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Automated assays to study longevity in *C. elegans*

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Abstract

The nematode *Caenorhabditis elegans* is excellently suited as a model for studying the genetic and molecular genetic basis of aging, and to test chemical compounds that interfere with the aging process. Mutants of factors in both the insulin and target of rapamycin (TOR) signalling pathways have been shown to extend life span of the worm. Phenotypic similarities among those mutants suggested that, exploiting the corresponding phenotypes in a semiautomated way, may increase the speed of investigating life span and aging in *C. elegans*. Here, we discuss several methodological approaches to automate longevity assays in the nematode.

Keywords: *C. elegans*; Automation; Genetics; *daf-2*; Insulin signalling; TOR signalling; *pep-2*

1. Mutants in the insulin signalling pathway affect longevity in *C. elegans*

Inhibition of the insulin signalling in worms, flies, yeast and rodents leads to extended life spans and results in several developmental and metabolic changes (Bluher et al., 2003; Clancy et al., 2001; Fabrizio et al., 2001; Kenyon et al., 1993; Tatar et al., 2001). In *Caenorhabditis elegans*, a block in the insulin signalling pathway, induced by scarce nutrients, high temperature or mutations in signalling components, results in retarded postembryonic development, constitutive arrest at the dauer larval stage, increased fat storage, defective egg-laying, extended life span, and increased stress tolerance for heat, oxidation and various chemicals (Kimura et al., 1997; Paradis et al., 1999). *C. elegans* insulin-like signalling involves insulin-like ligands, the DAF-2 insulin receptor-like protein, the AGE-1 PI3K-like catalytic subunit and the AAP-1 adaptor subunit, the DAF-18 PTEN lipid phosphatase, and the serine/threonine kinases PDK-1, AKT-1, AKT-2 and SGK-1 (Fig. 1). The latter three transduce the AGE-1/PI3K signals via PDK-1 to control the intracellular localization and activity of the forkhead transcription factor DAF-16/FOXO by phosphorylation (Hertweck et al., 2004; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997; Paradis and Ruvkun, 1998; Wolkow et al., 2000). DAF-16, the major downstream effector of DAF-2 signalling, is negatively regulated by its upstream components. When DAF-2 signalling is abrogated, DAF-16 migrates to the nucleus to modulate transcription. Multiple DAF-16 transcriptional targets are thought to mediate the diverse functions of the insulin signalling pathway. Candidate gene approaches, comparative genomics and DNA microarray analysis revealed several genes, encoding catalases (*ctl-1*, *ctl-2*), superoxide dismutases (*sod-1*, *sod-3*), FK506 binding proteins (*dao-1*, *dao-8*, *dao-9*), nucleolar phosphoprotein (*dao-5*), transmembrane tyrosine kinase (*old-1*), metallothioneins (*mtl-1*, *mtl-2*), heat shock proteins HSP70, HSP90, HSP16, HSP20, and several other genes that are up- or down-regulated by DAF-16 (for review, see Munoz, 2003; Patterson, 2003; Tatar et al., 2003; Wolkow, 2002). The genes affected by differential regulation of DAF-16 are currently under intense investigation (Murphy et al., 2003). The central biological function of *C. elegans sgk-1* in the DAF-2 pathway was recently described (Hertweck et al., 2004) (Fig. 1). Loss of *sgk-1* results in a ~25% extended generation time, defective egg-laying (~70% of the animals die from a bag of worms phenotype at 25 °C), increased stress tolerance to heat, paraquat and H_2O_2, and a ~63% extended life span, similar to worms with *daf-2* mutations.

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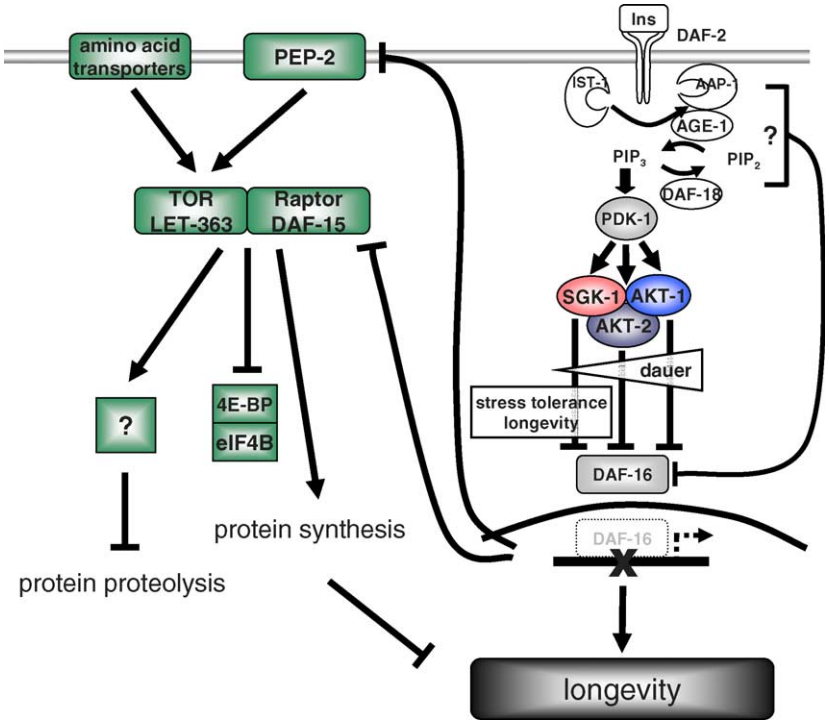

Fig. 1. Proposed cross-talk between insulin signalling, TOR signalling and nutrient sensing in *C. elegans*. The DAF-2 insulin signalling pathway regulates metabolism, development, stress response and life span via the forkhead transcription factor DAF-16. The serine/threonine kinases AKT-1, AKT-2 and SGK-1 suppress DAF-16 function by direct phosphorylation, yet are activated by different cues in the signalling pathway. LET-363/TOR signalling (green) mediates various cellular processes, including growth and translation. The intestinal peptide transporter PEP-2, LET-363/TOR and DAF-15/raptor act in parallel or downstream of the DAF-16 downstream effector of insulin signalling to regulate *C. elegans* life span. See text for details.

SGK-1 acts in parallel to the Akt/PKB kinases AKT-1 and AKT-2, with which it forms a multimeric protein complex. All three kinases in the complex are able to suppress DAF-16 function by direct phosphorylation, yet have different functions in the signalling pathway. While AKT-1 together with AKT-2 are more important for regulating dauer formation, SGK-1 is crucial for the control of the postembryonic development, stress response and life span. Tissue specificity (Libina et al., 2003) and parallel signalling pathways (Paradis and Ruvkun, 1998) contribute to the complexity of the DAF-2 signalling to influence the localization and function of DAF-16. At least one additional, still unknown branch of the DAF-2 pathway seems to exist that is independent of AKT-1, AKT-2 and SGK-1 (Hertweck et al., 2004).

2. The TOR pathway responds to variations in nutrition

The target of rapamycin (TOR) kinase contributes to a signalling cascade that regulates various cellular processes, including initiation of translation, ribosome synthesis, expression of metabolism-related genes, protein degradation, and autophagy in response to nutrients and hormone-dependent mitogenic signals in many species (Abraham, 2002; Chou and Blenis, 1995; Schmelzle and Hall, 2000). It was recently shown in worms and flies that TOR signalling has a function in aging control (Kapahi et al., 2004; Vellai et al., 2003). *C. elegans let-363*/TOR together with *daf-15*/raptor (regulatory associated protein of mTOR) appears to act downstream or in parallel to DAF-16 as mediators of life span regulation by insulin signalling and nutrient sensing (Fig. 1) (Jia et al., 2004; Vellai et al., 2003). This clearly represents a link between nutrition, metabolism, larval development and life span.

Our lab recently characterized *C. elegans pep-2* that was shown to affect both insulin and TOR signalling (Meissner et al., 2004) (Fig. 1). PEP-2 functions in the intestinal absorption of peptide-bound amino-acids for overall protein nutrition of the organism. Loss of *pep-2* results in an abolished uptake of peptides from the gut lumen, ~80% retarded postembryonic development, ~64% reduced progeny, ~28% reduced body size, and increased heat and oxidative stress tolerance. Furthermore, the *pep-2* mutant enhances the developmental, longevity and stress tolerance phenotype of the insulin receptor mutant *daf-2,* and intensifies all aspects of the *let-363*/TOR RNA interference phenotype including development and life span. Therefore, *pep-2* has a predominant role for the delivery of di- and tri-peptides for growth and development which consequently affect signalling pathways that regulate metabolism and aging.

3. Longevity screens in *C. elegans*—future prospects

In the past, several longevity genes belonging to the insulin signalling pathway were found in EMS mutagenesis large-scale screens searching for mutants that constitutively form dauer larvae at higher temperatures (Ailion et al., 1999; Larsen et al., 1995; Malone and Thomas, 1994). Although the DAF-2 pathway regulates both dauer formation and life span, the regulation of both is uncoupled from one another. The pathway regulates diapause during development, whereas aging is influenced during adulthood (Dillin et al., 2002). Thereby, the downstream effector DAF-16 functions tissue-specifically in different so-called signalling centers (Libina et al., 2003). This corresponds to our recent observation that SGK-1 is crucial for the regulation of life span but not or only marginally important for diapause. In contrast, the kinases AKT-1 and AKT-2 acting in parallel to SGK-1 are mainly involved in dauer control, and only marginal in the regulation of life span. Therefore, novel large-scale EMS and RNAi screens are necessary to identify both dauer-dependent and -independent genes that control *C. elegans* life span.

sgk-1 and *pep-2* mutants both have a pleiotropic phenotype which is very similar to the phenotypes of other insulin signalling mutants, including *daf-2*, *age-1* or *pdk-1* (Table 1) (Gems et al., 1998; Morris et al., 1996; Paradis et al., 1999). Some of the *sgk-1* and *pep-2* phenotypes listed in Table 1 are very suitable to perform genome-wide RNAi screens for the identification of suppressors and/or activators of *sgk-1* and *pep-2*. Each of the phenotypic aspects is in principle suitable for screening purposes, yet individual inspection of mutants is tedious and labor-intensive. The identification of new upstream regulators or downstream targets of *sgk-1* and *pep-2* should give new insight into the great complexity as well as the cross-connections between DAF-2 signalling, TOR signalling and nutrient sensing.

In the last 4 years, RNAi screening methods have emerged that allow the genome-wide screening for modifier genes. These were successfully performed on NGM agar plate media or in liquid culture (Fraser et al., 2000; Kamath and Fraser, 2003; Ashrafi et al., 2003; Lee et al., 2003; Nollen et al., 2004). Compared to agar plates, screens on liquid culture medium are generally faster and easier to handle, which makes them better suited for automation. However, the lack of standardization in detecting phenotypes, such as complex behaviour and longevity, has thus far constrained the application of liquid-based cultures. Further improvement of phenotypic assays in this field, including their automation, are therefore of critical importance.

4. Dispensing worms in liquid cultures

In general, handling of worms in liquid cultures can be automated easily using pipeting robots that simplify both the preparation of bacteria cultures (the *C. elegans* food source) and the distribution of worms to individual wells. Distribution of an equal number of animals per well is not trivial, since it requires first collecting the worms in a rather low volume of buffer (to allow the distribution of 10–50 animals per 5–20 μl droplet), and second a vigorous swirling of the culture prior to pipeting in order to avoid the worms from settling to the ground.

Dispensing worms using a FACS like sorting robot greatly facilitates the equal distribution of animals to individual wells. Such a device is now commercially available (COPAS, Union Biometrica) and, in addition, allows in its simplest (and least expensive) version even the distribution of animals at certain developmental stages, due to their sorting by size (Fig. 2). The COPAS machine also facilitates the detection of phenotypic aspects when combined with a fluorescence—laser detection system, and thus became a rather versatile instrument during phenotypic screenings. First use of this technology was recently demonstrated in a quantitative image analysis of an innate immunity gene fused to GFP (Couillault et al., 2004). The large collection of GPF/YFP expression markers now available for *C. elegans* further extends the involvement of such a machine in mutant screens and phenotype detections.

5. Automated tracking of locomotion

Phenotypic aspects that require monitoring behaviours are more difficult to automate, since the optical variations to

Table 1
List of phenotypes seen in several *C. elegans* insulin signalling mutants compared to the phenotypes of *sgk-1(ok538), sgk-1(RNAi)* or *pep-2(lg601)*

Phenotype of several insulin signalling mutants	*sgk-1*(*ok538*) or RNAi	*pep-2*(*lg601*)
Extended life span	+	(−)[a]
Constitutive dauer formation (Daf-c)	(−)[b]	−
Increased stress tolerance	+	+
Extended generation time	+	+
Reduced brood size	+	+
Egg-laying defect (Egl)	+	−
Increased fat storage	−	−
Longer body (Lon)	Thinner/clearer	Shorter

+: Similar phenotype observed, −: not observed.
[a] *pep-2* enhances the life span phenotype of *daf-2* and *let-363*/TOR mutants.
[b] *sgk-1* enhances the Daf-c phenotype of other insulin signaling mutants.

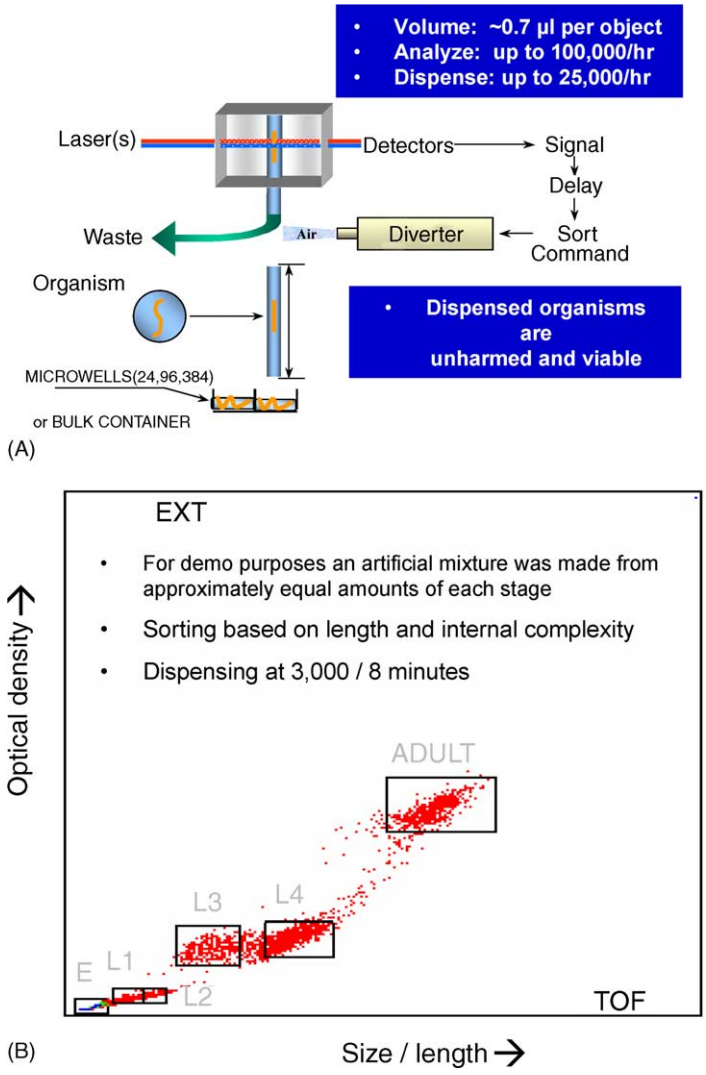

Fig. 2. A FACS-like sorting machine to dispense *C. elegans* animals. (A) Principles of detection and sorting. Individual animals are recognised by altered absorption/transmission of a laser and then either discarded or distributed into the individual wells of a motorised microtiter plate holder. (B) Example for sorting a mixed stage culture of *C. elegans* by length. Rectangular boxes represent collections of animals used for resorting. E, L1-4, Adult: individual developmental stages of animals recognized by length. TOF: time of flight. Figure provided by courtesy of Union Biometrica, Belgium.

be followed are more diverse and cannot easily be standardized. The process flow for an automated detection of mobility can be as follows: a frequently applied method to detect motion in a prerecorded video movie involves the analysis of pixel-flip events. For this purpose, two consecutive images of a film sequences are compared to detect those events where an individual picture pixel swaps from light to dark or vice versa (Fig. 3). Neighboring pixel-flips are then collected to define the size of the moving object. If the size and shape of the recognized object corresponds to the predefined settings, they are defined as a moving worm, and front end (anterior), rear end (posterior) and center can be detected. Next, the movement of this vectorized object ''worm'' are followed to record direction, speed and responses, such as a reversal, when the worm bumps into an obstacle (Fig. 3).

6. Automated scoring of egg-laying behavior

Defective egg-laying behaviour is a frequent phenotypic aspect observed in *C. elegans* mutants. Several mutants of genes in the insulin signalling pathway display retention of eggs in the uterus (and are, thus, egg-laying defective) or lay a reduced number of eggs. Typically, an Egl phenotype is assayed qualitatively by visual inspection of aging adults (which then frequently display a bag-of-worms phenotype, because development of the embryos inside their protective egg-shell proceeds, although the eggs are not laid. This results in embryos hatching inside the mother as a terminal phenotype. Quantification of this phenotype is possible by scoring the (reduced) progeny of the animals and by counting *eggs*—in utero in individual animals under the dissecting scope. The latter is a rather tedious and time-consuming method.

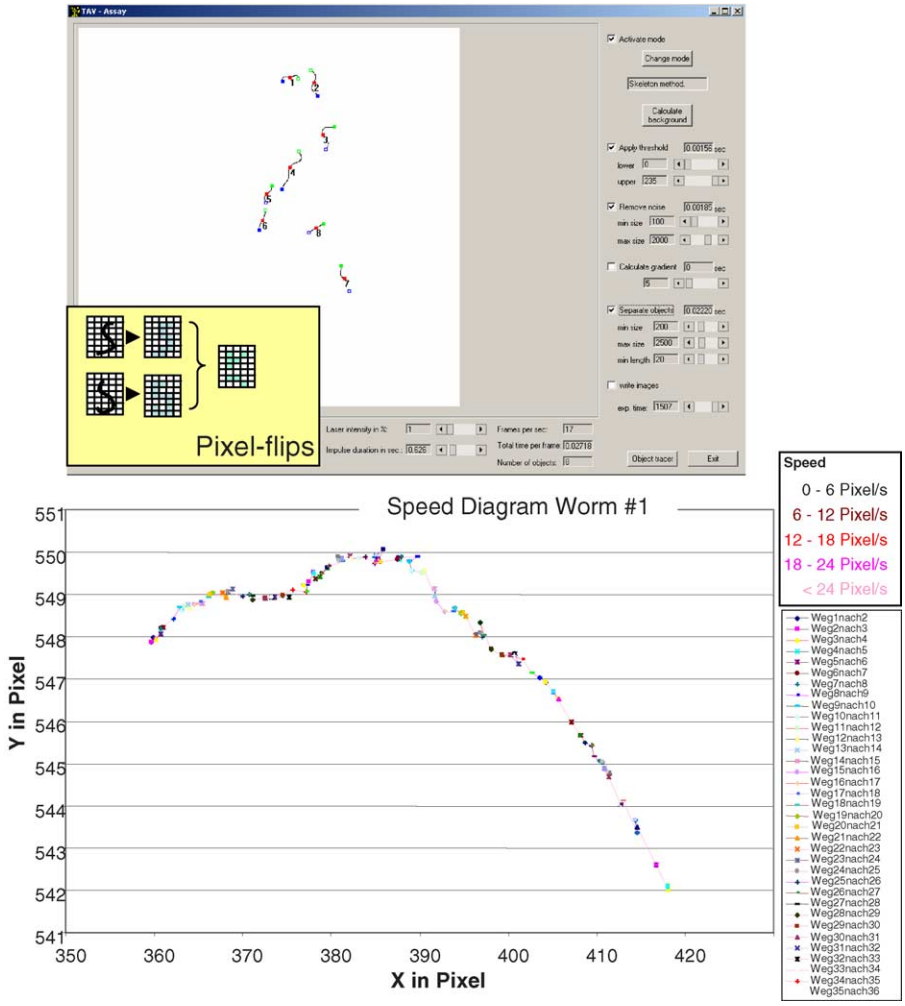

Fig. 3. Automated detection of worm movements. Upper part: screen image of motion detection software. Eight *C. elegans* animals were recognized by movements and subsequently vectorized. Front, rear ends and center of the animals was determined mathematically by the software and is indicated. Insert: principles of detection pixel-flips provoked by animal movements. Lower part: quantification of direction and speed of an individual animal is stored in a spreadsheet table. Data are graphically depicted.

Attempts to automate egg-laying are complicated by the fact that, so far, software-based visual detection of eggs is inferior to detection by the trained eye of an experienced researcher. However, staining of eggs in utero, as well as using transgenic GFP marker genes that are expressed early in embryogenesis, facilitate optical detection. Secondly, layed eggs can be quantitated after detection of individual eggs in the medium. Although automation of egg-laying would be very useful for a number of purposes [for example, to score for anti-Alzheimer drugs using presenilin mutants that display a very strong Egl phenotype (Levitan and Greenwald, 1995), this method is still in its infancies.

7. Scoring complex behaviours and response to environmental stimuli

C. elegans has long been known as an ideal test model to analyze the genetic and neuronal components of an animal to external stimuli (mechanical, chemical, thermal). Aged animals, as well as animals with mutants in age-related disease genes, frequently show a reduced response, e.g. to mechanical stimulation, and loss of mechanosensory responses is used as an indicator of age-related decay (Link, 1995; Parker et al., 2001; Syntichaki et al., 2002).

As an example for automation, we describe here the thermal avoidance response of *C. elegans* [response to stressful heat, applied locally via a laserbeam (Wittenburg and Baumeister, 1999)]. The process flow for such an automated behavioural assay may be as follows: (A) recognition of a moving animal with a high-speed camera. (B) Tracking speed and direction of this animal. (C) Targeting the sensitive areas of the animal (either head or tail) with the laser beam. (D) Recording of latency. (E) Recording of movement changes. (F) Analysis—classification of the reaction.

As in the previous example, the identification of pixel-flips provides the basis for the quantification of movements.

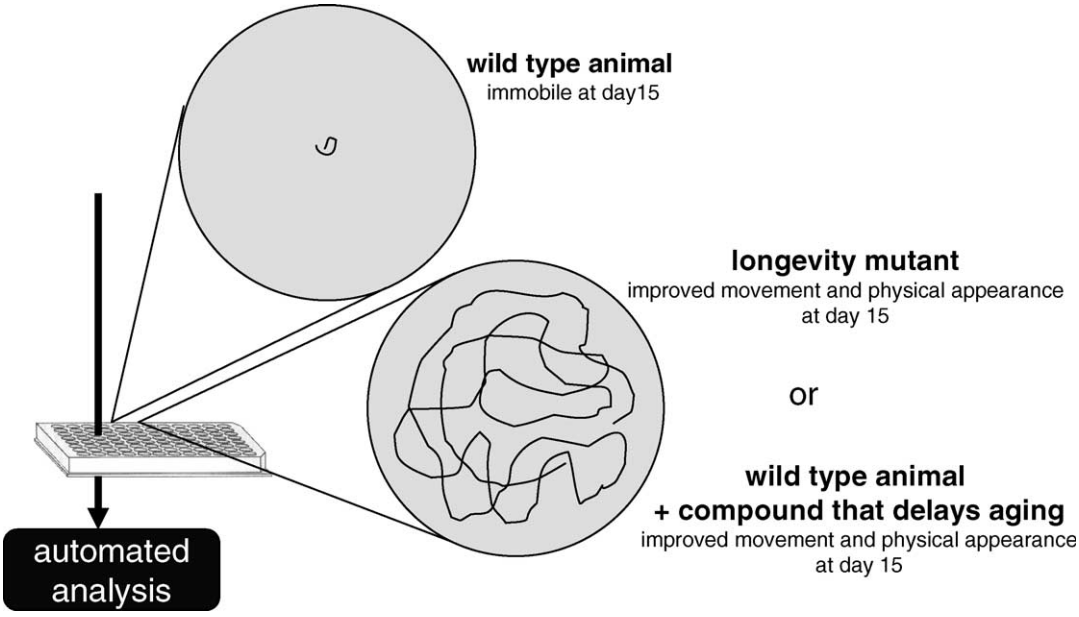

Fig. 4. Principles of a simple longevity assay in liquid culture. Worms are grown in microtiter format and are subjected to automated analyses after mutagenesis or after 2 weeks of exposure to drugs. Whereas wild type animals are typically immobile (dead) at day 15, longevity mutants or animals in which a longevity program was activated chemically survive and move actively.

The determination of front/rear ends of worms is facilitated by the use of GFP markers that are expressed solely on one end of the animal, e.g. a *myo-3::gfp* marker that preferentially stains the pharynx. A laserbeam with a wavelength above 670 nm is then targeted to the recognised pharynx via a galvano-scanner, which is basically a set of motor-driven mirrors whose tilt is controlled by computer software. Exposure to the laser-beam should be kept to a minimum, so that it does not interfere with the latency period that is a useful measure of the response rate of mutant or drug-treated animals. Moreover, alterations of movements as a consequence of laser exposure also serve as indicators for thermal avoidance (Wittenburg and Baumeister, 1999). Typically, old animals react slower and with reduced vigor than young animals.

8. Pharmacological assays to find compounds that prolong longevity

There are two main incentives for designing automated assays to monitor *C. elegans* aging. First, these techniques facilitate the identification of mutants with life span extending phenotypes. Second, *C. elegans* is perfectly suited as an in vivo animal model for testing chemicals that interfere with aging, and may perhaps be the only multicellular organism suitable for whole-animal high-throughput assays in aging research. A typical setup for such an assay is shown in Fig. 4. A defined number of synchronised animals, preferably at L4 or young adult stage, is seeded into each well of a (96- or 384-well) microtiter plate. This can easily be accomplished with the help of a dispensing robot (compare Fig. 2). Compounds are added to each well and worms are incubated in liquid culture for an extended time period. Genetic or pharmacological manipulation is advisable to prevent egg-laying of the test animals, since self-progeny in the wells would interfere with the readout. Typically, after 14–20 days wild type animals kept at these conditions die, as indicated by the lack of movements of individual animals. Under the influence of life span extending compounds, aging is delayed, indicated by the motility of animals under these conditions. Therefore, the automated analysis of motility as a surrogate marker for vibrancy and health may identify in high-throughput novel mutants/compounds that interfere with the aging process.

9. Conclusions

Most aspects of worm handling, as well as the detection of many phenotypic variants of mutants, can be automated using imaging software and robots. Particularly in a research field like in aging research, where vertebrate assays and cell culture are difficult to apply in large quantities, standardization of techniques allows a high throughput of probe analyses, and is, thus, of particular interest for drug discovery.

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