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A Morphological Identification Cell Cytotoxicity Assay Using Cytoplasm-localized Fluorescent Probe (CLFP) to Distinguish Living and Dead Cells

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Abstract

Cell cytotoxicity assays include cell activity assays and morphological identification assays. Currently, all frequently used cytotoxicity assays belong to cell activity assays but suffer from detection limitations. Morphological identification of cell death remains as the gold standard, although the method is difficult to scale up. At present there is no generally accepted morphological identification based cell cytotoxicity assay. In this study, we applied previous developed cell cytoplasm-localized fluorescent probe (CLFP) to display cell morphologies. Under fluorescence microscopy, the fluorescence morphology and intensity of living cells are distinct from dead cells. Based on these characters we extracted the images of living cells from series of samples via computational analysis. Thus, a novel cell morphological identification cytotoxicity assay (CLFP assay) is developed. The performance of the CLFP assay was similar to cell activity assay (MTT assay), but the accuracy of the CLFP assay was superior when measuring the cytotoxicity of active compounds.

Graphical Abstract

Keywords

Cytotoxicity Assay, Cell Death, Cytoplasm-localized Fluorescent Probe, Cell Morphological Identification, Computational Analysis

Introduction

Evaluating cell cytotoxicity of the chemicals is important in biomedical research.[1,2,3,4,5,6] Researchers often need to screen the cytotoxicities of a big library of chemicals with diversified biological and physicochemical properties.[7,8,9] Thus, the ideal cell cytotoxicity assay should have pan-applicability towards all types of compounds. In addition, the assay should be simple, fast, accurate, cheap and easy to scale up to high-throughput screening.

In general, cell cytotoxicity assays can be classified into two main categories. The first type is cell activity assays, which measure biochemical activity or the abundance of cellular components.[10,11,12,13] Currently, this type of cell cytotoxicity assay is most commonly used in research; however, the screened chemicals may affect the detection of the activity and abundance. For example, the MTT assay is unsuitable for determining the cytotoxicity of reactive compounds.[14,15,16] It is no doubt that the performance of other biochemical assays has the potential to be interrupted by certain classes of chemicals. The second type is morphological identification assays. Morphological changes denote the start of cell death, and morphological identification of cell death remains as the gold standard.[17] Scientists are actively applying new physical and chemical techniques to develop the novel methods to morphologically identify cell death.[18] However, at present there is no generally accepted morphological identification based cell cytotoxicity assay because of the difficulty in processing large numbers of samples. For instance, morphological identification of cell death by optical microscopy is accurate and general for any types of cell death. However, this method is unsuitable for screening numerous samples.

The emergence of high-content screening (HCS) systems has provided the opportunity for developing morphological identification assays suitable for cytotoxicity screening.[11,19] An HCS apoptosis assay has been developed by commercial companies to measure cell nuclear shrinkage quantitatively, and finally evaluate the rate of cell apoptosis.[20,21] However, this assay was limited to measuring cell death by apoptosis. Moreover, during the experiments many dead cells will detach from culture plates, which prevents the accurate comparison of the nuclear areas of control cells with drug treated cells.

Cell cytoplasm-localized fluorescent probe (CLFP) refers to a series of fluorescent dyes that only stain cell cytoplasm of living cells. Owing to the absent exploration of CLFP application, no commercial CLFP is available, although some fluorescent probes have been reported with the characters of being the CLFPs.[22,23,24,25] In our previous study, we have reported two CLFPs, which have been successfully used to image cell multinucleation.[26] The structures of them are shown in Figure S1. Because cell cytoplasm occupies the majority of cells, morphological changes in the cell cytoplasm are the most obvious. Thus, instead of evaluating nuclear shrinkage as the morphological identification factor, in this study we are interested to developing a morphological identification cell cytotoxicity assay by using CLFP to display the different fluorescent morphologies between living and dead cells.

Materials and Methods

Reagents and materials. Human cancer cell lines: A2780 (Sigma, 93112519), HepG2 (ATCC: HB-8065), MDA-MB-453 (ATCC: HTB-131), MDA-MB-468 (ATCC: HTB-132) were obtained from cell resource center, Peking union medical college. All the chemicals used in this study were purchased from commercial sources: Sigma-aldrich, J&K® Chemical corporation, Beijing ouhe reagents corporation with a purity > 95%. The synthetic protocol of probe 1 and 2 was described in previous research.

Cell staining with CLFP. The CLFPs were reported previously,[26] the experiment condition of CLFP 1 is 25 $\mu\text{M} \times 30$ min, UV excited; and probe 2 is 1 $\mu\text{M} \times 10$ min, blue fluorescence excited. The fluorescence microscopy was performed with high content screening system (Operetta-PerkinElmer) and Olympus IX71. Various cancer cell lines were cultured in 24 well cell plates. When the cells reached around 50% confluence, cells were treated or untreated with various drugs for certain time. CLFP probe was added into the each well for incubation. After PBS washing, cells were imaged under fluorescent microscopy.

Computation analysis. Original images are converted into gray-scale images. Images are processed by opening operator from mathematical morphology so that noise can be removed. Contrast will be enhanced by histogram equalization, thus the difference between healthy and dead cells will be more obvious to be observed. Next, we removed the background by a thresholding method (e.g. Otsu's method)[27] for the high-contrast

images so that the area of all cells is extracted. For the image of all cells, using the thresholding method again to obtain a new threshold value and use it to convert the image to a binary one, where dead cells have the gray value of 1 while healthy cells have the value of 0. The data acquisition was performed through counting the number of pixels of the two images (the image of all cells and the binary cell) we get from above step. The number of pixels represents the area of all cells and dead cells, from which we can compute the ratio of dead cells among all cells in the image.

The CLFP assay. HepG2 cells were cultured into 24-well plates and treated with various concentrations of three cytotoxicity drugs (Paclitaxel, Staurosporine, and Olaparib) and two reducing compounds (Quercetin and Chlorogenic acid) for 24 or 48 h. Cells were then incubated with CLFP probe for 30 min. After washing with PBS for three times, the samples with same treated conditions were evaluated using both MTT assay and CLFP assay. MTT assays were carried out following the standard protocol. For CLFP assays, the cells were imaged under fluorescent microscopy of HCS system. HCS system recorded numerous images from different point of the whole well. In our process, for each sample, 5 to 7 fluorescent images were chosen from various areas of the wells, these images were analyzed via computation methods. Finally, the middle three pixel values of healthy cells from all obtained images were selected, and averaged them to get a final pixel value to represent the cell viability of this sample. The IC_{50} or inhibition rate was calculated.

Results

Fluorescence morphologies of living and dead cells.

Staining cells with both CLFP probes 1 and 2 (different fluorophore) can obtain reliable and high quality cell images with cytoplasm fluorescent staining, although the experiment conditions for each CLFP probe are different. Once the cell images were recorded, they were all gone through the same computational analysis. We analyzed the cell images obtained from probe 1 staining. The relevant information of probe 2 staining was shown in the supporting information (Figure S1-S4).

Next, we need to visualize CLFP stained cell images in various situations to summarize a general morphological character that can be used to computationally discriminate living and dead cells. Various

cytotoxic drugs (paclitaxel, staurosporine, triptolide, geldanamycin, and doxorubicin) and non-cytotoxic drugs olaparib were incubated with HepG2 cells to induce different modes of cell death. HepG2 cells were stained with probe 1. After washing, cells were imaged by fluorescence microscopy. Figure 1A show the morphologies of dying cells in the early phase as bright, solid fluorescent spheres (white arrows). The intensity of these fluorescent spheres was stronger than that of living cells. Figure 1B shows that in the late phase of cell death, many dead cells and cell debris could not be fluorescently stained (white arrows) because they had broken membranes. Other dead cells or membrane-bound bodies were stained as various solid fluorescent shapes. In addition, we also stained the nuclei of healthy cells and paclitaxel-treated HepG2 cells (Figure 1C) with Hoechst dye. At this early stage of apoptosis, it was difficult to distinguish the shrinkage in nuclear area between healthy and dead cells. Lastly, in addition to cylindrical HepG2 cells we also examined the difference in fluorescence morphology and intensity between dead cells and living cells for three round and irregular cells (Figure 1D). Probe 1 can well display the morphological difference between living and dead cells in all cylindrical, round and irregular shapes of cells.

Figure 1. Fluorescence microscope images (HCS system) of (A) HepG2 cells stained with probe 1 and treated with cytotoxic drugs: paclitaxel, staurosporine, doxorubicin, triptolide, geldanamycin, and the non-cytotoxic drug olaparib to show the early phase of cell death (white arrows denote dying cells). (B) HepG2 cells were treated with paclitaxel, then stained with probe 1 and visualized with bright-field and fluorescence microscopy, and HepG2 cells were treated with staurosporine and visualized with bright-field and fluorescence microscopy. (White arrows show cells with broken membranes and cell debris, that cannot be stained with CLFP). (C) HepG2 cells untreated control, treated with paclitaxel. Both of them were stained with Hoechst dye to show nuclear shrinkage. This shows the small differences of nuclear shrinkage between dead cells and healthy cells. (D) MDA-MB-468 cells untreated control, or treated with paclitaxel, MDA-MB-453 cells untreated control or treated with paclitaxel, A2780 cells untreated control or treated with paclitaxel, they were stained with probe 1 and imaged under fluorescent microscope. (White arrows denote the dead cells which has different morphology compared with healthy cells).

Computational analysis.

Based on our microscopy results, we concluded that two characteristics are useful for computational discrimination of living and dead cells. (1), For one type of cell line, living cells have a single CLFP staining morphology, whereas dead cells have various fluorescent morphologies. Thus, a matched filter program can be used to extract the unique shape of living cells. (2), Living cells have weaker fluorescence intensity than dead cells. Therefore, a threshold method can be applied by choosing an appropriate fluorescence intensity threshold to separate areas of healthy cells and dead cells.

According to character (1), we exploit the difference in shapes between living and dead cells. A template function with a hollow round shape is used to match and extract the living cells, considering the CLFP staining morphology. By applying a convolution operator of a pre-processed image and the template function, we obtain the convolution value for each image pixel. Then, all pixels with a value higher than an appropriately chosen standard are identified as living cells. Based on character (2), we combine an auto-threshold method with multiple image processing techniques, which is a simple strategy that can also produce reliable results in these experiments (Figure S5). First, the images are converted to grayscale to make them easier to process. Next, the mathematical morphology opening operator [28] is used for noise removal, and histogram equalization [29] is used to enhance the contrast if necessary. With the black background of the high-contrast images, the area of all the cells is extracted from the background by choosing an appropriate threshold. The dead cells are then distinguished from healthy cells by characteristic (2), namely that dead cells and membrane bodies usually have stronger fluorescence intensity than healthy cells. A number of thresholding methods can be used for this purpose. An auto-threshold method based on Otsu's method [27] is efficient and accurate for our technique. Finally, the area of living cells in a series of samples is analyzed to evaluate cell viability.

Applicability of the CLFP assay.

To evaluate the applicability of the CLFP assay, we measured the cytotoxicity of drugs paclitaxel and staurosporine, and non-cytotoxic drug olaparib using both CLFP and MTT assays. The MTT assay was chosen for comparison because it is a common cell activity assay and its detection limits are well defined. Figures 2A–F show the ratio of healthy cells to dead cells in different concentrations of paclitaxel-treated HepG2 cells.

As the concentration of paclitaxel increased, fewer healthy cells were observed, whereas the number of dead cells initially increased to a maximum (Figure 2D), and then decreased (Figures 2E and 2F). Both methods provided similar IC_{50} values for paclitaxel, staurosporine, and olaparib for HepG2 cells (Figures 2G–2I). The inhibition graphs for the two assays fully or partially overlapped. Thus, the CLFP assay shows excellent detection of cell cytotoxicity, comparable to the MTT assay.

Figure 2. (A–F) Fluorescence microscopy images of the morphologies of HepG2 cells treated with a series of concentrations of paclitaxel. The graphs of the IC_{50} calculations for (G) paclitaxel, (H) staurosporine, and (I) olaparib using the CLFP and MTT assays.

Next, we measured cell viability for two reducing compounds, quercetin and chlorogenic acid against HepG2 cells using CLFP and MTT assays. The cell viability of the CLFP assay was much lower than that of the MTT assay. For quercetin, the cell viability obtained by CLFP was 62%, and that obtained by MTT was 85%. Similarly, for chlorogenic acid, cell viability obtained by CLFP was 43%, whereas that obtained by MTT was 75% (Figure 3). The higher cell viability obtained by the MTT assay (23% higher for quercetin and 35% higher for chlorogenic acid) was caused by the reduction of MTT by the reducing compounds. Thus, the CLFP method is more accurate for screening reducing compounds.

Figure 3. Cell viability assay results for two reducing compounds, quercetin (QCT) and chlorogenic acid (CGA), obtained with CLFP and MTT assays. Before the cell viability was determined, 100 μ M quercetin and chlorogenic acid were incubated with HepG2 cells separately for 48 h.

Discussion

In biomedical research, researchers often screen the cytotoxicity of a library of compounds, which have diversified properties. Thus, the ideal cell cytotoxicity assays should be suitable to test any types of compounds. Cell activity based cytotoxicity assay is common used to screen cell cytotoxicity, while the performance can be affected by the screened compounds. For instance, the MTT assay is unsuitable for determining the cytotoxicity of reducing compounds. In comparison, morphological identification cell cytotoxicity assay is feasible to test any types of compounds. But this type of assay is inefficient to deal with a big number of samples, and there is no well accepted morphological identification assay. It is desirable to develop a generally accepted morphological identification assay. Companies, such as, Molecular devices and PerkinElmer have developed morphological identification assays by evaluating nuclear area with high content screening (HCS) system.[20,21] Unfortunately, this method is problematic to correctly evaluate nuclear area.

Cell cytoplasm occupies the majority of cells, morphological changes in the cell cytoplasm are the most obvious. We are the first to use Cytoplasm-localized Fluorescent Probe (CLFP) to display cell morphologies of dead cells and living cells. The distinct distributions of CLFP in living and dead cells are likely to be explained

as follows. Because living cells are under relatively stable physiological conditions, when they are stained with CLFP, the probes penetrate the plasma membrane via passive diffusion, and accumulate in the cells until they are saturated. Inside the cells, the probes bind to soluble cytosolic proteins, which prevent the probes from crossing the nuclear pore complexes (NPC) and entering the cell nucleus. Indeed, NPCs allow small molecules ($MW < 5 \text{ kDa}$) to diffuse freely in and out of the cell nucleus. In addition, cationic and planar molecules show preferential nuclear accumulation, the physicochemical properties of CLFP probe can prevent the probe to enter cell nucleus. Thus, healthy cells are imaged as a uniform phenotype with bright cytoplasm and a hollow, dark nucleus (Figure 4A).

In contrast to living cells, dead cells cannot control the diffusion of CLFP. Dead cells show various fluorescent phenotypes corresponding to different cell death modes. In the early phase of cell death, a higher concentration of CLFP accumulates in dead cells owing to the changes in cell shape and the increase of plasma membrane permeability. Meanwhile, the cell matrix proteins become condensed and degraded, and a large amount of CLFP cannot bind with cytosolic proteins. Free CLFP crosses the nuclear membrane via NPCs and stain both the cytoplasm and nucleus. Thus, dead cells that have intact plasma membranes are imaged as solid fluorescent shapes with no differentiation between the cytoplasm and nucleus, and the fluorescence intensity of dead cells is higher than that of living cells (Figures 4B and 4D). In the late phase of cell death, some membrane-bound bodies, such as apoptotic bodies and cell debris, are formed. If these small bodies have intact bound membranes, they will be fully stained by CLFP (Figure 4C). Conversely, if the dead cells, including necrotic cells, cell debris and other membrane bodies, have broken membranes, CLFP does not remain in the broken cells and is removed during washing. They are not visible by fluorescence microscopy (Figures 4C and 4E).

Figure 4. Cartoons of CLFP-stained healthy cells (A) and dead cells in the early phase of apoptosis (B), apoptotic bodies (C), cells in the early phase of necrosis (D), and cells with broken plasma membranes (E).

Computation analysis has particularly advantages to process large number of data.[30] In this study, we applied

the computation analysis to scale up the morphological identification. A matched filter program can be used to extract the unique fluorescent shape of healthy cells, which is distinct from the fluorescent morphology of dead cells. A threshold method is applied to separate fluorescent areas of healthy cells and dead cells by choosing an appropriate fluorescence intensity threshold. Both strategies are common computation analysis methods, and can meet up the needs in the data process of the CLFP assay. Particularly, for practical applications, software will be developed where the cell type can be defined to make the analysis more accurate, and either method or a combination of the two can be selected to process the images. In addition, we also found that in cytotoxicity screening experiments dead cells are partially detached from the wall of the culture plate owing to the change in cell shape and the degradation of the membrane proteins. Thus, dead cells will be removed during washing. Moreover, the remaining dead cells with broken plasma membranes are not visible by fluorescence microscopy. Thus, only some dead cells and all living cells can be imaged by fluorescence microscopy. This aids of recognizing living cells.

The features of CLFP and MTT assays are summarized as follow:

- (1), The CLFP assay has inherent advantages in accuracy compared with cell activity methods. CLFP is a morphological identification assay, it detects the cells themselves instead of the product of a biochemical reaction; therefore, it is not affected by the screened compounds. For example, CLFP can accurately detect the cell viability for reducing compounds, whereas the MTT assay cannot.
- (2), The CLFP assay takes less time to stain cells, which is much faster than processing detectable biochemical reactions. In comparison, the MTT assay detects the mitochondrial activity of healthy cells. However, as the number of living cells decreases, it takes a longer time for the MTT assay to produce a detectable colour change.
- (3), The CLFP counts living cells. During screening, dead cells become partially detached from the culture plate, and they can be washed away. In addition, in the late phase of drug-induced cell death, most dead cells have broken membranes. Thus, they are not stained by CLFP. This greatly simplifies the computational counting of healthy cells.

(4), The CLFP assay can be integrated into HCS systems, which becomes a common facility in many research centers. CLFP shows the morphological difference in the fluorescent shapes and intensity between living cells and dead cells, and the advanced computational analysis accurately recognizes healthy cells based on these two characteristics.

Conclusion

We used CLFP to stain cells and clearly show the morphological difference between living and dead cells, which can be recognized by computational analysis. This overcomes the difficulty in processing large numbers of samples in a morphological identification assay. The CLFP assay is universal for detecting cell viability for any mode of cell death, and for any type of cell shapes, and it is simple, fast, and accurate, making it suitable for general use as a next-generation drug screening assay.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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