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

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Running title: Computerized chemical tests of Tetrahymena

Abstract

An apparatus and method for rapidly determining chemical toxicity has been evaluated. 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 device, called the Motility Assay Apparatus (MAA) is tested for 30 second determination of 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, in addition to agreeing for all chemicals with previous manual evaluations of single cell motility.

1. Introduction

For pollution monitoring, chemical testing and pharmaceutical approval, existing whole animal procedures are expensive, time consuming and 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 collaboration⁹ with Avon, Inc., Silverman⁸⁻⁹ demonstrated that hostile chemicals could 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 lab technicians performing a subjective evaluation of swimming behavior and scoring their opinions of regular vs. irregular swimming patterns⁷. Subsequent industrial interest has focused on finding more 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 seconds) aggregate to give a characteristic signature pattern (polygonal net). The honeycombed patterns change repeatedly when chemicals alter the culture media (water) and using image analysis of the aggregation patterns, chemical toxicity could be scored accordingly. This advance took away the capital investment in microscopes and technician time required by Silverman's test⁹, 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 labs or for field tests where microscopic observation and individual cell counting might prove impractical.

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While these innovative alternatives using single biological cells have shown promising results when compared to animal tests, they generally have suffered from a non-uniform procedure for scoring toxicity. Either microscopic observation of cell swimming patterns⁹ has required subjective and time consuming scoring by two lab technicians or alternatively, macroscopic observation of cell swimming patterns⁵ has required lengthy culture preparation. Industrial labs have therefore sought a more rapid and low cost device for implementing single cell monitoring on a wide scale.

Here we design and evaluate an automated method for computer aided scoring of single cell responses; 20 chemicals consisting of organics and salts are examined using *Tetrahymena* as the test organism. Changes in cell swimming velocity serve as the test's monitor, while computerized cell tracking provides the non-subjective evaluation. The method differs from previous approaches in its relative speed, reliability and operator ease. In several minutes, multiple chemicals can be either tested as they act alone or synergistically.

2. Materials and Methods

2.1. Organisms and culture conditions

The ciliate, *Tetrahymena pyriformis* (American Tissue Type Collection, Md., USA), were grown in (autoclaved) 2% proteose-peptone-yeast medium¹⁰. The organisms were cultivated axenically in a temperature-controlled (22° C) clean room (Class III). The protists were grown in 1 L glass containers without additional gassing or agitation.

T. pyriformis was pursued as a target organism because its chemical sensitivity has been well-characterized previously¹. Its short generation times and thoroughly investigated biology make it what one researcher⁷ called a "biochemical star." As a result, for more than 40 years, *T. pyriformis* has been the organism of choice¹ for assaying carcinogens, insecticides, fungicides, petroleum products and organics, mycotoxins, antimetabolites and heavy metals.

2.2 Laboratory preparations

Instructions for lab preparations of cell cultures include: 1) transfer freeze-dried *Tetrahymena* cells into rehydration fluid vial; 2) incubate 10 minutes at 35-37° C; 3) decant entire constituents into empty petri dish with either a dilution blank or previously measured concentration of chemicals.

2.3. Determination of pattern formation

For direct comparison with computerized scoring of single cell swimming, macroscopic aggregation patterns³⁻⁶ were also monitored at the same chemical dosages. Cell suspensions were prepared as dense, shallow cultures (3.2×10^5 cells mL⁻¹, culture depth, 4 mm). Assuming typical cell parameters (20-50% g carbon per g dry weight, 80% water content by weight, and cell specific gravity of 1.05 g cm⁻³), this cell population corresponds approximately to 1 g carbon per cubic meter.

Vertical migration and bioconvection patterns of the ciliate were determined in a rectangular polystyrene flask (33x77x15 mm inner dimensions). Since average pattern dimension was 1-2 mm, the effect of container dimensions on pattern formation was minimal. Different sized flasks did not affect either characteristic pattern size or overall morphology.

The flask was placed on a clear stage and illuminated (4000 lux, Edmund Scientific, USA, light meter) using two collimated incandescent beams angled at 45°. A continuously cooling fan maintained thermal control of the light source; each lamp was remotely placed on a gooseneck wand and focussed using 24 cm optical fibers. Thermal convection was not observed in either non-motile (dead) cells of *T. pyriformis* or with 0.1 mm aluminum flakes in water.

2.4 Image analysis

a. Single cells

Component parts of the signal detection system for single cells include: 1) compatible computer with printer; 2) 2 high-resolution black and white video monitors with black and white video camera; 3) compatible microscope with phase contrast or dark field optics and objectives; 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 and as applicable, *Tetrahymena* concentration, motile velocity and linearity data. A hard copy printout of test results can be made or optionally, stored as data in ASCII files. The assay device is able to recognize 50 micron swimming cells and to distinguish them from other biologicals based on their size, luminosity and motion.

b. Aggregation patterns

For comparison with single cell results, aggregation patterns were evaluated and reported for identical chemical conditions. The image of bioconvection patterns was recorded by a b/w 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 x 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 significance¹¹ of these results has been discussed elsewhere under the heading of statistical crystallography⁵.

3. Results

The potential importance of single cell swimming changes and chemical detection has been discussed previously^{3,7,8}. We therefore presently consider the computer-scored effects of chemical loading on single cell swimming and compare these results using geometric analysis on lab simulations which can include this aggregation mechanism for scoring chemical toxicity. Thus the comparative framework for chemical detection involves four different tests: 1) the present computerized scoring of single-cell swimming; 2) manual scoring of single cell swimming; 3) monitoring of many cells macroscopically through their chemically-hindered aggregation patterns (bioconvection)³⁻⁴; and 4) traditional whole animals, *in vivo* tests.

20 chemicals were tested for irritancy. Dosages are reported for organics (alcohols, ketones) as well as salts. To compare different assay results, we adopt the scoring formalism developed by Silverman⁹. Tolerated doses are found based on whether a particular applied chemical yields 10% (low dose) or 90% (high dose) of the cells immobile. In this way, a direct comparison is feasible between lab technician scoring of abnormal swimming vs. computerized cell tracking. 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. All assays are effectively measuring cell swimming, whether the endpoint is direct single-cell trajectories or the overall indirect indicator of cell aggregation.

As a function of chemical concentration, a representative organic (methanol) and salt (DMSO) are tested using the computerized assay. The curve of concentration (g/kg) generally follows an increasing trend for both the percent of cells immobilized and the percent of swimming inhibition. Higher concentrations

generally react biologically to hinder cell movement. No saturation effect at high concentration (up to 100 g/kg) appears in the *Tetrahymena* system. A summary table for all the 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, Figs. 5-6 compare toxic thresholds for previously developed assays. Both the computerized and aggregation assays were carried out on identically grown cultures. Lab technician scoring was evaluated directly from previous results¹ and compared with standard *in vivo* results. A comprehensive report for 5 representative chemicals is shown in Fig. 7. The enlarged shaded region indicates that a lower average dose can be evaluated for toxic detection using the computerized assay compared to aggregation methods. The computer scoring method likewise signals positively at lower doses compared to scoring by the lab technician method.

The most illustrative comparison between different methods can be constructed using a simple three-outcome score. For dilution factors of 0-100 %, if a chemical reaction (toxic response) occurs only for high (0-30%) dilutions, then the chemical is scored as mild. Alternatively for medium dosages (30-60% dilutions), a reaction indicates moderate toxicity. Finally for low dosages (greater than 60% dilution) then 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% of cells mobile and the low dose leaving 90% of cells mobile. The computerized assay delivers these averages in an automated fashion and final results are shown in Fig. 8.

4. Discussion and conclusions

Using computerized scoring of cell motility, the present method differs from previous approaches in its speed (just 10 minutes from refrigeration to incubation to computerized evaluation of chemical toxicity), its ease-of-use (as easy as aqueous transfer) and laboratory stability (several month shelf life). Using computerized evaluation provides a documented report of toxicity in a labor-free (completely automated) and low cost (pennies per test) apparatus.

Advantages of the Motility Assay Apparatus (MAA) include: 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 toxicity 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) and finally reliability, including high enough speed evaluations to make many repetitions of results realistic to perform.

Alternative applications of the MAA include: 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 anti-microbial preservatives and disinfectants; d) microbial limit tests; e) media quality control in biotechnology, clean room testing, clinical, environmental, food and beverage, industrial, pharmaceuticals and cosmetic tests.

To summarize, the present results have surveyed 3 alternative embodiments for testing chemical toxicity on single cell swimming behavior. The alternatives gave similar results to existing *in vivo* results when adapted to a 3-tiered scoring scheme (mild, moderate and severe). In all chemicals tested (both organics and salts), the computerized assay gave equal or lower thresholds for detecting toxicity (reported as dosages which immobilize a percentage of cell activity). Given the potential time and money saving possibilities of an automated method, the computerized assay should receive further consideration as a scientifically competitive evaluator of chemical toxicity.

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Table 1. Rank order toxicity from computerized assay. Toxicity scores shown as the tolerated dose (dilution factor) which immobilized a) dhigh, 90% of the swimming cells (high dose); b) dlow, 10% of the swimming cells (low dose); c) the average does 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.

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 lab technicians (Silverman), aggregation patterns (bioconvection assay). Single cell results are compared to standard Draize tests (in vivo) as reported in Silverman.

Figure captions

Fig. 1 Schematic of assay procedure. A 5 ml sample of *Tetrahymena* cells (density 2.5×10^3 /ml) is diluted with 5 ml 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).

Fig. 2 Effect of chemical addition on *Tetrahymena* swimming patterns. Test results in aqueous media as a function of methanol concentration between 0-100 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 methanol concentration between 0-100 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 computerized assay results. Dilution factors (x) of 1: x which immobilized 10% and 90% of the motile cells are reciprocally summed in agreement with Silverman. Chemicals which immobilize for dilutions less than 30 fold are mild, 30-60 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 computerized 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 or 1000.)

Fig. 6. Graphical comparison of assay sensitivity for *Tetrahymena*. The circle perimeter correspond to an average tolerated dilution of 100% (no chemical), the central point to 0%. 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 lab technician (Silverman). The exception is a more sensitive aggregation test for acetone.

Fig. 7 Graphical comparison of assay sensitivity for *Tetrahymena* vs. 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 20 chemicals which score in that test rating (eg. mild). Ideal correspondence between in vivo and in vitro results would fill the central diagonal with chemicals but leave the non-diagonal 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. Identical performance for all assays would correspond to similar rankings in a single row.

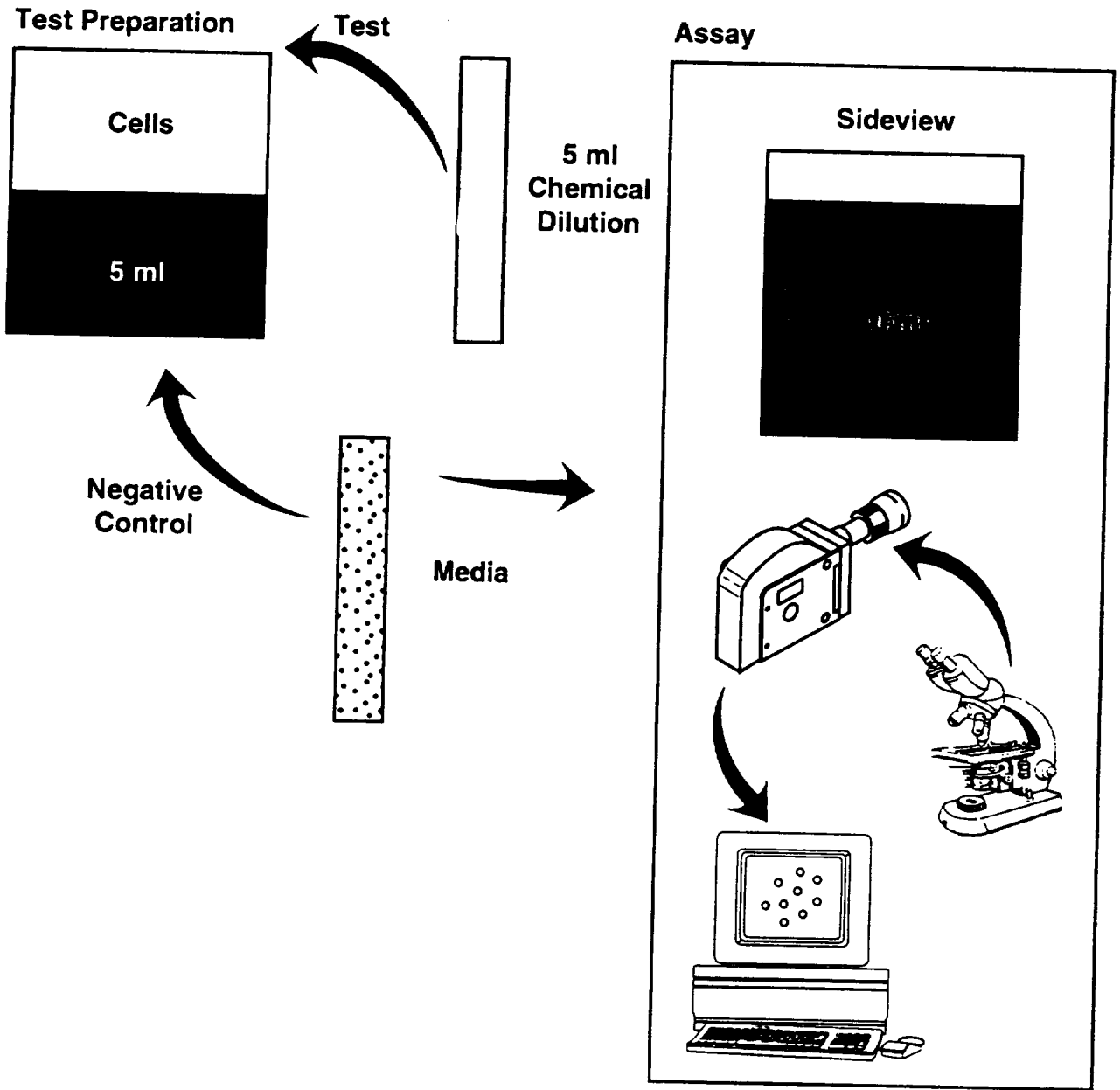
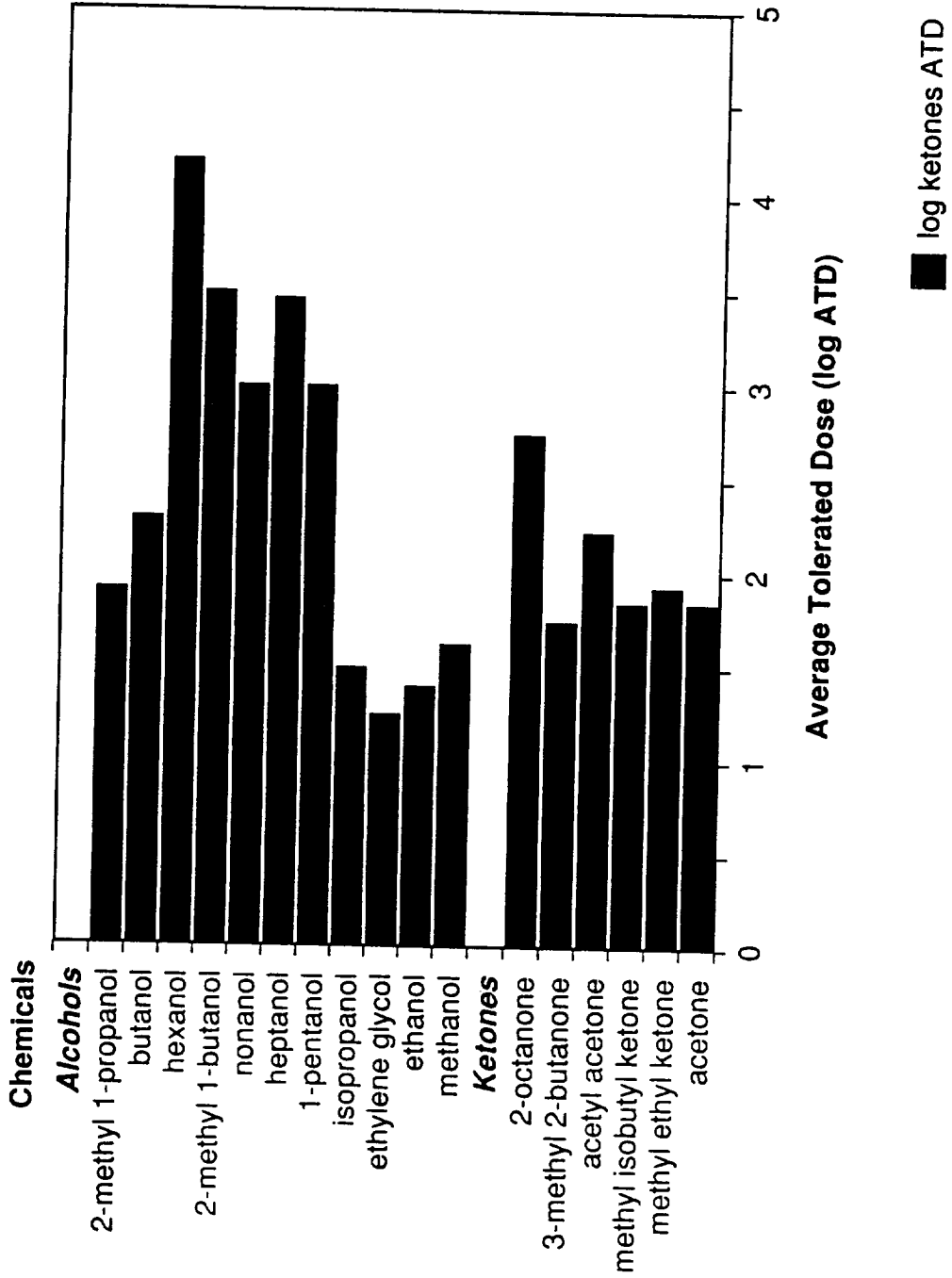


Figure 1. Schematic of Assay

in vivo in vitro	Mild	Moderate	Severe
Mild	1	0	0
Moderate	3	0	0
Severe	1	2	5



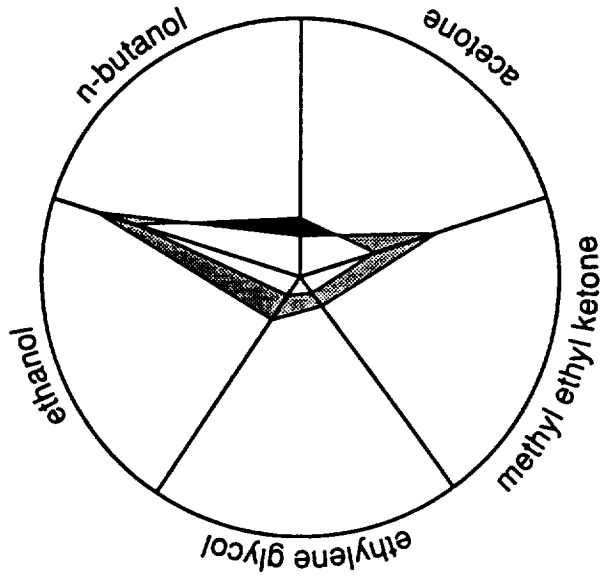


Figure a. Computerized results vs. swimming aggregation results.

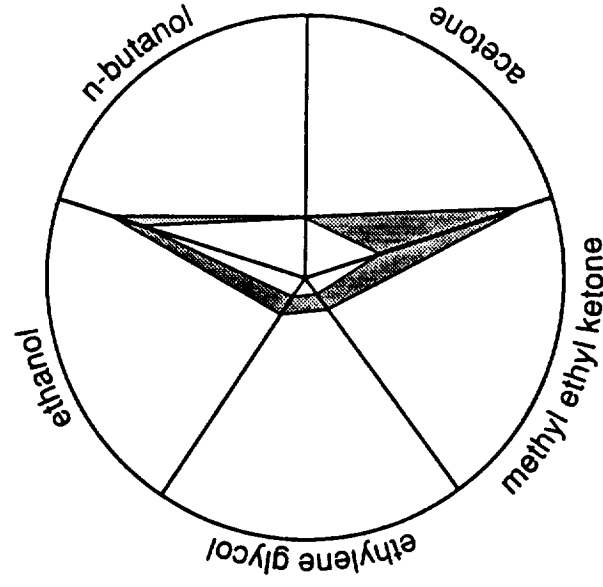
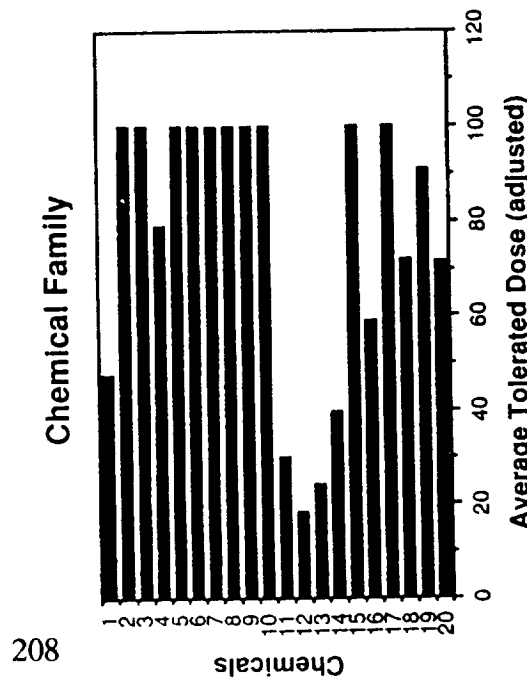
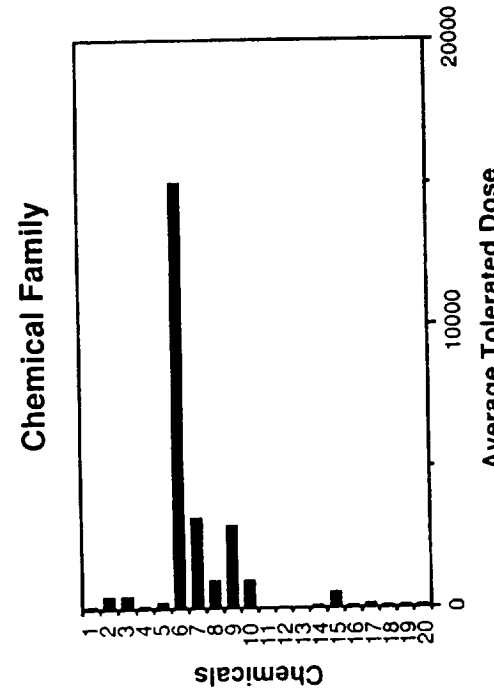
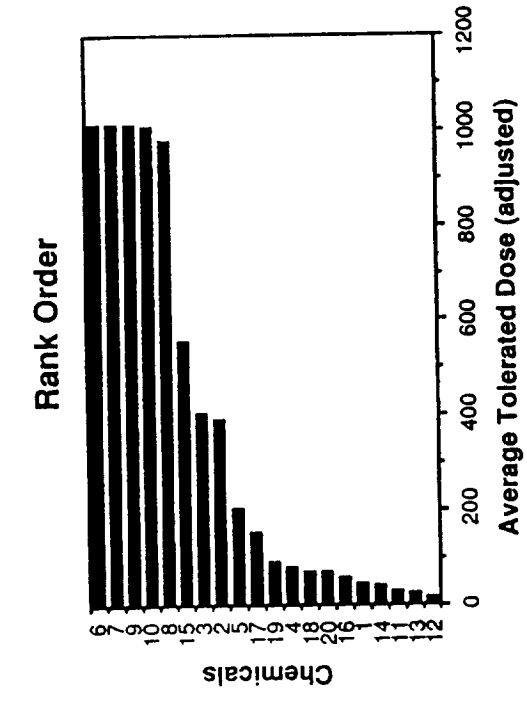
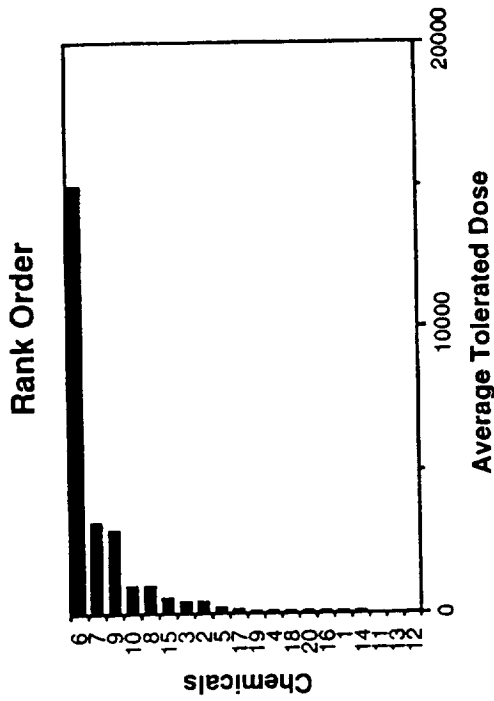


Figure b. Computerized results vs. Silverman results.

Irritancy

Chemical	Score	Computerized	Swimming	Silverman	in vivo
DMSO	47.500	○	○	○	○
bleach	391.000	●			
diethylanolamine	404.000	●			
2-methyl 1-propanol	79.000	●	●	●	●
butanol	203.000	●	●	●	○
hexanol	15000.000	●	●	●	●
2-methyl 1-butanol	3238.000	●	●	●	●
nonanol	979.000	●	●	●	●
heptanol	2944.000	●	●	●	●
1-pentanol	1008.000	●	●	●	●
isopropanol	30.300	○			
ethylene glycol	18.500	○			
ethanol	24.100	○	○	○	○
methanol	39.900	○	○	○	○
2-octanone	558.000	●	●	●	●
3-methyl 2-butanone	58.900	○	○	○	○
acetyl acetone	153.000	●			
isobutyl ketone	72.100	●			
methyl ethyl ketone	90.700	●	●	●	○
acetone	71.400	●	○	○	○





Legend

1	DMSO
2	bleach
3	diethylaniline
4	2-methyl 1-propanol
5	butanol
6	hexanol
7	2-methyl 1-butanol
8	nonanol
9	heptanol
10	1-pentanol
11	isopropanol
12	ethylene glycol
13	ethonal
14	methanol
15	2-octanone
16	3-methyl 2-butanone
17	acetyl acetone
18	methyl isobutyl ketone
19	methyl ethyl ketone
20	acetone