C. elegans	Mutants: *unc-52*, homologue of human perlecan	Progressive paralysis	Rogalski *et al.*, 2001
D. melanogaster	Mutants: drosophila delta-sarcoglycan	Reduced life span and locomotor defects	Allikian *et al.*, 2007
D. rerio	Mutants: zebrafish laminin a2 (sapje or candy½oss)	Progressive detachment of muscle ¼bres	Ingham, 2009
	Mutants: zebrafish ryr1b, homologue of human RYR1	Locomotor defects (slow swimming behaviour)	Ingham, 2009
Cancer			
D. rerio	Transgenics: mouse c-myc expression	Lethal acute lymphoblastic leukemia	Langenau *et al.*, 2003; Langenau *et al.*, 2005a
	Transgenics: human *BRAF* melanocyte-specific expression	Malignant melanoma	Patton *et al.*, 2005
	Chemical carcinogen exposure	Assorted tumours (sarcoma, seminoma . . .)	Beckwith *et al.*, 2000; Spitsbergen *et al.*, 2000
Fat metabolism			
C. elegans	Mutant: *daf-2*	Fat accumulation	Ashrafi *et al.*, 2003
D. melanogaster	Mutants: *Adp* (Adipose)	Fat accumulation and sterility	Schlegel and Stainier, 2007
D. rerio	Transgenics: AgRP overexpression (agouti-related Protein)	Fat accumulation	Song and Cone, 2007
Pathogen/immunity			
C. elegans	A variety of human pathogens	Premature death	Sifri *et al.*, 2005
D. melanogaster	Tuberculosis-like disease (*mycobacterium marinum*)	Premature death	Dionne *et al.*, 2003
D. rerio	Streptococcal infections (*streptococcus iniae*)	Premature death	Neely *et al.*, 2002

used in large-scale screens to identify enhancers or suppressors of tumours (Stern and Zon, 2003). Similarly, even if zebrafish do not spontaneously develop cardiovascular diseases analogous to those seen in humans, some related mechanisms may be modelled in zebrafish embryos such as cardiomyopathy, thrombosis, arteriogenesis and cardiac regeneration (Chico *et al.*, 2008). Unfortunately, at this day, the lack of appropriate tools for automated manipulation

and analysis limit the throughput of screens based on the zebrafish model.

Finally, in comparison with *D. melanogaster* and *D. rerio*, an advantage of the *C. elegans* model for modelling molecular disorders is the possibility to interfere with almost any gene function at any time in its life cycle by delivering RNAi by feeding (Timmons and Fire, 1998). Therefore, chemical screens with essential genes are also possible with this model. For this purpose, a *C. elegans* RNAi feeding library may be obtained from the Geneservice company (Cambridge UK). This library consists of 16 757 bacterial strains dispensed in 384-well plates, which cover 87% of *C. elegans* genes. As RNAi is delivered by feeding, it is easy to incorporate it an HTS process in combination with chemical compounds. One has to keep in mind that RNAi is poorly active on the nervous system of *C. elegans*, whereas a lot of molecule may act in this way. However, a recent study showed that this limitation could be overturned by using a specific strain (Kennedy *et al.*, 2004). Indeed, Seiburth *et al.* used an *eri-1; lin-15B* strain to perform a large-scale RNAi screen that identified more than 100 novel genes involved in synaptic transmission (Sieburth *et al.*, 2005).

Options in process design

Defining a relevant model is a crucial step for the success of a screening campaign. In addition, to identify hits among the multitude of tested compounds, a screen must generate comparable, homogeneous and reliable data. Moreover, it needs to be performed in an automatic manner to allow a high-throughput (Zhang *et al.*, 1999). Even if small animal models have already proved their versatility for large-scale studies, scientists usually manipulate these organisms by manually collecting, sorting and transferring individual animals. As a result, large-scale screens often take months or even years, increasing the risk of generating heterogeneous and non-quantitative data. Indeed, whole-animal screens are much more difficult to automate than *in vitro* screens. Animals size is not suitable with traditional HTS equipment, and culture volumes are often incompatible with large-scale chemical screens. However, emerging methods where culture is performed in minute volumes in 96- or 384-well plates, coupled with the development of robotics, open the way to new possibilities (Table 1) (Lehner *et al.*, 2006; Murphey and Zon, 2006; Pulak, 2006; Vogt *et al.*, 2009). For example, the development of the Complex Object Parametric Analyzer and Sorter (COPAS) Biosort (Union Biometrica, MA, USA), which allows the automatic dispensing of precise numbers of animals into multiwell plates, minimize the initial manipulation of individual animals and therefore dramatically increases the quality and the throughput of the screen. Indeed, the COPAS, like a flow-through sorter, can rapidly sorts objects based on several criteria including length, optical density and several channels of fluorescence (Pulak, 2006). Although all *C. elegans* stages can be manipulated with this machine, only eggs and embryos of *D. melanogaster* and *D. rerio* can.

Another important point of the assay design is the compounds concentration. This question is well debated for *in vitro*- or cell-based assays. On the contrary, in the case of whole animals, as the target is not always known and the treatment usually delivered through the media, it is difficult to accurately predict the range of doses which have to be tested. Compounds penetrate by both ingestion and diffusion through the animal epiderm (Kaletta and Hengartner, 2006). Indeed, the concentration of a given compound within the animals cannot be predicted and it varies depending on the chemical properties of the compound. As a consequence, negative results cannot be interpreted because it is hardly possible to determine if a negative result is due to poor penetration, docking problems or a true absence of biological activity in the model. It is particularly true for the *C. elegans* model, which is surrounded by a thick cuticle which provides protection from environmental chemicals (Page and Johnstone, 2007). A recent study evaluated that the absorbed concentration of the 5-hydroxy tryptophane is approximately 100–1000× lower than in the medium (Carre-Pierrat *et al.*, 2006). To avoid missing hits, a conceivable approach may be to test compounds at several concentrations, defined by pre-existing data and by the molecular properties of the chemical library. If the number of assays are limited, the compounds may be tested at a high concentration in the medium. With this approach, a lot of of compounds will display a toxic activity, but they could be further re-tested at a lower concentration.

Options for automatic and quantitative read-out?

Another crucial step which has a profound effect upon the quality of the information produced, and upon the throughput, is the output measure (the readout). As a machine is not appropriate to detect the unsuspected, whole animal screening have often relied on laborious observation and manual scoring by small teams of highly trained students and post-docs (Evanko, 2006). Moreover, data generated were often non quantitative and ambiguous. For example, a recent study described a valuable procedure to screen molecules for their ability to induce a phenotype, nevertheless, their throughput was hampered due to the manual interpretation of the data (Burns *et al.*, 2006). In this study, *C. elegans* larvae were deposited using the COPAS biosort into 24-well plates on top of the agar-chemical mixture. Three to 5 days later, images of the wells were acquired and archived using a HiDI2100 automated imaging system. With this automated method, up to 2400 compounds per week could be screened. Unfortunately, in absence of robust statistical methods to extract data, phenotypes had to be characterized from the archived images which was not better or more rapid than manually scoring phenotype at the dissection microscope. Moreover, the manual interpretation of the data was ambiguous and non quantitative. There is thus a need for more rapid, and more consistent, methods for scoring phenotypes. It is obvious that the ultimate goal of the automatization will be to attempt a comprehensive description of all 'observable perturbation' using a large list of numerical parameters. This approach is very complex, it will be highly demanding of programming skills and need considerable progress of the current automated imaging systems. However, several methods already

Table 3 Example of automated phenotypic observation

Example of observation	*Example of automatic readout*	*References*
C. elegans		
Global picture of well	Automated imaging of 24-well plate (solid media)	Burns *et al.*, 2006
Life span/death	Measurement of fluorescent dye SYTOX (marker of nematode death)	Gill *et al.*, 2003
Egg-laying	Quantification of the chitinase released into the culture medium	Ellerbrock *et al.*, 2004
Locomotion	Measurement of average speed and paralysis into 35 mm petri plates (solid media)	Tsibidis and Tavernarakis, 2007; Ramot *et al.*, 2008
	Measurement of swimming into multiwell plate (liquid media)	Restif and Metaxas, 2008; Tsechpenakis *et al.*, 2008
Fluorescent cells or tissues	Automated profiling of individual animals into multiwell plate (liquid media)	Pulak, 2006; Rohde *et al.*, 2007
D. melanogaster		
Behaviour and locomotion	Quantification of locomotor activity rhythms	Branson *et al.*, 2009
	Measurement of jump reflex (habituation/learning)	Sharma *et al.*, 2009
	Measurement of aggression and courtship	Dankert *et al.*, 2009
	Measurement of locomotion and social behaviours	Reiser, 2009
Fluorescent cells or tissues	Automated imaging and analysis of Drosophila embryos	Pulak, 2006; Peng *et al.*, 2007
D. rerio		
Histology overview	Automated process which generate digital larval slides for review and annotation	Sabaliauskas *et al.*, 2006
Locomotion/behaviour	Analysis of location and orientation of zebrafish larvae into multiwell plate	Winter *et al.*, 2008; Creton, 2009
Fluorescent cells or tissues	Automated imaging and analysis of zebrafish embryos into multiwell plate	Burns *et al.*, 2005; Tran *et al.*, 2007; Vogt *et al.*, 2009

exist at this day to measure the switch between abnormal phenotype to wild-type phenotype, or reciproquely (Table 3).

As an example, Gill *et al.* have developed a method to study the life span of worms. This 'survival assay' combine automated worm-handling technology with automated real-time fluorescence detection (Gill *et al.*, 2003). To measure nematode death, they added to the culture the fluorescent dye SYTOX, a nucleic acid stain that binds to DNA in damaged cells. Then, fluorescence was measured using a traditional fluorescence plate reader, allowing the quantification of the amount of dead worms per well. This approach may, in principle, be applied to the identification of pharmacological agents that extend life span of worms or kill them, as well. Additionally, with addition of pathogens in the culture, this method may be used to screen for anti-microbial or anti-fungal compounds. The same approach could be developed for *D. melanogaster* et *D. rerio* (Kang *et al.*, 2002; Gerhard, 2007).

With traditional equipment, several other events may be measured in an automatic manner. For example, to measure egg-laying behaviour of *C. elegans*, a pharmaceutical company has developed an indirect method, called the 'chitinase assay' (Ellerbrock *et al.*, 2004). It consists of measuring the chitinase activity that is released from the eggs by hatching larvae. Therefore, this activity reflects the amount of hatching larvae per well. As many disorders models, like neurodegenerative disorders, affect egg-laying behaviour of *C. elegans*, this rapid measure could be used to monitor the efficiency of the compounds (Smialowska and Baumeister, 2006; Liau *et al.*, 2007; Briese *et al.*, 2009). Nevertheless, one has to keep in mind that the more indirect the observation is, the more false negative are expected.

Additionally, neuro-degenerative, neuro-muscular and several other disorders often result in locomotion or behavioural defects in these models (Table 2). As this type of injury is particularly relevant to human disease, different teams have developed systems to monitor locomotion and behaviour in an automatic and quantitative manner (Tsibidis and Tavernarakis, 2007; Buckingham and Sattelle, 2008; Ramot *et al.*, 2008; Restif and Metaxas, 2008; Tsechpenakis *et al.*, 2008). As an example, a worm-tracker system called the 'thrashing assay' is used to evaluate the effect of drugs and mutations on locomotion (Tsechpenakis *et al.*, 2008). Nevertheless, these worm-trackers avoid excessive computational demand by extracting a limited set of features and are time-consuming at this day. In parallel, similar efforts are being made by *Drosophila* and Zebrafish laboratories (Hicks *et al.*, 2006; Rosato and Kyriacou, 2006; Flinn *et al.*, 2008; Winter *et al.*, 2008; Branson *et al.*, 2009; Creton, 2009; Dankert *et al.*, 2009; Reiser, 2009; Sharma *et al.*, 2009).

Additionally, the optical transparency of the *C. elegans* body, the *D. melanogaster* embryos and the *D. rerio* larvae allows *in vivo* vizualization and tracking of their cells, tissues and fluorescent markers. Furthermore, the known and invariant cell lineage of *C. elegans* may be used to establish robust and accurate measurements. Indeed, *C. elegans* is composed of 959 somatic cells including muscles cells (111), neurons (302), intestine (34 cells), epidermis (213 cells), which are not able to regenerate (Harris *et al.*, 2004). Although this last feature is advantageous for quantitative measurements, it may also be seen as a drawback because many diseases may be treated through the regeneration process.

As example of this approach, mutations in the *C. elegans* homologue of the human dystrophin, a protein involved in the Duchenne muscular dystrophy, result in a progressive paralysis phenotype (Gieseler *et al.*, 2000). As this paralysis is due to a muscle degenerescence, the use of fluorescent markers under the control of a muscle-specific promoter allow

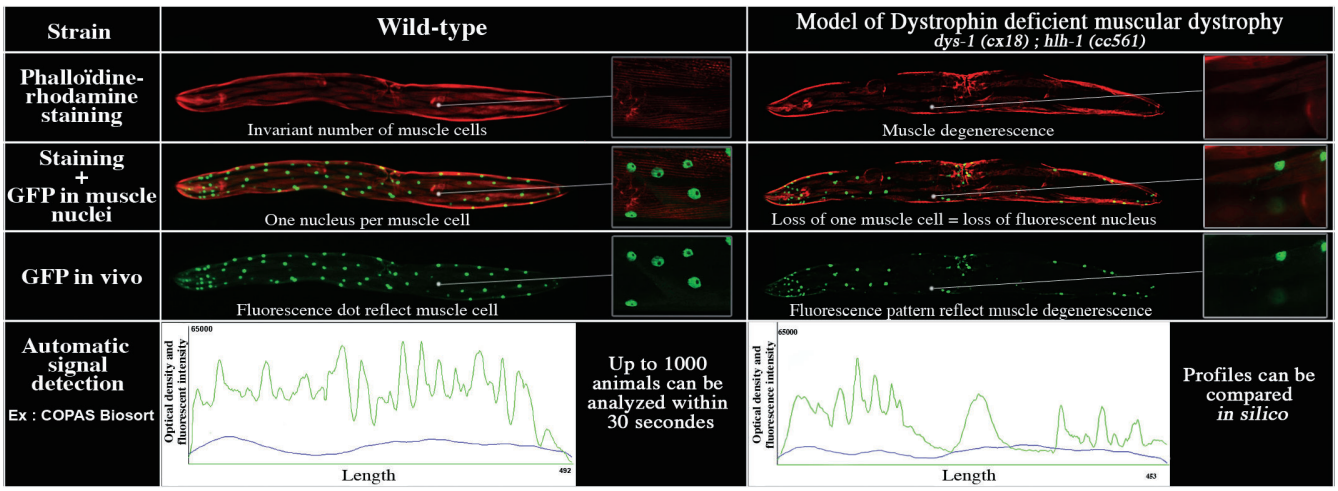

Figure 3 Example of disease model and engineering with *C. elegans*. Mutations in the *C. elegans* homologue of the human dystrophin result in a progressive paralysis phenotype due to a muscle degeneresecence. Traditionally, this phenotype is quantitated by manually scoring the number of absent muscle cells after labelling of actin fibres. This approach is laborious and time-consuming. However, the use of a fluorescent marker under the control of tissue-specific promoter allows the *in vivo* quantification of the disorder. Phenotype comparison is possible with automated imaging system like the COPAS Biosort which records the fluorescent profile of individual animals (bottom panels).

to monitor, *in vivo*, the degree of injury without having to estimate the locomotion or to perform a muscle-specific staining (Figure 3). Then, with appropriate equipment, the fluorescent pattern of each animals may be collected to establish a quantitative measurement of the muscle degeneresence (Figure 3). Two systems are available at this day to monitor the fluorescent profile of *C. elegans*. The first one is the multi-well plate reader of the COPAS biosort which has been previously described (Pulak, 2006). The second one consists in a high-speed microfluidic analyser that can isolate and immobilize *C. elegans* animals in a well defined geometry. Then, fluorescent profiles may be recorded at a subcellular resolution in physiologically active animals (Rohde *et al.*, 2007). Although this recent technology has been designed for sorting, it is attractive for phenotypic analysis at a cellular and subcellular level.

The same strategy may be applied to *D. melanogaster* and *D. rerio* in their early stages (Table 3). For example, several teams use fluorescent marker coupled to automated imaging systems and artificial intelligence-based image analysis to track the angiogenesis process and find compounds which modulate it (Tran *et al.*, 2007; Vogt *et al.*, 2009). Furthermore, their results demonstrated that it is feasible to adapt image-based high-content screening methodology to measure complex whole organism phenotypes in an automatic manner. Another example is described by Burns *et al.* who developed a rapid assay to quantify the effect of novel drugs on heart rate by generating transgenic zebrafish with fluorescent cardiomyocytes. Embryos were distributed in 96-well plates and the heart rate of individual animals was measured by an automated imaging system (Burns *et al.*, 2005).

After the screen?

One of the concerns of using small animal models for drug discovery is the determination of the effective dose for a lead compound. Dose-response experiments are easily feasible but one has to keep in mind that the compounds are provided to the animals through the media (Kaletta and Hengartner, 2006). To meet this challenge, the results obtained with these models will have to be linked to data obtained on laboratory rodents and, when possible, in humans. It is too early to establish if the range of doses to be tested in mammals can be extrapolated from data obtained on *C. elegans*, *D. melanogaster* and *D. rerio*. Furthermore, it may be different from drug to drug. At this day, the confirmation in mammals remains essential. Indeed, mammalian models will remain necessary in drug development to answer fundamental questions of drug pharmacology and toxicity.

For the future of drug discovery, the identification of novel targets seems critical. Then, the flexibility of small animal models is a powerful tool to rapidly understand the mechanism of action of each lead at a low cost (Artal-Sanz *et al.*, 2006; Kaletta and Hengartner, 2006). Several approaches may be used to determine the targets and modes of action of active compounds, such as candidate-based approaches, affinity chromatography coupled to mass spectrometry, micro-array technologies and more global approaches like genome-wide RNAi screen, random mutagenesis or metabo-profiling studies (Kaletta and Hengartner, 2006; Lehner *et al.*, 2006; Blaise *et al.*, 2007; Sleno and Emili, 2008; Blaise *et al.*, 2009).

For example, a screen of a collection of 1000 already approved compounds conducted on the *C. elegans* model of dystrophin-deficient muscular dystrophy led to the identification of several new active compounds (L. Segalat, unpublished results). Two of the most active hits obtained in this screen were methazolamide and dichlorphenamide, which are sulfonamides. As these chemicals are known to be strong inhibitors of human carbonic anhydrase enzymes, implication of these enzymes was investigated in the worm (Giacomotto *et al.*, 2009). For this purpose, RNAi experiments against all putative carbonic anhydrase of the worm were

performed, and conducted to identify CAH-4 as the sole target among the six carbonic anhydrases of the worm.

Alternatively, in the absence of candidate genes, the use of a global approach such as large-scale mutagenesis or genome-wide RNAi screens is an option which may rapidly conduct to the identification of potential targets. For example, as RNAi can be delivered by feeding in *C. elegans*, a secondary RNAi screen – based on the process used for hit identification – may be run in which *C. elegans* genes are tested one by one for their ability to modify the response to the drug. Then, candidate genes are identified by their ability to induce a resistance or a hypersensivity to the hit when inactivated (Burns *et al*., 2006). The major drawback of this method is that the process needs considerable amount of chemicals.

Translation to human

How predictable are the findings made on model organisms such as *C. elegans*, *D. melanogaster* or *Danio rerio*, when it comes to treating humans?

One has to keep in mind that the anatomical and molecular differences of small model organisms with humans may cause the elimination of a significant fraction of the hits generated. At this date, the question of hit predictability in this strategy cannot be answered because there is not enough feedback. One may imagine that the number of compounds which can be translated to human may not be much higher than the attrition rate seen with cellular screens. Along the same lines, the targets identified in these models might help to understand normal and disease biology of these tiny animals but not necessarily be relevant for human.

The lead-to-drug bottleneck, which remains a critical problem of today's drug discovery, will likely not be alleviated by screening on small model organisms. These models should be viewed as complementary alternatives to cellular or in-vitro screening devices, rather than as universal shortcuts to human treatments. Their limitations are numerous. However, the glass should be seen as half-full rather than half-empty. As it was already demonstrated in a few cases, the real added value of these models is more in their ability to reveal targets and pathways that would be missed by more conventional devices. Thus, they feed the downstream pipeline of drug discovery.

Conclusion

All in all, whole-animal screening based on *C. elegans*, *D. melanogaster* and *D. rerio* appears as a new tool in the drug discovery process. Their added value for drug discovery varies from disease to disease, and mainly depends on what alternative options are. Indeed, they are complementary to *in vitro* and cellular systems because their small size and their culture conditions fulfil the requirements for large-scale screens. Moreover, such approaches may permit the selection of potential therapeutic molecules with an improved safety profile earlier in the drug discovery phase, saving both time and funds. Furthermore, the versatility of these small animals for genetic studies potentially allows the rapid target identification of each lead at a low cost. Such new target may eventually feed the traditional HTS strategy based on target binding or function.

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Conflicts of interest

The authors state that they have no conflict of interest.

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