

# An Evaluation of the *In Vitro* Cytotoxicities of 50 Chemicals by using an Electrical Current Exclusion Method versus the Neutral Red Uptake and MTT Assays

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**Summary** — According to the 2001 National Institutes of Health guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity, the performance of the electrical current exclusion method (ECE) was studied for its suitability as an *in vitro* cytotoxicity test. In a comparative study, two established *in vitro* assays based on the quantification of metabolic processes necessary for cell proliferation or organelle integrity (the MTT/WST-8 [WST-8] assay and the neutral red uptake [NRU] assay), and two cytoplasm membrane integrity assays (the trypan blue exclusion [TB] and ECE methods), were performed. IC<sub>50</sub> values were evaluated for 50 chemicals ranging from low to high toxicity, 46 of which are listed in Halle's Registry of Cytotoxicity (RC). A high correlation was found between the IC<sub>50</sub> values obtained in this study and the IC<sub>50</sub> data published in the RC. The assay sensitivity was highest for the ECE method, and decreased from the WST-8 assay to the NRU assay to the TB assay. The consistent results of the ECE method are based on technical standardisation, high counting rate, and the ability to combine cell viability and cell volume analysis for detection of the first signs of cell necrosis and damage of the cytoplasmic membrane caused by cytotoxic agents.

**Key words:** *alternatives to animal testing, CASY model TTC, cell counting, cell proliferation, cell viability, cytotoxicity, electrical current exclusion, MTT, neutral red uptake, trypan blue exclusion.*

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## Introduction

Halle's Registry of Cytotoxicity (RC), and the introduction of the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) concept of Björn Ekwall, made major contributions to our understanding of the correlation between *in vitro* cytotoxicity and *in vivo* lethality (1–3). Therefore, the development and validation of reliable and easy to handle techniques for analysing the viability and status of the cells in culture became an important issue.

The aim of this study was to introduce and validate the electrical current exclusion (ECE) method (Figure 1), as an additional sensitive method for *in vitro* cytotoxicity testing. To evaluate the performance and reliability of the method, the cytotoxicities of 50 chemicals from different toxic classes were tested with mouse L-929 fibroblasts, including the 11 reference chemicals recommended for the validation of a new cytotoxicity test for use with Halle's RC prediction model, in accordance with the recommendations of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM; 4).

The ECE method was tested in direct comparison with the two recommended and established methods,

involving the uptake of neutral red into lysosomes (the NRU assay [5, 6]) and the conversion by mitochondrial and cytoplasmic reductases of tetrazolium salts into coloured formazan dyes (the MTT/WST-8 assay [WST-8; 7]). Additional studies involving the trypan blue exclusion (TB) assay (8–10) were conducted occasionally. The results (IC<sub>50</sub> values) from the various tests were compared with each other and with the *in vitro* data of Halle's RC (IC<sub>50x</sub> values; 2).

## Materials and Methods

### Cell culture

L-929 mouse fibroblasts were chosen, because this adherent-growing cell line can also be easily adopted to grow in suspension. In addition, a rodent cell line seemed to be appropriate for comparing *in vitro* test data with *in vivo* lethality data for mice and rats. Two different methods were used to culture the L-929 cells.

1. For the NRU and WST-8 assays, adherent-growing cells were cultured as monolayers in polystyrol 96-well plates, in Dulbecco's modified Eagle's Medium (DMEM), antibiotic-free and supplemented with 10% fetal calf serum (FCS), 1mM

sodium pyruvate, 2mM L-glutamine, 4mM NaHCO<sub>3</sub> and 20mM HEPES, at 37°C in an atmosphere of 1% CO<sub>2</sub>.

- For the ECE and TB methods, the cells were adapted to grow as a single cell suspension by transferring them into hydrophobic polystyrol 96-well plates. Such cells should not be trypsinised before counting, because this procedure might interfere with the cytotoxic effects of the chemicals to be tested. To avoid extensive aggregation, the suspension culture was grown in RPMI-1640, antibiotic-free and supplemented with 10% FCS, 1mM sodium pyruvate, 13mM NaHCO<sub>3</sub> and 2mM L-glutamine, at 37°C in an atmosphere of 5% CO<sub>2</sub>.

No differences in cell proliferation and sensitivity to the chemicals were detected in the two different media. The viability of the control cells was always greater than 90%, and both growth rate and viable cell size distribution were similar. The absence of mycoplasma contamination was regularly checked with the DAPI-staining test (8, 9).

All the assays were performed according to the recommendations for cytotoxicity test protocols (4). The cells, routinely cultured to 80% confluence in 75cm<sup>2</sup> T-flasks (Sarstedt AG, Sarstedt, Germany), were subcultured twice after thawing, prior to use in the assays. Cells in the exponential growth phase were plated in 96-well plates (Sarstedt AG, Sarstedt, Germany) and incubated for 24 hours to enable them to settle and to recover from the subculture treatment. Then they were incubated with different concentrations of the test chemicals for another 24 ± 1 hours before the endpoint measurements were assessed. Seeding concentrations (optimised for each test system and kept constant), and the number of independent experiments of the assays, are given in Table 1.

## Materials and chemicals

The 50 chemicals tested covered the range from high to low cytotoxicity, and were of the highest available

purity (Table 2). 46 chemicals were selected from the RC list or from the MEIC list (2, 3), including the 11 reference chemicals recommended for the evaluation of a new cytotoxicity test for use with the RC prediction model (4). An additional 4 chemicals of general interest in the cell culture context are referred to other literature surveys (7, 8, 11–16).

Water-soluble chemicals were preferentially used, in order to avoid interaction with the solvent. Sterile, filtered stock solutions of the cytotoxic agents were diluted in cell culture medium, taking into account pH and osmolality. Seven test concentration levels, covering at least three logs, were prepared, in order to obtain concentration–response curves ranging from no effect to maximum cytotoxicity.

The test chemicals were purchased from Merck AG (Darmstadt, Germany) and Sigma Aldrich Ltd. (Taufkirchen, Germany). The WST-8 cell counting kit was purchased from Probiol Ltd. (Munich, Germany). The NRU-stock solution and all the other chemicals and media used were obtained from Sigma Aldrich Ltd. CASY Model TTC and all the other materials necessary for running the ECE method were provided by Schaerfe System Ltd. (Reutlingen, Germany).

## Assay characteristics

### *NRU, WST-8 and TB assays*

The NRU assay is a well-established cytotoxic test for basal cytotoxicity (5, 6), and was performed according to standard operation procedures (7, 8). The results (IC<sub>50</sub> values) are expressed as the concentration of the test chemical which induced a 50% reduction in neutral red uptake into lysosomes, compared with untreated cells.

The WST-8 assay, an advanced version of the MTT assay (9, 10), was performed according to the standard protocol provided by the supplier. The absorption effect of the phenol red in the culture medium was allowed for in the calculations.

**Table 1: Key features of the assay**

Assay	Seeding concentration (cells/ml)	Substrate concentration	Number of replicates
ECE	4.0E +05	7	4
NRU	5.0E +05	7	10
WST-8	1.0E +05	7	10
TB	4.0E +05	7	1

*ECE = electrical current exclusion method; NRU = neutral red uptake assay; WST-8 = WST-8 assay; TB = trypan blue haemocytometer assay; substrate concentration = number of concentrations per assay; number of replicates = number of independent experiments. All seeding concentrations were measured by using the CASY model TTC.*

The TB exclusion assay was conducted according to the standard operation procedures (7, 8), with an improved Neubauer-Tuerk haemocytometer (volume 0.9 $\mu$ l). The TB was used only in range-finding and to provide additional information for some of the test chemicals. It was conducted once only for 35 of the 50 chemicals, so it cannot be assumed that the results obtained were statistically representative.

#### *Electrical current exclusion method (measurement and data evaluation)*

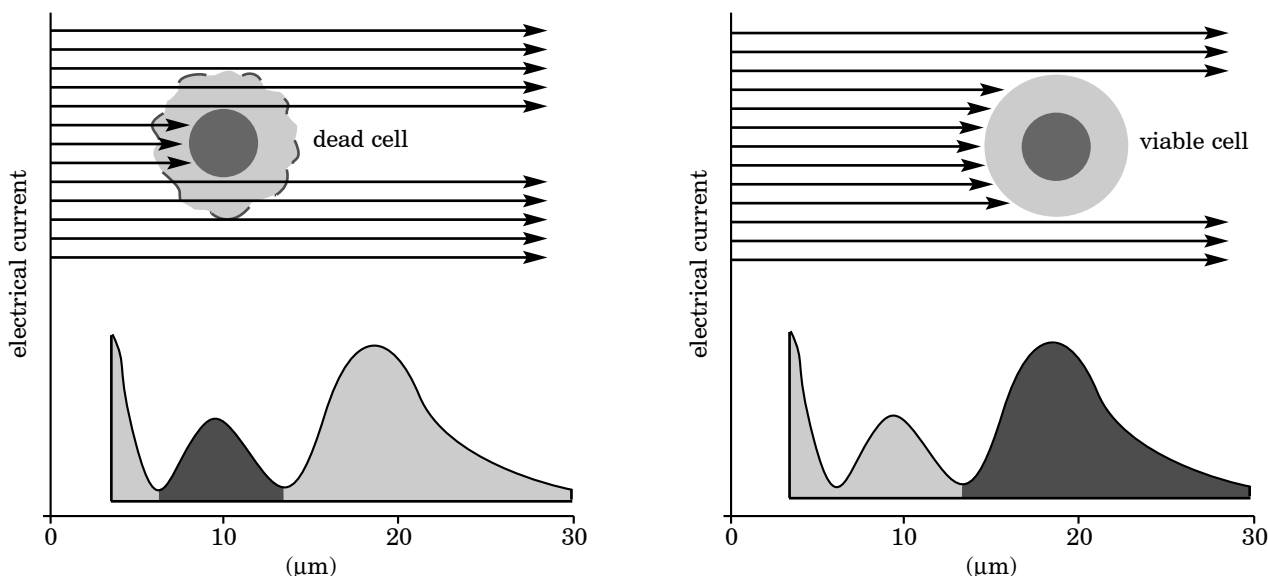
The ECE method is based on the different electrical properties of viable and dead cells suspended in CASYton, an isotonic and iso-osmotic electrolyte (Schaerfe System Ltd.). For analysis, the suspended cells were drawn cell-by-cell through an aperture with a defined geometry (150 $\mu$ m in diameter and length), then exposed to a low voltage field and cycled with 1MHz. The number of electrical signals generated by the cells is described as the cell count. The individual electrical signal is proportional to the respective individual cell volume. Each cell was analysed several hundred times when passing through the aperture. All the results related to an individual cell were processed by Pulse Area Analysis, an advanced digital pulse processing technique developed by Schaerfe System Ltd, and accumulated in a multi-channel analyser which offered more than 524,000 distinct volume linear size classes.

The resulting high-resolution cell-size distribution is characteristic for the individual cell population analysed and the actual status of the cell culture, acting somewhat like a fingerprint. The cell-size distribution permits a precise quantification of the cell concentration, cell volume, cell viability and cell aggregation, within 20 seconds.

Viable cells with an intact membrane act as electrical insulators, and are analysed by their true cell volume. Affected or dead cells have, or develop, a permeable membrane, which is especially permeable to the electrical current. They were identified by the remaining electrical insulating matrix of the cell, roughly the volume of the cell nucleus. Following the basic rule that a cell cannot become smaller than its own nucleus, dead and live cells can easily be differentiated by size. In analogy with the common dye exclusion assays for measuring cell viability, this method is called the Electrical Current Exclusion (ECE) method (Figure 1). Unlike dye exclusion assays, such as the TB assay, the ECE method counts viable cells and also, free nuclei, dying cells and debris.

Before conducting an assay with a new cell line, the gating between cell debris, nuclei, and living cell (including aggregates) had to be defined once for each cell line, and stored in a cell-specific set-up. The region for live single cells was defined by the rise of the peak of the size distribution and the end of the peak, where the range of aggregated cells began, up to a size of 40 $\mu$ m (Figure 2). Dependent

**Figure 1: Principle of the electrical current exclusion (ECE) method**



*Viable cells, with an intact membrane, act as an electrical insulator and exclude the electrical current, whereas dead cells have a membrane permeable to the electrical current. Cell debris is depicted on the left side as an additional peak.*

**Table 2: Comparison of IC50 values**

RC No.	Chemical	Registry of cytotoxicity				Assays performed in this study				Variation of IC50 values	
		Literature IC50 (mM)	ECE IC50 (mM)	NRU IC50 (mM)	WST-8 IC50 (mM)	TB IC50 (mM)	ECE versus literature	ECE versus assays performed			
361	methanol	930	1930	1805	996	1872	0.32	0.11			
131	glycerol <sup>a</sup>	624	67.3	453	509	—	-0.97	-0.85			
359	acetone	444	394	586	677	704	-0.05	-0.22			
130	ethanol	379	155	1008	876	—	-0.39	-0.78			
358	acetonitrile	368	1116	1043	1126	982	0.48	0.03			
129	dimethylsulphoxide	252	66	9604	101	58.8	-0.58	-0.10			
351	dimethylformamide <sup>a</sup>	114	420	298	261	601	0.57	0.07			
349	ethylmethylketone	104	365	164	315	551	0.55	0.08			
348	1-propanol	96.5	1.91	2.88	1.65	—	-1.70	-0.06			
344	sodium chloride	75.9	138	194	114	192	0.26	-0.07			
343	magnesium II chloride × 6H <sub>2</sub> O	70.4	63.9	50.8	56.1	68.9	-0.04	0.04			
338	1-butanol	52.5	50.3	75.3	128	77.7	-0.02	-0.26			
337	pyridine	46.9	87.1	71.7	76	66.6	0.27	0.09			
333	lithium I chloride	38.6	13	31.6	9.18	—	-0.47	-0.12			
304	calcium II chloride	12.4	0.41	33.5	11.6	8.53	-1.48	-1.56			
300	antipyrine <sup>a</sup>	11.6	71.6	67.1	31.6	65.2	0.79	0.14			
299	imidazole	11.5	21.6	41.6	37.2	44.1	0.27	-0.28			
123	isoniazid	7.49	30.3	114	nc	—	0.61	-0.58			
291	aniline	6.9	23.3	21.2	8.91	28.8	0.53	0.12			
290	sodium sulphite	6.78	84	17.6	18.4	40.7	1.09	0.55			
279	thioacetamide	4.17	139	140	237	158	1.52	-0.10			
272	salicylic acid <sup>a</sup>	3.38	0.35	23.9	4.53	21.7	-0.98	-1.58			
116	cyclophosphamide × H <sub>2</sub> O	3.12	36.1	19.7	9.14	28.5	1.06	0.32			
266	potassium hexacyanoferrate III	2.82	0.85	1.57	5.44	7.78	-0.52	-0.68			
107	acetylsalicylic acid	2.27	30.5	19.7	8.23	59.5	1.13	0.16			

Comparison of the IC50 values (mM) of the assays performed in this study with the reference IC50 (IC50X) values from the RC (1). The chemicals are listed according to decreasing RC values.

<sup>a</sup>The reference chemicals recommended for validation of a new cytotoxicity assay (NIH, 2001 [4]). <sup>b</sup>Chemicals which are not part of the RC, with other literature references.

ECE = electrical current exclusion method; WST-8 = advanced MTT assay; NRU = neutral red uptake assay; TB = trypan blue exclusion haemocytometer assay; nc = no IC50 value calculable; — = no assay performed; nv = no IC50 value available in literature.

Variation of ECE results versus literature and versus the assays performed is given as log10 of the quotient of the ECE result and the corresponding geometric mean values.

Table 2: continued

RC No.	Chemical	Registry of cytotoxicity			Assays performed in this study			Variation of IC50 values		
		Literature IC50 (mM)	ECE IC50 (mM)	NRU IC50 (mM)	WST-8 IC50 (mM)	TB IC50 (mM)	ECE versus literature	ECE versus assays performed		
261	ferrous sulphate	1.85	1.78	8.85	4.17	8.53	-0.02	-0.58		
106	sodium I fluoride	1.85	1.29	6.78	1.47	—	-0.16	-0.39		
105	theophylline	1.83	1.77	nc	5.21	nc	-0.01	-0.47		
102	acrylamide	1.61	2.09	13.3	8.63	—	0.11	-0.71		
100	L-ascorbic acid	1.52	31.2	16.1	6.96	—	1.31	0.47		
99	nalidixic acid <sup>a</sup>	1.5	3.26	10.3	5.47	7.68	0.34	-0.37		
252	potassium cyanide	1.12	24.7	21	20.8	19.5	1.34	0.08		
233	ibuprofen	0.52	0.36	4.03	1.57	—	-0.16	-0.84		
231	tween 80	0.49	0.04	1.14	0.97	2.77	-1.09	-1.56		
81	cupric sulphate × 5H <sub>2</sub> O	0.33	0.04	0.6	0.58	0.24	-0.92	-1.04		
75	trichlorfon <sup>a</sup>	0.27	25	0.82	0.41	1.39	1.97	1.51		
76	sodium dodecyl sulphate	0.27	0.46	0.18	0.2	0.21	0.23	0.37		
56	manganese II chloride × 4H <sub>2</sub> O	0.13	0.2	2.37	0.23	0.16	0.19	-0.35		
54	DL-propranolol × HCl <sup>a</sup>	0.13	0.22	0.71	0.17	—	0.26	-0.20		
42	p-aminophenol	0.062	0.44	0.46	0.17	—	0.85	0.20		
180	p-phenylenediamine (4) <sup>a</sup>	0.05	0.55	19.1	9.15	—	1.04	-1.38		
31	chloroquine diphosphate	0.017	0.03	0.16	0.05	—	0.25	-0.47		
27	chlorpromazine	0.014	0.013	0.041	0.092	0.091	-0.03	-0.73		
20	cadmium II chloride (4) <sup>a</sup>	0.0064	0.041	0.042	0.031	0.044	0.81	0.03		
144	sodium dichromate VI × 2H <sub>2</sub> O <sup>a</sup>	0.00093	0.0001	0.0027	0.0025	0.0173	-0.97	-1.69		
13	cycloheximide	0.00059	0.0004	0.0432	0.0152	—	-0.17	-1.81		
	hydroxyethyl metacrylate (12–15) <sup>b</sup>	3.8	1.94	2.01	1.14	nc	-0.29	0.11		
	gentamycin (7, 8) <sup>b</sup>	nv	nc	6.2	6.2	nc	nv	nc		
	ciprofloxacin (11) <sup>b</sup>	nv	0.62	3.02	0.71	—	nv	-0.37		
	polyethyleneimine (13) <sup>b</sup>	nv	0.0026	0.0019	0.009	0.0027	nv	0.19		

Comparison of the IC50 values (mM) of the assays performed in this study with the reference IC50 (IC50X) values from the RC (1). The chemicals are listed according to decreasing RC values.

<sup>a</sup>The reference chemicals recommended for validation of a new cytotoxicity assay (NIH, 2001 [4]). <sup>b</sup>Chemicals which are not part of the RC, with other literature references.

ECE = electrical current exclusion method; WST-8 = advanced MTT assay; NRU = neutral red uptake assay; TB = trypan blue exclusion haemocytometer assay; nc = no IC50 value calculable; — = no assay performed; nv = no IC50 value available in literature.

Variation of ECE results versus literature and versus the assays performed is given as log10 of the quotient of the ECE result and the corresponding geometric mean values.

on the actual cell density in the test, 4000 to 15,000 cells were analysed for each sample.

The ECE method combines two independent parameters: change in cell viability, calculated as the ratio of total live cells to total cells, and the change in total cell volume (biovolume) of the viable cell fraction. Cytotoxic effects on cells are revealed by a comparison of the cell-size distributions of treated cells and untreated controls. The “Electrical Current Exclusion Index of Cytotoxicity” (ECE-IC) was calculated as the product of the biovolume of the live cells and cell viability, standardised to the untreated control value. The IC50 value of an agent was calculated as the concentration of the chemical with an ECE-IC of 50% of the control value.

$$\text{ECE-IC} = \frac{\text{biovolume (sample)} \times \text{viability (sample)} \times 100}{\text{biovolume (control)} \times \text{viability (control)}}$$

## Results

### Determination of the cell size distribution by the ECE method

The cell size distribution of untreated L-929 mouse fibroblasts consisted of mainly viable single cells, gated in the range 12.6µm to 19.4µm, corresponding to a volume-range from 1047µm<sup>3</sup> to 3823µm<sup>3</sup>. This included cells at different states of the cell cycle. Live cell aggregates ranged from 19.4µm up to a size of 40µm (Figure 2a). The size range for dead cells at different stages of fragmentation was between 3.8µm and 12.6µm (Figure 2b). Size classes smaller than 3.8µm consisted of cell debris, for example, vesiculated membrane particles.

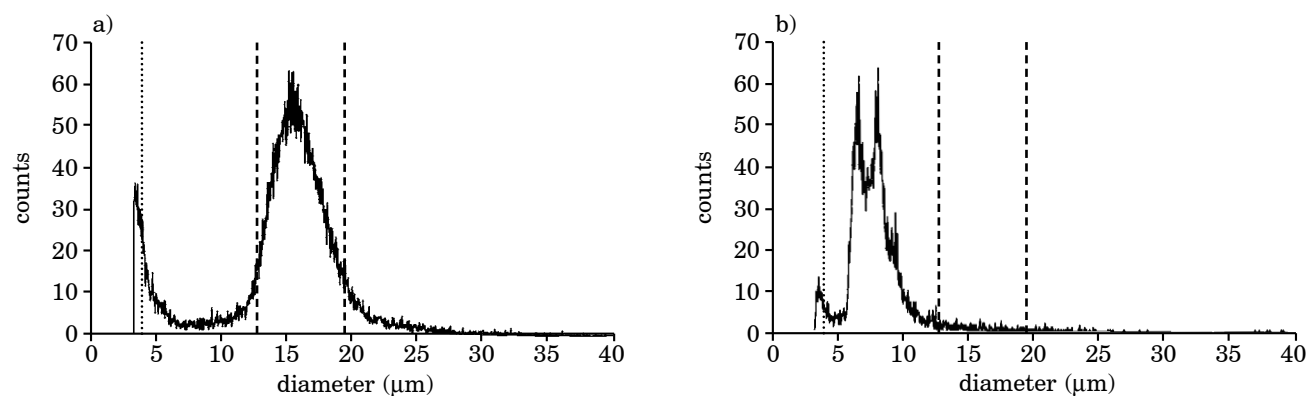
### Effects of cytotoxic compounds, detected by the ECE method

Depending on the mechanism of cytotoxic action, all the test chemicals showed the combined effects of decreasing viability (disruption of the cell membrane), decreasing cell count and biovolume (inhibition of cell proliferation), and cell swelling (the first signs of necrosis), reflecting the actual state of the ongoing process toward cell death. The time-course and intensity of each of these effects can be completely different. The ECE cytotoxicity index combines these effects, providing results with an excellent agreement with the findings of the reference WST-8 and NRU assays (Figures 3 to 6).

Taking potassium hexacyanoferrate as an example (Figure 3), these effects can easily be demonstrated at the concentration of the IC50 value reported in the RC (2.82mM) and at a maximum concentration (76.1mM; 1). It is evident that the cell count and biovolume had already decreased dramatically at 2.82mM, indicating a cessation of cell proliferation, whereas cell viability was relatively unaffected. With further increases in the concentration of the chemical, cell viability decreased dramatically, as indicated by the shift of diameter distribution into the region of the cell nucleus and debris. The combination of both effects in the ECE-IC results in a concentration–response relationship indicating an IC50 value of 0.85mM (Figure 4).

Taking acetonitrile as a further example (Figure 5), cell count and biovolume decreased only slightly at a medium concentration (56.5mM) and even at highest concentration (1525mM). However, it is obvious that necrosis had started. This can be seen by the swelling of the cells at the medium concen-

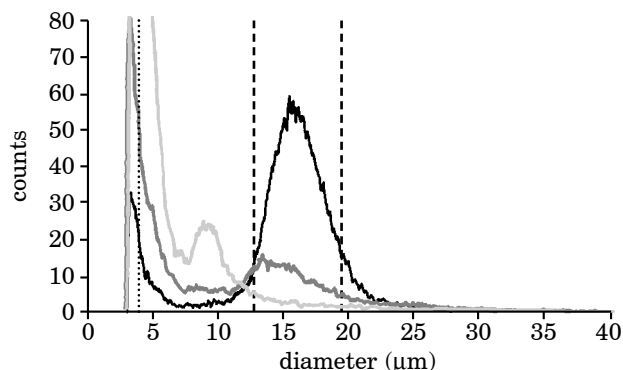
**Figure 2: Typical size distribution of an L-929 control sample**



a) Size distribution of an L-929 control sample with a viability > 90%;

b) a sample of dead L-929 cells with a viability < 10%. Cursors are used to define the gating between cell debris/dead cells (3.8µm), dead cells/viable cells (12.6µm) and viable cells/aggregates (19.4µm).

**Figure 3: Change in cell count and size distribution of L-929 cells exposed to increasing concentrations of potassium hexacyanoferrate**



Black trace = control; dark grey trace = concentration of 2.82mM; light grey trace = maximum concentration of 76.8mM.

tration, and the concomitant reduction in diameter distribution at the highest concentration, indicating a decrease in cell viability. Also in this case, the ECE-IC showed excellent agreement with the findings with the two reference assays (Figure 6).

### Comparison of the results

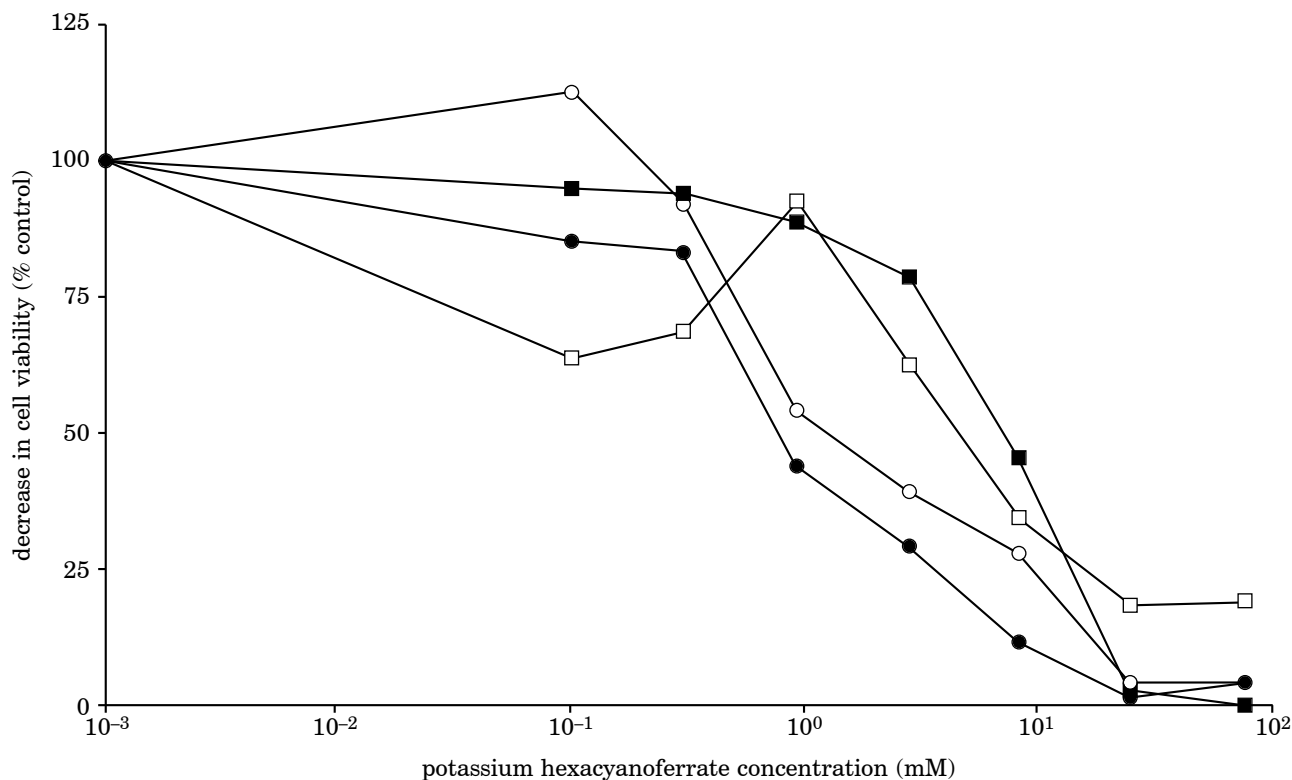
Table 2 lists all the 50 test chemicals with IC50 values from the four different assays. There was good agreement between the IC50 values determined by the ECE method and the literature data (1, 4) and with the results of the other assays performed. The comparison is given by the formula:

$$\text{factor} = \log_{10}(\text{ECE result}/\text{IC50}_{\text{mean}})$$

In the case of a negative factor, the ECE method is more sensitive compared to the average values from the other methods, whereas a positive factor indicates a lower sensitivity.

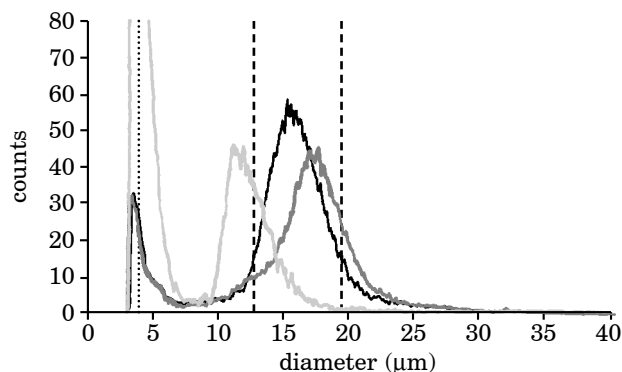
Only 11 ECE results differ by a factor of more than 10 from the IC50 values of the RC. For three of them, the IC50 values from the ECE method were lower than those listed in the RC, while for the

**Figure 4: Concentration response curves for the effect of potassium hexacyanoferrate on the viability of L-929 cells**



Analysed with the ● = ECE method; □ = WST-8 assay; ○ = neutral red uptake assay (NRU) and ■ = trypan blue exclusion assay (TB).

**Figure 5: Change in cell count and size distribution of L-929 cells exposed to increasing concentrations of acetonitrile**



Black trace = control; dark grey trace = mean concentration of 56.5mM; light grey trace = maximum concentration of 1525mM.

other seven chemicals, the RC values were lower. Only 8 ECE results differed by a factor of more than 10 from the mean values obtained with the other assays performed. In no case did differences with a factor of 100 or more occur.

Four of the test chemicals are not listed in the RC, but two of them are of importance, since they are used as antibiotics with cell cultures (gentamycin and ciprofloxacin; 7, 8). While ciprofloxacin was not previously tested on cells *in vitro*, it was found to be toxic to protozoans with an IC<sub>50</sub> value of 3.9mM (11). The ECE method was more sensitive than the WST-8 and NRU assays. The IC<sub>50</sub> value for gentamycin was 6.2mM in the WST-8 and NRU assays, but no IC<sub>50</sub> value could be determined with the ECE method. The other two chemicals are monomers for the production of medical devices (2-hydroxyethylmethacrylate; 12, 13) responsible for cell transfection (polyethylenimine with a  $M_r$  between 800 and 2000; 17).

The cytotoxicity of isoniazid could not be detected with the WST-8 assay, and that of theophylline could not be determined with the NRU assay. The exact reasons for this were unclear, but some chemicals may interfere with the assays themselves. Nevertheless, the overall sensitivity was best for the new ECE method, followed by the WST-8 assay, then the NRU, then the TB assay.

## Discussion

Acute systemic toxicity testing is conducted to determine the relative health hazards represented

by chemicals and various products. Currently, this testing is mainly conducted by using animals. Studies published in recent years have shown a good correlation between acute toxicity *in vitro* and *in vivo*, and have suggested that *in vitro* methods may be helpful in predicting *in vivo* acute toxicity (18–21).

In accordance with the aim of developing and validating appropriate cell culture-based *in vitro* cytotoxicity tests as alternatives to animal testing, this study tested the cytotoxicities of 50 chemicals with L-929 mouse fibroblast cells. As a result of this direct comparison to two already established and recommended reference assays, NRU and WST-8, and additional measurements with the TB assay, the ECE method, based on a widely introduced cell counting and analysing system (CASY model TTC), has been shown to be a useful new *in vitro* cytotoxicity test system.

The three established *in vitro* assays used are all histochemical methods, but vary in their endpoints (22–25).

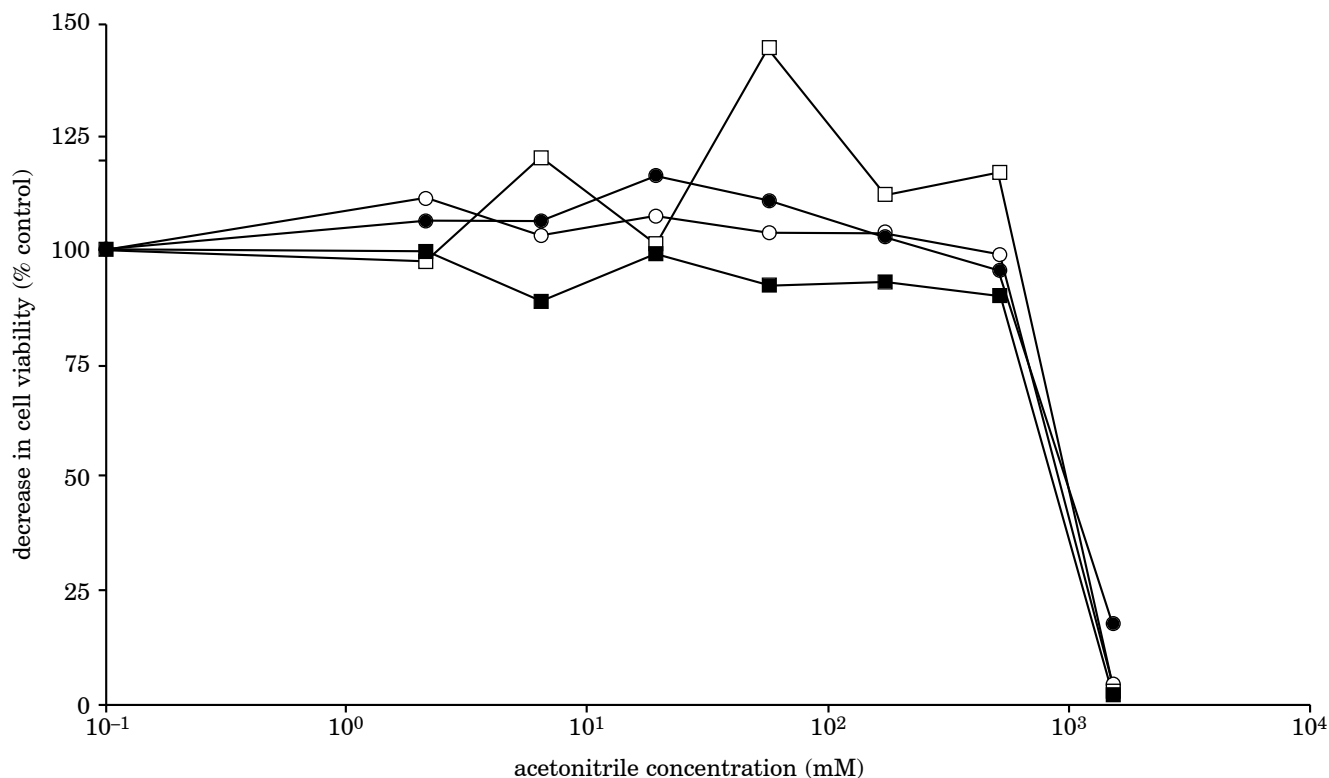
*The NRU assay:* the small neutral red vital dye molecules enter the cells and are trapped in the lysosomes of live cells only (neutral red uptake); viable cells are stained bright red, while dead cells remain unstained.

*The WST-8 assay:* in this advanced version of the MTT assay, the colourless, water-soluble tetrazolium salt, WST-8, enters the cells and becomes reduced to coloured formazan by the mitochondrial activity of viable cells only; viable cells and the culture medium become red to blue coloured, while dead cells are not stained.

*The TB exclusion assay:* the large, charged trypan blue molecules are not able to pass across the intact cell membranes of viable cells; dead cells, with permeable membranes, have a blue-stained cytoplasm.

The ECE viability assay is based on the different electrical properties of viable and dead cells. All cell lines show a characteristic cell size distribution. Viable cells are identified according to their true cell volume, whereas dead cells with a membrane permeable to an electrical current, are identified by the size of their nuclei. The consistent results of the ECE method are based on high technical standardisation, a very high counting rate, and the ability to combine cell viability and cell volume analysis for detecting the first signs of cell necrosis and damage to the cell membrane caused by cytotoxic agents.

Concentration–response experiments for 50 chemicals (including 46 selected from the RC [1]) were conducted multiple times with the four cytotoxicity assays. Compared to the results from the reference WST-8, NRU and TB assays, the ECE-IC values are highly consistent. Furthermore, the

**Figure 6: Concentration response curves for the effect of acetonitrile on the viability of L-929 cells**

Analysed with the ● = ECE method; □ = WST-8 assay; ○ = neutral red uptake assay (NRU) and ■ = trypan blue exclusion assay (TB).

assay-to-assay variation of the IC<sub>50</sub> values found in this study, compared directly with the reference IC<sub>50</sub> values in the RC (1, 2), was less than factor 10 for 80% of the test chemicals. The IC<sub>50</sub> values of the ECE method were the most sensitive and were closest to values recorded in the literature (1–3, 17, 18). The assay-to-assay variation of the results was greater than factor 10, but smaller than 100, for only 8 of the test chemicals (16%).

This study clearly shows that each of the test systems used was able to monitor the cytotoxicity of the respective chemicals in a reliable and reproducible manner. It was also found that some chemicals are not appropriate for some assays, because of chemical interference with the functional mechanism of the assay concerned.

The ECE assay has a decisive advantage. All the results can be directly stored as an electronic record, compliant with the quality rule for scientific records released by the Food and Drug Administration (FDA, 21CFR Part 11). Any change of an original result is traceable by audit trails. The data can easily be networked or transferred into common spreadsheet programs.

In conclusion, the ECE method, based on an advanced electronic cell counting and analysis system, was shown to be an excellent and suitable *in vitro* cytotoxicity assay system for cells cultured in suspension, whereas the NRU and WST-8 assays are preferable for adherent-growing cells. However, the study also demonstrated, that adherent-growing cell lines, such as L-929 mouse fibroblasts, can easily be adapted to growth in suspension by changing the cell culture medium, thus enabling the direct use of the CASY-Technology. A direct comparison with the recommended dye based assays (4) is possible.

Furthermore, the new ECE test system can detect the complex influence of toxic compounds on the first and most sensitive barrier of animal cells, the cell membrane. Although 24 hours was selected as the time for incubation with the toxic compounds (16), it is obvious that progressive changes in the state of dying cells can be monitored with this technology. Signs of necrosis can be seen, indicating the transition from viable cells with an intact membrane to dead cells which have lost their membrane integrity. This transition can be seen by the appear-

ance of cell nuclei and cell debris and the concomitant reduction in the viable cell peak.

Sophisticated technology, such as Allen-Video-Enhanced-Microscopy provides information about cell organelle movements and their subtle relationships to the influence of cytotoxic chemicals on living cells (26). A combined multispectral cytometric approach can be used for the analysis of apoptotic versus necrotic cells (27), but these systems are highly sophisticated and not suitable for routine analysis. No other comparable, reliable and simple assay system can detect these delicate, complex toxic influences of chemicals on living cells according to the guidance procedures for evaluation and validation of *in vitro* cytotoxicity assays (4). The ECE method proved to be a very suitable, reliable and sensitive assay for characterising cell cultures and for determining or predicting the cytotoxic potentials of chemical compounds.

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