



COMPUTERIZED *IN VITRO* TEST FOR CHEMICAL TOXICITY BASED ON *TETRAHYMENA* SWIMMING PATTERNS

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ABSTRACT

An apparatus and a method for rapidly determining chemical toxicity have been evaluated as an alternative to the rabbit eye irritancy test (Draize). The toxicity monitor includes an automated scoring of how motile biological cells (*Tetrahymena pyriformis*) slow down or otherwise change their swimming patterns in a hostile chemical environment. The method, called the Motility Assay (MA), is tested for 30 s to determine the chemical toxicity in 20 aqueous samples containing trace organics and salts. With equal or better detection limits, results compare favorably to *in vivo* animal tests of eye irritancy.

INTRODUCTION

For pollution monitoring, chemical testing, and pharmaceutical approval, existing whole-animal testing procedures are often expensive, time consuming, and are being increasingly restricted by federal law. Thus, a combination of public pressure and high costs has stimulated the \$3 billion chemical testing market to look for alternatives to whole-animal research.

In collaboration¹ with Avon, Inc., Silverman¹⁻² has demonstrated that hostile chemicals can change the swimming behavior of single biological cells (*Tetrahymena*) in a controlled and reliable way. For 21 chemicals and pharmaceuticals, he found equal or better results for the toxic response of single cells compared to alternative whole-animal tests (e.g., FDA's Draize rabbit eye test). His method relied on two laboratory technicians performing a subjective evaluation of swimming behavior and scoring their opinions of regular versus irregular swimming patterns. Subsequent industrial interest has focused on finding reliable and rapid ways to improve the toxicity evaluation.

Other experiments³⁻⁴ have demonstrated that for many (*Tetrahymena*) cells suspended in shallow culture dishes, the cells rapidly (20 s) aggregate to give a characteristic signature pattern (polygonal net). These dense, honeycombed patterns of rising and sinking cells change repeatedly when chemicals alter the culture media (water). Image analysis of aggregation patterns showed that chemical toxicity could be scored objectively. This

advance took away the capital investment in microscopes and technician time required by Silverman's test,¹ but nevertheless demanded an extended time for culturing cells to high enough densities (million cells/mL). The assay's aim was to make the advantages of the single-cell method more widely accessible to smaller laboratories or for field tests where microscopic observation and individual cell counting might prove impractical.

While these innovative alternatives using single biological cells have shown promising results when compared to animal tests, they generally have suffered from a nonuniform procedure for scoring toxicity. Either microscopic observation of cell swimming patterns¹ has required subjective and time-consuming scoring by two laboratory technicians. Alternatively, macroscopic observation of cell swimming patterns⁵ has required lengthy culture preparation. Industrial laboratories have, therefore, sought a more rapid and low-cost device for implementing single-cell monitoring on a wide scale.

The goal of this research was to design and evaluate an automated method for computer-aided scoring of single-cell responses. Twenty chemicals, including major organics and salts, were examined using *Tetrahymena* as the test organism. Changes in cell swimming velocity served as the test's monitor, while computerized cell movement tracking provided a nonsubjective evaluation. The method differs from previous approaches in its relative speed, reliability, and operator ease. In several minutes, multiple chemicals can be tested as they act alone or synergistically.

MATERIALS AND METHODS

Test Materials

Twenty water-soluble chemicals (Sigma Chemicals, St. Louis, MO, USA) were tested. Each chemical was dissolved in deionized (DI) water with further purification. One blank (negative control of cells and media only) and two reference substances (acetone p.a., Merck and methanol p.a., Merck) were included in each experiment.

Test System

The ciliate, *Tetrahymena pyriformis* (American Tissue Type Collection, MD, USA), was grown in (autoclaved) two-percent proteose-peptone-yeast medium.⁶ The organisms were cultivated axenically in a temperature-controlled (22 °C) clean room (class III). The protists were grown in 1-L glass containers (media volume) without additional gassing or agitation.

Chemical Treatment

The test materials were all applied in dilute form. A 1M solution was pipetted into test tubes of known *Tetrahymena* concentration (varying between 10² and 10⁴ cells/mL) and vortexed (low speed, 40 r/min) for 30 s. Five hundred mL of liquid was withdrawn from the mixed solution of cells and chemicals. Four samples of 50-μL suspensions of *Tetrahymena* were placed in a glass depression slide (square) and covered with a microscope cover slip (acetate) to form a 100-μm depth observation chamber (fig. 1). Half a minute later the culture and chamber were assessed for 50 cells per area of each video frame using an automated CellSoft cell-tracking computer system manufactured by CRYO Resources, Ltd. (Montgomery, NY). The observed number of swimming cells and average linear velocity were reported for each scheduled chemical application. The observed effects were graded as described below and compared to existing scoring schemes. Replicates of four chemicals were used for each test material.

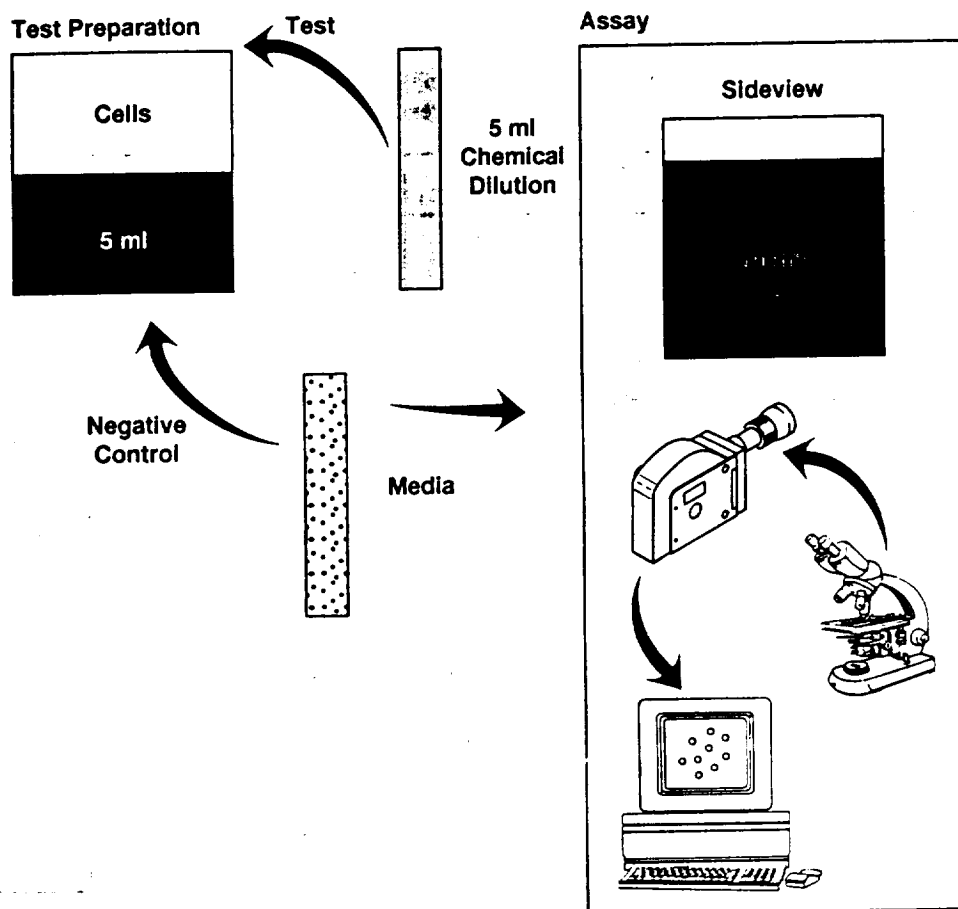


Fig. 1. Schematic of assay procedure. A 5-mL sample of *Tetrahymena* cells (density $2.5 \times 10^3/\text{mL}$) is diluted with 5 mL of yeast media and test chemical at the desired concentration. 0.1 mL of the 10-mL preparation is placed on a 100-micron observation chamber, videotaped under microscopic observation and then analyzed for cell-tracking parameters (velocity and number of cells motile).

Dose-Response Assessment

Dose-response curves provided a check for assay inhibition. Biological responses were registered as a reduction in the number of moving cells (percentile motile) and linear velocity. The effects of each test substance on *Tetrahymena* swimming speed and direction were scored as described by Silverman and are shown in Tables I and II. Four sets of 50 cells each frame determined standard deviations.

To compare different assay results, the present motility assay adopted the scoring formalism developed by Silverman.¹ Dilution factors are reported for organics (alcohols, ketones) as well as salts. Tolerated doses were found based on whether a particular applied chemical yields 10-percent (low dose) or 90-percent (high dose) of the cells immobile. In this way, a direct comparison is feasible between laboratory technician scoring of abnormal swimming versus computerized cellsoft cell tracking. All assays effectively measure cell swimming, whether the endpoint is direct single-cell trajectories or the overall indirect indicator of cell aggregation.

Table I. Rank order toxicity from computerized assay. Toxicity scores shown as the tolerated dose (dilution factor) with immobilized (a) d_{high} , 90 percent of the swimming cells (high dose); (b) d_{low} , 10 percent of the swimming cells (low dose); (c) the average dose as the reciprocal sum of the high and low dose ($1/d_{avg} = 1/d_{high} + 1/d_{low}$). Rank orders shown for 20 organics (alcohol, ketones, ethers, esters) and salts.

| Chemical | 10 Percent Motile | 90 Percent Motile (HTD) | Computer (ATD) | Rank Order | Silverman |
|------------------------|-------------------|-------------------------|----------------|------------|-----------|
| Ethylene glycol | 1.000 | 17.500 | 18.500 | 1 | 11.000 |
| Ethanol | 5.310 | 18.800 | 24.100 | 2 | 13.000 |
| Isopropanol | 78.000 | 22.500 | 30.300 | 3 | |
| Methanol | 1.000 | 38.900 | 39.900 | 4 | |
| DMSO | 1.000 | 46.500 | 47.500 | 5 | 9.300 |
| 3-methyl 2-butanone | 18.900 | 40.000 | 58.900 | 6 | |
| Isobutyl acetone | 1.000 | 79.400 | 71.400 | 7 | 19.300 |
| Methyl isobutyl ketone | 13.800 | 58.300 | 72.100 | 8 | |
| 2-methyl 1-propanol | 1.000 | 78.000 | 79.000 | 9 | |
| Methyl ethyl ketone | 34.800 | 55.900 | 90.700 | 10 | 60.000 |
| Acetyl acetone | 31.200 | 122.000 | 153.000 | 11 | |
| Butanol | 33.000 | 170.000 | 203.000 | 12 | |
| Bleach | 44.600 | 346.000 | 391.000 | 13 | |
| Diethylanoamine | 120.000 | 284.000 | 404.000 | 14 | |
| 2-octanone | 250.000 | 308.000 | 558.000 | 15 | |
| Nonanol | 178.000 | 801.000 | 979.000 | 16 | |
| 1-pentanol | 321.000 | 687.000 | 1,008.000 | 17 | |
| Heptanol | 694.000 | 2,250.000 | 2,944.000 | 18 | |
| 2-methyl 1-butanol | 963.000 | 2,275.000 | 3,238.000 | 19 | |
| Hexanol | 2,000.000 | 13,000.000 | 15,000.000 | 20 | |

Table II. Comparison of irritancy rankings (mild, moderate, severe) between the four assay methods. Results refer to single-cell swimming behavior of *Tetrahymena* scored with cell tracking (computerized assay), microscopic method using laboratory technicians (Silverman), and aggregation patterns (bioconvection assay). Single cell results are compared to standard Draize tests (*in vivo*) as reported in Silverman.

| Chemical | Score | | | Irritancy | | | <i>In Vivo</i> |
|---------------------|----------|-------------|-----------|--------------|-------------|-----------|----------------|
| | Computer | Aggregation | Silverman | Computerized | Aggregation | Silverman | |
| N-butanol | 203.000 | 168.000 | 64.000 | Severe | Severe | Severe | Moderate |
| Ethanol | 24.100 | 38.000 | 13.000 | Mild | Mild | Mild | Mild |
| Ethylene glycol | 18.500 | 29.000 | 11.000 | Mild | Mild | Mild | Mild |
| Methyl ethyl ketone | 90.700 | 111.000 | 71.000 | Severe | Severe | Severe | Moderate? |
| Acetone | 71.400 | 30.000 | 19.300 | Moderate | Mild | Mild | Moderate |

The most illustrative comparison between different methods can be constructed using a simple three-outcome score. For dilution factors of 0 to 100 percent, if a chemical reaction (toxic response) occurs only for high dosages (0- to 30-percent dilutions), then the chemical is scored as mild. Alternatively, for medium dosages (30- to 60-percent dilutions), a reaction indicates moderate toxicity. Finally, for low dosages (greater than 60-percent dilution), the chemical toxicity scores as severe. To evaluate an average tolerated dose, reciprocal dilutions are summed for high and low values which deliver toxic reactions, with the high dose leaving 10 percent of the cells mobile and the low dose leaving 90 percent of the cells mobile.

Image Analysis of Single-Cell Motility

Component parts of the CellSoft signal detection system (CRYO Resources Ltd., Montgomery, NY, USA) for single cells include: (1) CellSoft processor with printer, (2) two high-resolution black and white video monitors with a black and white video camera, (3) compatible microscope with phase contrast or dark field optics and objectives, and (4) 100-micron deep observation chamber. A live or videotaped image of 50 or more single cells of *Tetrahymena* is centered and delivered into the computer. The image is analyzed for cell identification (CellSoft-KASA) and, as applicable, *Tetrahymena* concentration, percent motility, motile velocity, and linearity data. A hard copy printout of test results can be made or, optionally, stored as data in ASCII files. At the magnification selected, the system is able to recognize 50-micron swimming cells in each image and to distinguish them from other biologicals based on their size, luminosity, and motion.

Image Analysis of Aggregation Patterns

For comparison with single-cell results, aggregation patterns⁵ were evaluated and reported for identical chemical conditions. The image of bioconvection patterns was recorded by a black and white camera (Nikon FM-2, lens, Medical Nikkor 120 mm) mounted above the observation flask. The photographic images were digitized by manually tracing their pattern boundaries (e.g., regions of high relative organism density), then scanned (Albaton 300S Scanner, CA, USA) with a spatial resolution of 512 by 512 pixels. The digital images were further analyzed for geometric parameters of aggregation patterns using a main image analysis program (Image Analyst, CA, USA) written in the computer language C.

The outline and position of each pattern (polygon) was determined using a chain coding algorithm and analyzed spatially as a best-fitted centroid. For each polygon, the geometry was stored in the form of area, perimeter, and average radius (arbitrary pixel units) as well as the number of polygonal sides, then calibrated (normalized) to the average value for all polygons. As the pattern changed with chemical addition, the geometric measures of polygonal area and perimeter were plotted as a function of cell sides. The physical significance⁷ of these results has been discussed elsewhere under the heading of statistical crystallography.⁵

Additionally, test results for the aggregation assay supplement this comparison, but instead of rendering a percentage of motile cells, the disappearance of macroscopic aggregation pattern signals the tolerated chemical dose. More toxic dosages disperse pattern formation by reducing cell mobility.

Draize Test

The *in vivo* results were obtained for comparison from reference sources. In brief, young adult albino rabbits of the New Zealand white strain, SPF quality, were used. The studies were carried out in conformity with the OECD Guidelines for Testing of Chemicals (1987). The test substance was applied in a single dose to one of the eyes of the animals; the untreated eye was used to provide control information. The degree of irritation was evaluated and scored at specific intervals and was further described to provide a complete evaluation of the toxic effects. For the *in vivo* data, a Draize score was calculated and the observations were classified according to the

scheme of Kay and Calandra. The 95-percent confidence interval for the *in vivo* data was dependent on the number of animals used.

RESULTS

The potential importance of single-cell swimming changes and chemical detection has been discussed previously.^{2,3,7} We, therefore, presently consider the computer-scored effects of chemical loading on single-cell swimming and compare these results against existing assays. Thus, the comparative framework for chemical detection involves four different tests: (1) the present motility assay of single-cell swimming, (2) manual scoring of single-cell swimming (Silverman¹), (3) monitoring of many cells macroscopically through their chemically hindered aggregation patterns (bioconvection),³⁻⁴ and (4) traditional whole animals, *in vivo* (Draize) tests.

As a function of chemical concentration, a representative organic (methanol) and salt (DMSO) were tested using the motility assay (figs. 2 and 3). The dose-response curve was found to be linear (positive correlation coefficient $R > 0.90$ for all chemicals tested) when the response was assessed for either the percent of cells immobilized and the percent reduction in linear velocity. Higher irritancy dosages were found to hinder cell movement in *Tetrahymena*. Irritation classifications were selected based on a modification of existing rating scales.¹ No saturation effect at high concentration (up to 100 g/kg) appears in the *Tetrahymena* system. A summary for all chemicals is shown in Table 1 for organics and salts and classified graphically by chemical family (fig. 3) and rank order toxicity (fig. 4).

To evaluate the assay results, figures 5 and 6 compare toxic thresholds with previously developed assays.

Both the motility and aggregation assays were carried out on identically grown cultures. Laboratory technician scoring was evaluated directly from previous results⁸ and compared with standard *in vivo* results.⁸ A comprehensive report for five representative chemicals is shown in figure 7. The enlarged shaded region indicates that a toxic effect can be detected at a lower dose (<15 p/m dry chemical in some cases) using the motility assay compared to aggregation methods. The automated CellSoft processor likewise signals positively at lower doses compared to scoring by the laboratory technician method. By sensitivity, we refer to the number of eye irritants identified correctly as irritants by MA divided by the number of eye irritants tested. The computerized CellSoft apparatus delivers these averages in an automated fashion, and final results are shown in figure 8.

DISCUSSION AND CONCLUSIONS

It has been suggested that the swimming behavior of protists (*Tetrahymena pyriformis*) could be used as an alternative to the rabbit eye test for irritation. *T. pyriformis* was pursued as a target organism because its chemical sensitivity has been well-characterized previously.⁸ Its short generation times and thoroughly investigated biology make it what one researcher⁹ called a "biochemical star." As a result, for more than 40 years, *T. pyriformis* has been the organism of choice⁸ for assaying carcinogens, insecticides, fungicides, petroleum products and organics, mycotoxins, antimetabolites, and heavy metals.

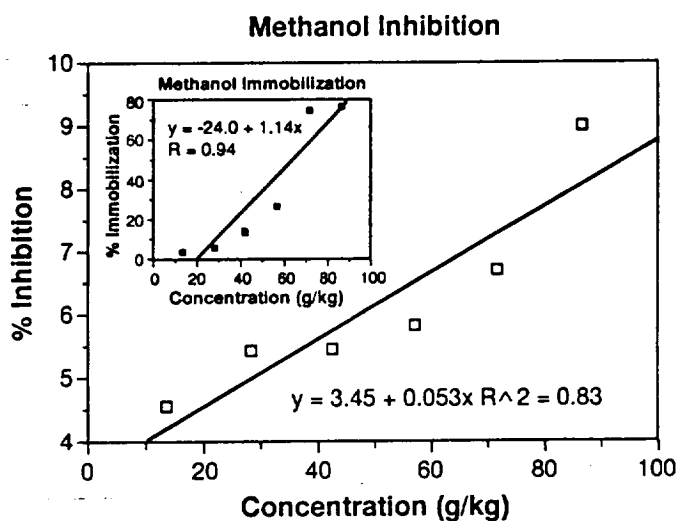


Fig. 2. Effect of chemical addition on *Tetrahymena* swimming patterns. Test results in aqueous media as a function of methanol concentration between 0 and 100 g/kg. (a) Percent inhibition is the calculated reduction in swimming (forward) velocity, $P = 100(v - v_c/v_c)$, where v_c is the control velocity with no chemical addition, and v is the measured velocity with methanol. (b) Percent immobilization is the calculated reduction in the number of cells swimming, $P = 100(n - n_c/n_c)$, where n_c is the control number of motile cells with no chemical addition, and n is the measured number with methanol.

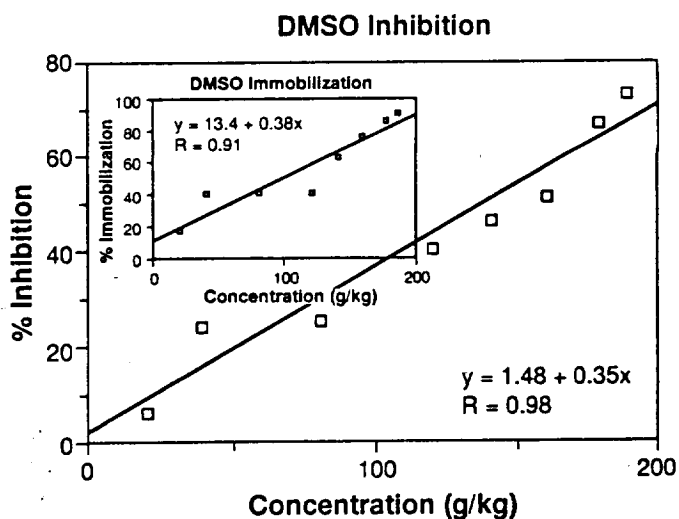


Fig. 3. Effect of chemical addition on *Tetrahymena* swimming patterns. Test results in aqueous media as a function of DMSO concentration between 0 and 100 g/kg. (a) Percent inhibition is the calculated reduction in swimming (forward) velocity, $P = 100(v - v_c/v_c)$, where v_c is the control velocity with no chemical addition, and v is the measured velocity with DMSO. (b) Percent immobilization is the calculated reduction in the number of cells swimming, $P = 100(n - n_c/n_c)$, where n_c is the control number of motile cells with no chemical addition, and n is the measured number with DMSO.

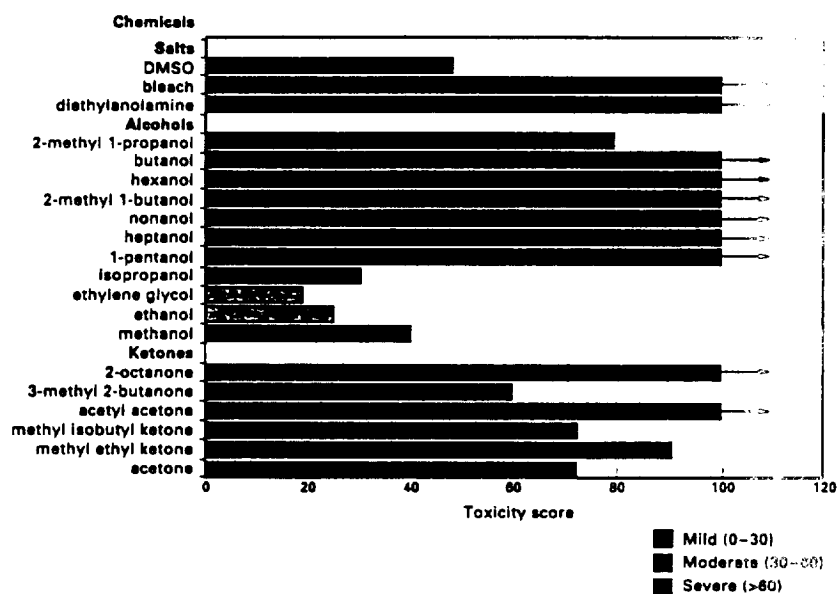


Fig. 4. Toxicity scores arranged by chemical families for motility assay results. Dilution factors (x) of 1:x which immobilized 10 and 90 percent of the motile cells are reciprocally summed in agreement with Silverman. Chemicals which immobilize for dilutions less than 30 fold are mild, 30 to 60 fold are moderate, greater than 60 fold are severe. Arrows indicate severe toxicity which immobilized at trace composition for dilutions greater than 60 fold in yeast media.

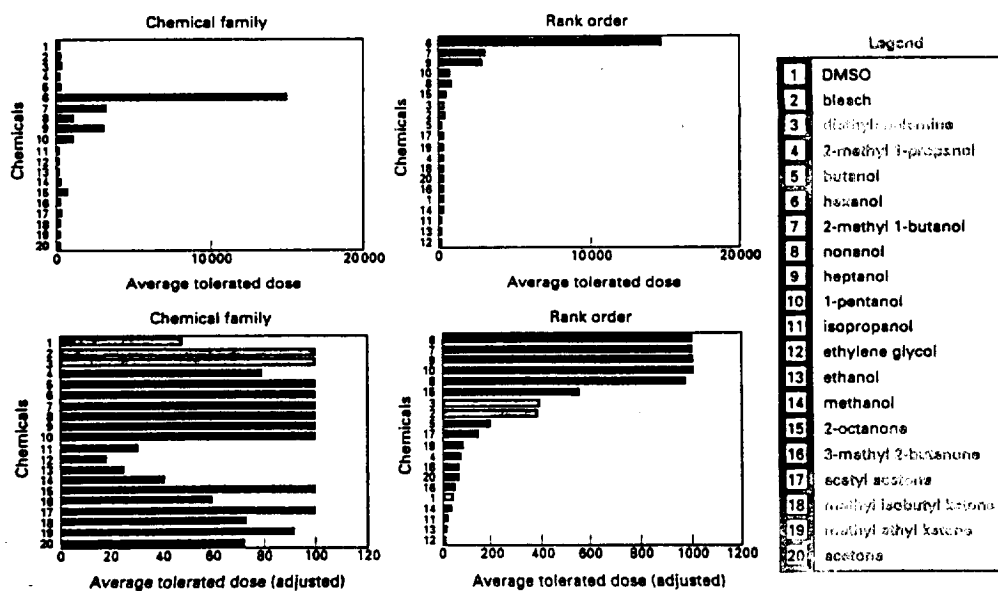


Fig. 5. Toxicity scores for 20 organics and salts arranged by chemical family and rank order from motility assay. Results shown as average tolerated doses with (lower) adjusted orders indicated in bottom graphs showing the finer details of chemical comparisons (e.g., thresholded higher toxicity like hexanol shown at arbitrary cutoff at 100 to 1,000).

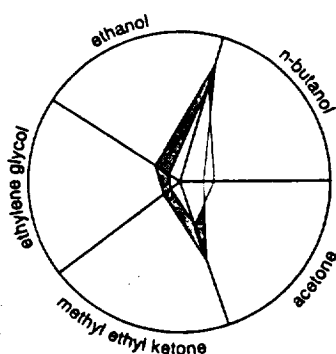


Figure a. Computerized results vs. swimming aggregation results.

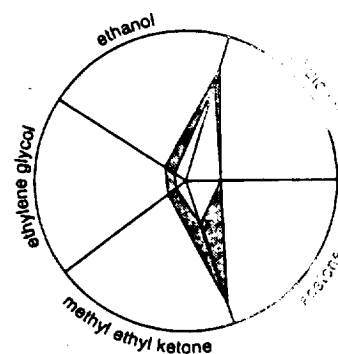


Figure b. Computerized results vs. Silverman results.

Fig. 6. Graphical comparison of assay sensitivity for *Tetrahymena*. The circle perimeter corresponds to an average tolerated dilution of 100 percent (no chemical), the central point to 0 percent. The shaded region (polygon) indicates that computerized results (larger polygon) has equal or better dose discrimination compared to models of either swimming aggregation (Noever) or laboratory technician (Silverman). The exception is a more sensitive aggregation test for acetone.

| In vivo \ In vitro | In vitro | | |
|--------------------|----------|----------|--------|
| | Mild | Moderate | Severe |
| Mild | 0 | 0 | 1 |
| Moderate | 0 | 0 | 3 |
| Severe | 5 | 2 | 1 |

Fig. 7. Graphical comparison of assay sensitivity for *Tetrahymena* versus *in vivo* rabbit irritancy. Three test outcomes (mild, moderate, and severe) are shown as a matrix. *In vitro* refers to the number of counts for the standard 100 chemicals which score in that test rating (e.g., mild). Ideal correspondence between *in vivo* and *in vitro* results would fill the central diagonal with chemicals but leave the nondiagonal elements equal to zero. Since the matrix results generally fill the higher columns and lower rows (a "bottom-heavy" matrix) then the *in vivo* tests can be understood to give a more sensitive assay.

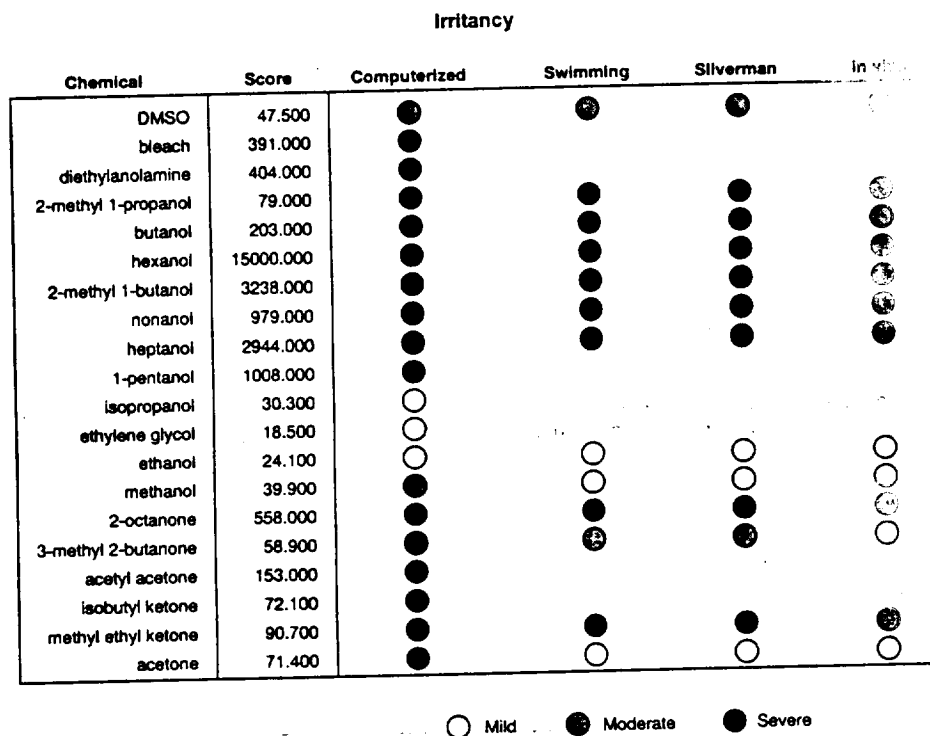


Fig. 8. Summary comparison for assay methods from the 20 chemicals. Toxicity scored as mild, moderate, and severe. Score reported as average tolerated dose for computerized assay with *Tetrahymena*. Ideal ranking of results for all assays would correspond to similar rankings in a single row.

Various authors have evaluated different methods for investigating the irritant potential of chemicals to *Tetrahymena*. We used the *Tetrahymena* system as described by Silverman¹ to assess the effects of 20 chemicals of known *in vivo* eye irritancy. Quantifiable effects on *Tetrahymena* included a reduced number of swimming cells (percent motile), diminished linear velocity, and halted motility. A comparison was made between the rank order of these effects on *Tetrahymena* and the known ranking for *in vivo* eye irritancy. In all cases, a positive correlation was found between *in vitro* and *in vivo* results. The *in vitro* assessment could distinguish between irritant chemicals and those of little or no irritancy for all 20 organics. Compared to previous *Tetrahymena* studies, the primary advance found in the present work was to provide an automated platform for chemical assessment with a 30-s irritancy determination. These data indicate that this method would have application as a screening procedure for eye irritation potential or as part of a battery of *in vitro* tests.

Using CellSoft's computerized scoring of cell motility, the present method differs from previous approaches in its speed (just 10 min from refrigeration to incubation to computerized evaluation of chemical toxicity), its ease-of-use (as straightforward as aqueous transfer), and its laboratory stability (several months shelf life). Using CellSoft computerized evaluation provides a documented report of toxicity in a largely labor-free (completely automated) setup at low cost (pennies per test). Table III shows the expected labor savings for automated test performance. These cost estimates do not include animal maintenance, capital investment in cages, etc., which do not have comparable expenses in an *in vitro* setting. Advantages and applications are summarized in Tables IV and V.

Table III. Cost comparison between *in vivo* and *in vitro* tests for labor time only in assessing chemical irritancy.

| | Cost and Time Savings | |
|------------------------------|------------------------------------|----------------|
| | Method | |
| | Conventional (<i>in vivo</i>) | Motility Assay |
| Preparation Time | months to days (2 to 3 days) | 10 min |
| Analyst Performance Time* | 22.5 min | 0.5 min |
| Materials | 2.50 | 0.05 |
| Supplies | 8.00 | 8.00 |
| Culture | | |
| Labor** | 9.45 | 0.21 |
| | 19.95 | 8.26 |

*Based on performance times of similar tasks as cited in Working Time Units Catalog, ILO, 1992.

**Assume analyst salary, benefits, and overhead of \$25 (W.S.)/hour.

Table IV. Advantages of the motility assay and CellSoft system.

- (1) Instant results in a ready-to-use quality-controlled system of microorganisms
- (2) Quantitative reporting which delivers a specific range of swimming changes upon chemical addition
- (3) Ease-of-use which requires only push-button effort to give documented toxic evaluation and reporting
- (4) Real economy, eliminating biological growth periods of several days. (No more trial-and-error dilution of suspensions to achieve desired cell counts, thus transforming lengthy technician scoring into a single objective result.)
- (5) Safety which minimizes technician handling and exposure to potentially hazardous chemicals
- (6) Finally, reliability, including high enough speed evaluations to make many repetitions of results realistic to perform

Table V. Alternative embodiments of the motility assay.

- (1) An alternative to Draize rabbit eye test for cosmetic testing.
- (2) Research tool for pollution monitoring in organic and heavy metal detection.
- (3) Chemical safety data for Federal monitoring.
- (4) A standardized test for (a) growth promotion, (b) bacteriostatis, (c) effectiveness testing of antimicrobial preservatives and disinfectants, (d) microbial limit tests, and (e) media quality control in biotechnology, clean room testing, clinical, environmental, food and beverage, industrial, pharmaceuticals, and cosmetic tests.

As currently conducted, the automated assay does not provide information on the time dependence of irritancy or on responses caused by physical irritancy or immunological mechanisms. Overestimation error in the study (false positives) arose in part because of synergistic reactions with culture media (precipitation, etc.). Underestimation of sample irritancy (false negatives) was generally associated with low solubility at higher concentrations and surfactant effects.

No fully formulated products such as shampoo or industrial detergent cleaners were tested. (The irritancy potential of each chemical was assessed by reference to literature sources). Disadvantages of the automated assay application procedure included the inability to qualify low solubility and highly colored materials.

To conclude, the present results have surveyed three alternative embodiments for testing chemical effects on single-cell swimming behavior. The alternatives give similar results to existing *in vivo* results when adapted to a three-tiered scoring scheme (mild, moderate, and severe). In all chemicals tested (both organics and inorganics), the motility assay gave equal or lower thresholds for detecting toxicity (reported as dosages which immobilized a percentage of cell activity). Given the potential time and money saving possibilities of an automated method, the motility assay should receive further consideration as a scientifically competitive evaluator of chemical toxicity.

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10. When considering an *in vitro* screening assay or an alternative to animal testing, the effects observed in the present animal test serve as a reference for evaluating the new assay's results. However, this animal test will not always make a faultless prediction for the irritancy in man. Thus, a less than perfect correlation between *in vivo* (animal) and *in vitro* tests does not make the screening test less predictive for effects in humans.