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# Summary in 96-Well Microplate-Based Cytotoxicity and Development of High-Content Image Analysis (HCIA) Assay with High Throughput and High Accuracy

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**Abstract**

Various cytotoxicity assays using 96-well microplate have often been performed by measuring biological indicators, such enzyme activity, uptake quantity of dye, or cellular ATP content, and as a matter of course, they have been reported in several manuscripts as a tool for expressing cell viability. However, recently reported that cytotoxicity assays, such as ATP and MTS, were underestimated cytotoxicity when chemicals such anti-cancer drug or mutagens induced cell hypertrophy with increasing intracellular ATP content. In addition, there are few studies using many chemicals to compare the cell viability of the cytotoxicity test using the abovementioned indicators and the actual cell counting. Therefore, the authors have revealed that cytotoxicity tests using biological indicators, such as those mentioned above, do not necessarily produce cell viability that accurately reflects the number of cells as target for 25 chemicals. Moreover, authors clarified that cell hypertrophy and cell cycle are correlated with the use of HCIA (high-content image analysis). Based on these results and the authors' experience, we summarized the features of the 96-well microplate-based cytotoxicity test. Finally, the authors demonstrated the usefulness of the HCIA assay, which can obtain a large number of cytological parameters at once from a single microplate by using cell cycle-specific inhibitors.

**Keywords:** MTT; NRU; WST-8; Alamar blue; ATP; HCIA; Cytotoxicity test; Cell hypertrophy

## Introduction

The cytotoxicity test is used to evaluate the influence of chemicals on cell death or cell growth inhibition. Because cells are the smallest units that make up an organism, cytotoxicity test is used in various fields, such as pharmaceuticals, medical devices, chemicals, cosmetics, and agricultural chemicals, as the first step in predicting human toxicity. In particular, in the field of medical devices, cytotoxicity test is registered in ISO10993-5 (Part 5: Tests for *in vitro* cytotoxicity) as a test necessary to be applied by authorities such as OECD. The degree of cytotoxicity test is basically expressed as cell viability by comparing the number of cells following chemical exposure with the number of of controls, so if adherent cells are used for cytotoxicity test, detaching the cells from the plate and counting the number of cells are necessary. However, this method is not practical because it not only uses a lot of chemicals and cells but also takes time. Therefore, by using a 96-well microplate and measuring a biological indicator that correlates with the number of cells by microplate reader, even with a small amount of chemicals, cell viability can be easily achieved in a short time [1].

## Methodology

Several comparative studies have examined various cytotoxicity assays that use microplates [2-7]. The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a reaction in which MTT is reduced to insoluble purple formazan by dehydrogenase in the mitochondria of cells. It has been used most often to evaluate cytotoxicity and cell growth inhibition for more than 30 years [8]. The NRU (neutral red uptake) assay utilizes the phenomenon that weak red cationic dye, NR is accumulated in lysosomes in cells and is not only recognized as a test required for the use of medical device (ISO10993-5) but also used in OECD phototoxicity test (OECD TG421). What is common to these two methods is that the operation of eluting insoluble formazan and NR accumulated in the cells is necessary and is thus complicated. Highly water-soluble tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), is reduced by NADH, which is required for dehydrogenase activities in the mitochondria, to give a water-soluble formazan dye (yellow color), which is soluble in the tissue culture media [9,10]. Alamar blue (resazurin), which is used in the Alamar blue assay, is a nonfluorescent blue redox dye that is converted into fluorescent red dye, resorufin, when the medium is reduced by cell growth. The fluorescence or colorimetric signal generated by the conversion is proportional to the number of living cells in the sample and can be used to detect cell viability [11-13]. Since the water-soluble formazan and resorufin produced in these assays are cell permeable and are easy to operate considering a cell lysis step is not required and have high detection sensitivity, these assays have recently become more popular than MTT and NRU.

The ATP (adenosine triphosphate) assay uses bioluminescence as an indicator of the number of living cells and represents a simple, rapid, and sensitive cytotoxicity test [14]. The ATP assay can be used to measure the functional integrity of living cells since all cells need ATP to stay alive. The ATP assay is especially useful because it is not limited to cancer cell lines [15] and has a 100-fold higher sensitivity than the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay for measuring the number of cell

lines [16,17]. Moreover, it has shown excellent linearity and a wide dynamic range (0 – 750,000 RLU) in RPMI-8226 cells [18]. Furthermore, a major advantage of the ATP assay over other microplate-based cytotoxicity tests is that the ATP assay can be used to assess cytotoxicity in cell suspensions and monolayers [19]. Therefore, ATP assay was utilized as cytotoxicity test associated with chromosome aberration test [20]. The high sensitivity, absence of artifacts, versatility, speed, and simplicity of the ATP assay make it a recommended routine cytotoxicity test [21].

However, Chan et al. and Tahara et al. reported that drug-induced increases in ATP content were associated with increased cell size by measuring the number and area of cells using a simple high-content imaging method [22]. This method used for image analyzing number and area of cells have not reported to compare with traditional method measuring suspended cells directly for ensuring accuracy. Since this method enables the measurement of the area of elongating cells that adhere to the plate in two dimensions, estimation of the size of cells may not be a substantive method and the size of cells suspended by enzyme treatment is considered original one. Therefore, in this study, the number and diameter of cells suspended by enzyme treatment were measured by automatic cell counter in three dimensions. We considered these values as standard and evaluated the accuracy of NRU, MTT, WST-8, Alamar blue, and ATP assay in 25 chemicals. In addition, the same evaluation will be conducted for the HCIA assay using the image analyzer that we developed, and its usefulness will be elucidated.

## 96-well microplate-based cytotoxicity test

The authors selected 25 chemicals from the cytotoxicity tests reported previously, and differences between cell viability calculated from 4 types of cytotoxicity tests (Alamar blue assay, WST-8 assay, ATP assay, and HCIA assay) and that calculated from automatic cell counter were compared. As a result, the correlation coefficient (r2) between each of the four cytotoxicity assays and automatic cell counter was low (r2<0.8, at least 2 out of 3 experiments) as follows: ATP assay, 7 (acetaminophen, catechol, 5-fluorouracil, p-phenylenediamine, daunorubicin, doxorubicin, and rotenone); WST-8 assay, 2 (acetaminophen and doxorubicin); Alamar blue assay, 3 (acetaminophen, p-phenylenediamine, and doxorubicin); and HCIA assay, 0. The common point of these seven chemicals was cell hypertrophy [22]. Image analysis revealed that cell hypertrophy is about 1.4 to 2 times larger than that of the control in the state of cell adherence to the plate. Regarding the area, the cell may have just expanded transversally and not swelled actually, so the cells became spherical after they were detached from the plate by enzyme treatment was measured by automatic cell counter. As a result, the diameter was 1.15 – 1.25 times that of the control [23], i.e., in terms of volume, confirming that the cells were enlarged [22]. Furthermore, when the cell cycle was analyzed with the image analyzer using the images of chemical treated-cells, those treated with acetaminophen, catechol, and 5-fluorouracil had an increased proportion of cell population in S phase (data not shown). Although no report was found on catechol, the results of acetaminophen and 5-fluorouracil are consistent with those reported in a study [24,25]. Daunorubicin, doxorubicin, p-phenylenediamine, and rotenone had an increased ratio of G2/M phase population and are consistent with the findings of some studies [26-29]. Among the 25 chemicals tested for cytotoxicity, as a representative example, two chemicals without cell hypertrophy (m-chloroaniline, nicotine) and two chemicals with cell hypertrophy (S phase arrest type, acetaminophen; G2/M phase arrest type, doxorubicin) are shown in Figure 1. For these chemicals, the cell viability calculated from seven types of cytotoxicity tests, including MTT and NR assays, in addition to automatic cell counter and WST-8, Alamar blue, ATP, and HCIA assays. As a representative photograph of cells treated with chemicals, a photograph of unchanged cells treated by nicotine or enlargement of cells treated by acetaminophen is shown in Figure 2.

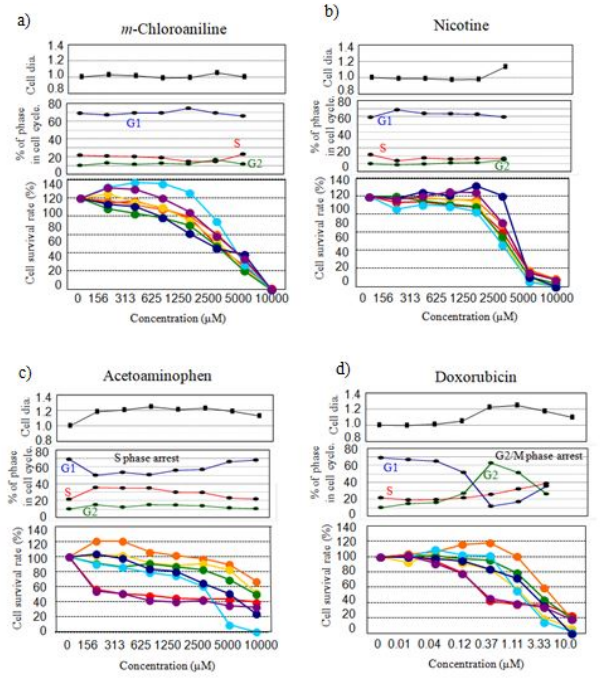


**Figure 1:** Comparison of cell diameter, cell cycle, and cell viability values obtained from seven cytotoxicity assays following treatment with four chemicals.

Cells ($4 \times 10^3$) were seeded into 96-well microplates for NRU, MTT, WST-8, Alamar blue, ATP, and HCIA assays. Cells ($4 \times 10^4$) were seeded into 12-well plates for automatic cell counting. After pre-culture for 24 h, cells were treated with four chemicals for 24 h, and viability was measured using seven cytotoxicity assays. Cell dia. indicates the mean diameter of cells (ratio of control). Cell cycle indicated the percentage of three populations ((%), G1 phase, S phase, and G2/M phase). Cell survival rate indicates the percentage of control (%):

NRU assay ( blue ),

MTT assay (light blue ),

WST-8 assay (yellow ),

Alamar blue (green ),

ATP (orange ),

HCIA assay (violet ),

Automatic cell counter (red ).

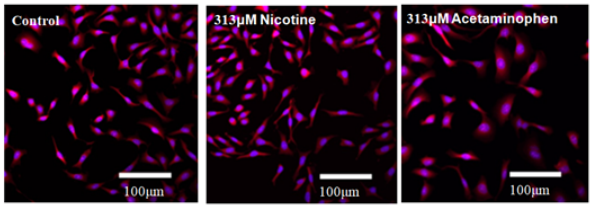


**Figure 2:** Photograph of cells treated by nicotine or acetaminophen. Fluorescence images showing staining with Hoechst 33342 (dark blue, nuclei) and Cell Mask (red, cytoplasm).

## Effect of cell cycle-specific inhibitor-induced cell hypertrophy on the accuracy of cytotoxicity tests

There are a few reports concerning chemically induced cell hypertrophy and increased ATP content through cell cycle. Methotrexate (MTX) was shown to induce an increase in K562 cell size with arresting S phase [30]. Reversine, a synthetic purine, has been shown to induce cell cycle arrest in G2/M phase followed by an increased cell size of PC3 cell [31]. Moreover, DNA damage caused by anti-cancer drug elevated cellular ATP content [32-34]. Oyama et al. reported that H2O2 and doxorubicin induced G2/M cell cycle arrest with an increase of p21 expression in H9c2 cells, which was related to cellular hypertrophy [35]. Moreover, in normal cells, there are also few reports concerning change in cell size or intracellular ATP through cell cycle. Barberis et al. reported the following: RNA and proteins increase exponentially, whereas the DNA content shows a typical double amount with increasing cell size until the cells divide to generate a newborn daughter from G1 phase to M phase [36]. Marcussen et al. indicated that the cellular ATP concentration varies through the cell cycle, reaching a peak at G2/M phase and minimum at late G1/early S phase [37]. Recently, Chan et al. reported the following: under low concentrations of repairable DNA damage, drugs can be arrested at S or G2/M phase that induces an increase in the size and amount of ATP content per cell [38].

Considering the findings of previous reports and the present study, because size, dehydrogenase activity, and ATP content per cell increase during cell division from G1 phase to G2/M phase in the cell cycle, increasing cellular ATP content and dehydrogenase activity can often cause cell hypertrophy when cells are treated with chemicals that can arrest them in S or G2/M phase (Figure 3). This phenomenon is thought to be the cause of the deviation in cell viability rates between the method using biological indicators (NRU, MTT, WST-8, Alamar blue, and ATP assay) and method using cell count (the automatic cell counting, HCIA assay).

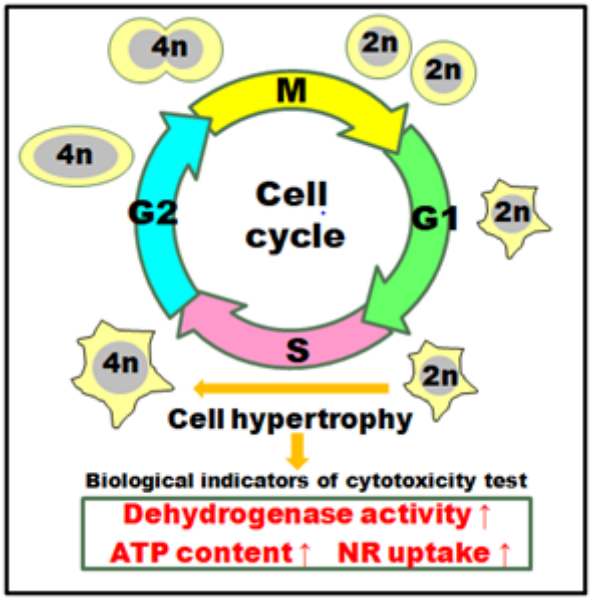


**Figure 3:** Relationship among cell cycle, cell hypertrophy, and biological indicators used in cytotoxicity tests during cell division.

## Summary for features of various 96-well microplate-based cytotoxicity tests

Table 1 summarizes the features of NRU, MTT, WST-8, Alamar blue, ATP, and HCIA assays, which are 96-well microplate-based cytotoxicity tests. In the NRU cytotoxicity test, neutral red is added to the medium and taken up into the cells for several hours. Then, the cells are washed, and the NR accumulated in the cells is eluted with alcohol, and the colorimetric concentration of NR is measured as absorbance. In my experience, the amount of NR uptake into cells may vary depending on the manufacturer, so care must be taken. In the same way as NRU, MTT is added to the medium and taken up by the cells for several hours, and then the cells are washed. Insoluble MTT-formazan reduced by dehydrogenase in the cells is eluted with alcohol. The colorimetric concentration of MTT-formazan is measured as absorbance.

| | NRU | MTT | WST-8 | Alamar blue | ATP | HCIA |
|---|---|---|---|---|---|---|
| Target indicater | Uptake quantity of NR | Dehydrogenase activity | Dehydrogenase activity | Dehydrogenase activity | ATP content | Number of whole cell |
| Detection Reagent | Neutral red | MTT (yellow) | WST-8 (yellow) | Alamar blue (resazurin) (blue) | Luciferase | Nuclear staining: Hoechst33342 Cytoplasmic stainingCellMask |
| Fixation time | --- | --- | --- | --- | --- | Yes (ethanol, 1530 min) |
| Reaction time (time) | 180240 min (uptake) | 180300 min | 60240 min | 60240 min | 10 min | 15 30 min (staining) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Reaction product | Neutral red | MTT formazan (insoluble, dark blue) | WST-8 formazan (water-soluble, orange) | Resorufin (red) | Light | Hoechst33342: Blue fluorescence CellMaskRed fluorescence |
| Extraxtion step | Yes (1030 min) | Yes (1030 min) | No | No | No (Simultaneous with reaction) | No |
| Measuring device | Plate reader | Plate reader | Plate reader | Plate reader | Plate reader | Image analyzer |
| Detection reagent | Absorbance (reference) | Absorbance (reference) | Absorbance (reference) | Fluorescence (Ex/Em) | Chemi-luminescence | Fluorescence (Ex/Em) |
| | 540 nm | 570 (650) nm | 450(650) nm | 560/590 nm | | H33342: 350/461 nmCellMask588/612 nm |
| Required time (one plate) | 5 h | 5 h | 3 h | 3 h | 0.5 h | 1.5 h |
| Operability | A little hassle | A little hassle | No hassle | No hassle | No hassle | No hassle |
| Correlation vs. Automatic cell counter | Good | Good | Good | Good | Good | Very Good |
| 1) There were some chemicals showing abnormal values 2) Correlation is low when chemicals induce cell hypertrophy | | | | | | |

**Table 1:** Summary for features of various 96-well microplate based cytotoxicity tests.

## Results and Discussion

The common problem with NRU and MTT is the low detection sensitivity, so that the incubation time between the detection reagent and cells is long, and the step of lysing the cells is necessary, so that the cells after the cytotoxicity test cannot be used for other purposes (data not shown). In this study of 25 chemicals, the authors initially examined the correlation between NRU or MTT assay and automatic cell counter, but there were some chemicals showing abnormal values (data not shown). This seems to have been caused by the cytotoxicity test having many steps, such as washing and elution. WST-8 and Alamar blue have the same measurement principles as the MTT assay, but because the reaction product produced by intracellular dehydrogenase is a soluble dye, there is no step to lyse cells, and the operation is simple, and abnormal values are not easily generated. In addition, since the reaction is highly sensitive, the incubation time between the detection reagent and cells is as short as 1 to 3 h. The Alamar blue method can detect not only fluorescence but also absorbance (OD: 560 nm), but in the case of absorbance detection, the correlation with the cell viability calculated from the automatic cell counter is low (data not shown). Authors recommend selecting fluorescence detection when using the Alamar blue method. The ATP assay is sensitive enough to be detected from several cells, and the operation can be finished within 30 min. Although it is easy to operate, since the cells are lysed at the time of detection, cells cannot be used for other purposes. HCIA assay is a cytotoxicity test method using an image analysis device developed by us. In this method, cells are fixed with alcohol, and then the nucleus and cytoplasm are stained simultaneously with two types of fluorescent dyes and automatically detected by image analyzer. This test method is highly correlated with the cell viability of the automatic cell counter, and the test procedure is relatively simple.

### HICA assay

We have shown that HICA assay is a very accurate method for assessing cell viability because it can count the number of cells without using biological indicator [22] Since HCIA assay uses image analysis technology, it can quantify various biological phenomena. When we conduct HCIA assay, in addition to counting the number of cells, we usually obtain data on nuclear area, cell area, micronucleus appearance rate, round cell appearance rate, and cell cycle at the same time [39]. As for the cell number and cell area, the results of the cytotoxicity test of the 25 chemicals [22] show that the cell number and cell area measured by HCIA are highly correlated with the cell number and cell diameter measured by automatic cell counter [22,23]. From the above, authors confirmed that the cell number and cell area measured by HCIA assay are correct. Although the nuclear volume has not been directly verified, it can still be measured correctly because the nuclei area changes in the same way as the cell area [22].The micronucleus appearance rate has been validated because authors have previously reported that genotoxicity potential chemicals can be detected by methods using an image analyzer [40].

Moreover, 6 chemicals (acetaminophen, catechol, 5-fluorouracil, p-phenylenediamine, daunorubicin, doxorubicin, and rotenone), which had a cell area of 1.5 times or more than that of the control in the cytotoxicity test of 25 chemicals conducted this time, appeared with obvious increase of micronuclei rate (data not shown). All these chemicals have been reported to be mutagenic [41-43]. When the cell area increased, nuclear enlargement was always observed, but the cell area increase was more obvious than the nuclear area increase (data not shown). Westmoreland et al. reported that carcinogen induced nuclear enlargement in HaLa S3 cell, and Takeshita et al. have reported that chemicals with inducing nuclei swelling cause structural aberration in chromosomal aberration tests, which is consistent with our report [44,45]. It is expected that chemicals which specifically arrest the cell cycle in S phase or G2/M phase and enlarge the cells will induce chromosomal aberration because they affect DNA synthesis and cell division. In most cases of inducing chromosomal aberration, since the cell diameter is 1.15 times or more than that of control, it may be used as a biomarker for chromosomal structural aberration test. In addition, the phenomenon that the cell morphology becomes round occurs when the cytotoxicity becomes strong, and it is an important parameter in evaluating the strength of the cytotoxicity. As a result of carrying out the cytotoxicity test of 25 chemicals [22], it was

confirmed that the result actually judged under the microscope and that of HCIA assay coincided (data not shown), but all rounded cells cannot be evaluated because most of them are detached during the washing process. As for the cell cycle, in order to verify whether HCIA assay can evaluate the cell cycle correctly, cells treated with cell cycle G1 phase inhibitor, rapamycin [46-48]; cell cycle S phase inhibitor, aphidicolin [49]; and G2/M inhibitor, Cytochalasin B [50] were co-stained with Hoechst 33342 and CellMask (Table 2). Rapamycin was confirmed to increase the ratio of G1 phase to 80% at almost all doses, whereas the G1 phase ratio of the control was 70%. Aphidicolin increased the population of G1 phase and S phase, consistent with the report of Costa et al. [51]. Cytochalasin B had a significantly higher population of S phase and G2/M phase, similar to the report by Gu et al.

| Chemicals | Dose | Cell viability (%) calculated from counting cell number | Area of nuclei (% of cont.) | Area of cell (% of cont.) | Micronucleus appearance (ratio of cont.) | % of round cell | Cell cycle (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | G1 | S | G2/M |
| | μM | Mean | Mean | Mean | Mean | Mean | Mean | Mean | Mean |
| Control | 0.00 | 100.0 | 100.0 | 100.0 | 1.0 | 3.5 | 70.0 | 21.4 | 7.8 |
| Rapamycin | 0.08 | 58.8 | 114.3 | 139.3 | 0.8 | 0.8 | 83.1 | 13.8 | 2.4 |
| | 0.16 | 71.4 | 112.5 | 136.6 | 0.8 | 1.2 | 84.8 | 12.7 | 1.9 |
| | 0.31 | 67.0 | 113.7 | 141.0 | 1.0 | 1.1 | 81.8 | 15.1 | 2.6 |
| | 0.63 | 61.2 | 118.6 | 144.9 | 0.9 | 1.1 | 83.4 | 13.5 | 2.6 |
| | 1.25 | 57.8 | 116.3 | 144.0 | 0.9 | 1.1 | 80.3 | 16.0 | 3.2 |
| | 2.50 | 58.4 | 114.9 | 143.2 | 1.0 | 1.3 | 76.6 | 18.4 | 4.4 |
| | 5.00 | 55.0 | 112.9 | 133.5 | 0.7 | 2.1 | 72.7 | 20.4 | 6.4 |
| | 10.00 | 35.9 | 92.4 | 91.1 | 2.1 | 10.5 | 61.4 | 21.1 | 16.2 |
| Aphidicolin | 0.08 | 57.3 | 115.0 | 142.4 | 1.4 | 1.8 | 76.8 | 18.5 | 3.9 |
| | 0.16 | 56.4 | 123.4 | 160.5 | 1.7 | 1.2 | 76.1 | 20.1 | 3.2 |
| | 0.31 | 47.1 | 140.2 | 190.4 | 3.0 | 1.2 | 70.3 | 25.3 | 3.5 |
| | 0.63 | 36.1 | 163.9 | 237.3 | 4.5 | 0.9 | 67.3 | 28.4 | 3.5 |
| | 1.25 | 31.8 | 194.9 | 293.9 | 3.4 | 0.5 | 76.1 | 20.6 | 2.3 |
| | 2.50 | 30.9 | 189.5 | 281.8 | 1.8 | 0.4 | 75.9 | 20.4 | 2.7 |
| | 5.00 | 29.4 | 185.6 | 280.7 | 1.6 | 0.7 | 80.0 | 16.8 | 2.1 |
| | 10.00 | 31.7 | 178.6 | 253.9 | 1.6 | 1.1 | 81.7 | 14.5 | 2.3 |
| Cytochalasin B | 0.08 | 70.6 | 100.0 | 110.4 | 1.4 | 1.3 | 75.7 | 19.1 | 4.4 |
| | 0.16 | 87.9 | 96.3 | 100.8 | 1.1 | 1.6 | 75.6 | 19.1 | 4.6 |
| | 0.31 | 66.8 | 93.1 | 92.2 | 1.5 | 1.6 | 77.5 | 17.0 | 4.8 |
| | 0.63 | 60.1 | 109.1 | 106.5 | 1.7 | 2.9 | 56.6 | 24.8 | 17.5 |
| | 1.25 | 36.2 | 139.1 | 148.1 | 2.1 | 1.5 | 34.2 | 30.9 | 33.1 |
| | 2.50 | 33.8 | 136.6 | 160.7 | 1.9 | 0.2 | 29.8 | 33.8 | 33.8 |
| | 5.00 | 32.8 | 124.1 | 167.2 | 2.8 | 0.3 | 33.8 | 37.1 | 27.9 |
| | 10.00 | 31.7 | 122.8 | 158.6 | 2.1 | 0.4 | 33.7 | 39.6 | 25.7 |

**Table 2:** Measurement of biological parameters using HCIA assay in CHL cell treated by representative.

## Conclusion

In this study, we showed that cytotoxicity assays, such as NRU, MTT, WST-8, Alamar blue, and ATP, are highly accurate cytotoxicity tests, and the results generated correlate well with those of automatic cell counter when chemicals do not induce cell hypertrophy. However, we also showed that these cytotoxicity assays underestimate

cytotoxicity when chemicals induce cell hypertrophy and suggested that this is caused by an increase in intracellular ATP and NADH content accompanied by an increase in cell and mitochondrial area. These cytotoxicity assays utilizing biological indicators, such NRU uptake and NADH and ATP content, are therefore not suitable for assessing cytotoxicity when chemicals possessing cell hypertrophy-inducing potential-like mutagens and carcinogens are measured for cytotoxicity. Therefore, we showed that the HCIA assay can directly count the number of cells by image analysis and evaluate cytotoxicity with high accuracy without being influenced by intracellular ATP or NADH content. Furthermore, since this assay can not only measure the area of cells and nuclei as well as count cell number but also evaluate cell circularity, micronucleus appearance ratio, and cell cycle phase, we can gain further insight from the cytotoxicity results. Moreover, when using a stacker system that can automatically take microplates in and out of an image analyzer, HCIA assay can automatically measure many microplates at once and is thus applicable for high throughput screening (HTS). As described above, the HCIA assay can identify not only the number of cells but also the information related to many other toxicity parameters from image information based on a principle different from those of NRU, WST-8, Alamar blue, and ATP assays, so it can be considered an extremely effective system for comprehensively evaluating toxicity.

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