More efficient homogeneous cell-based immunofluorescence assay using Light-Sheet Microplate Cytometer

Yiwen Wang[1], Natsumi Kato[2], Masumi Suzuki[3], Tokio Takagi[3], and Hirofumi Horai[2]. 1; Hamamatsu Corp. 2; Systems Division, Hamamatsu Photonics K.K. Japan, 3; Central Research Laboratory, Hamamatsu Photonics K.K. Japan.

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Introduction

Detection of apoptosis is an essential method in various fields, including the evaluation of drug efficacy for new compounds in drug discovery research and screening, and the analysis of survival mechanisms in tumor cells in cancer research. Common apoptosis detection methods include Annexin V staining assays and TUNEL assays. Among these, fluorescence antibody-based methods are particularly advantageous due to their high specificity, allowing direct detection of the expression and activity of specific proteins involved in apoptosis. However, fluorescence antibody-based methods often require washing steps to remove nonspecific antibody binding. These washing steps pose risks such as cell detachment and data variability, especially for fragile cells after apoptosis induction, which makes achieving accurate high-throughput screening difficult.

To overcome these challenges, we utilized CYTOQUBE®, a light-sheet microplate cytometer equipped with patented Zyncscan® technology, which performs high-speed fluorescent imaging and analysis for 2D and 3D cell cultures. This technology acquires XZ tomographic fluorescence images and removes background fluorescence in real time by three-dimensionally separating it from sample fluorescence. As a result, CYTOQUBE® effectively eliminates background fluorescence in fluorescent antibody-stained samples, enabling high-precision fluorescence imaging without requiring washing steps.

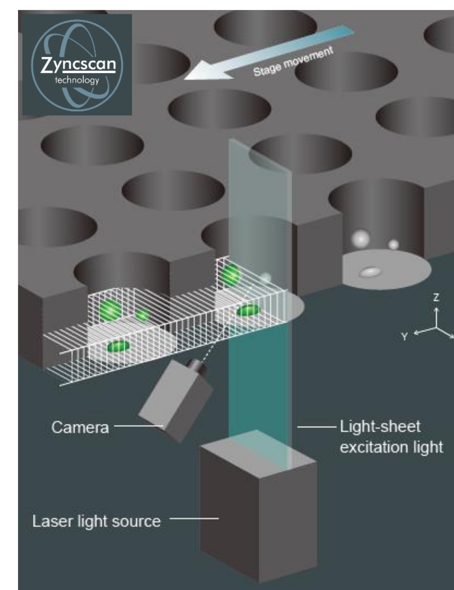
Using CYTOQUBE®, we successfully detected apoptosis in MCF-7 cells treated with staurosporine through Cleaved Caspase-3 antibody staining. The technology minimized cell loss and facilitated the generation of accurate dose-response curves.

These results highlight the potential of CYTOQUBE® as a robust tool for efficient and reliable drug discovery screening and toxicity evaluation, addressing limitations of conventional methods and ensuring high-precision data in apoptosis detection.

Light-Sheet Microplate Cytometer: CYTOQUBE®

Advantages of using CYTOQUBE® for 2D assays

- ✓ **High throughput**
  By acquiring 3D images, focus adjustment is unnecessary, enabling high throughput imaging across the entire well plate.
- ✓ **Easy setting**
  No focus setting or multiple threshold for cell recognition are required.
- ✓ **Parallel processing of scan and analysis**
  Analysis, including cell recognition and statistical processing, is performed in parallel with image acquisition, providing results immediately upon scan completion.



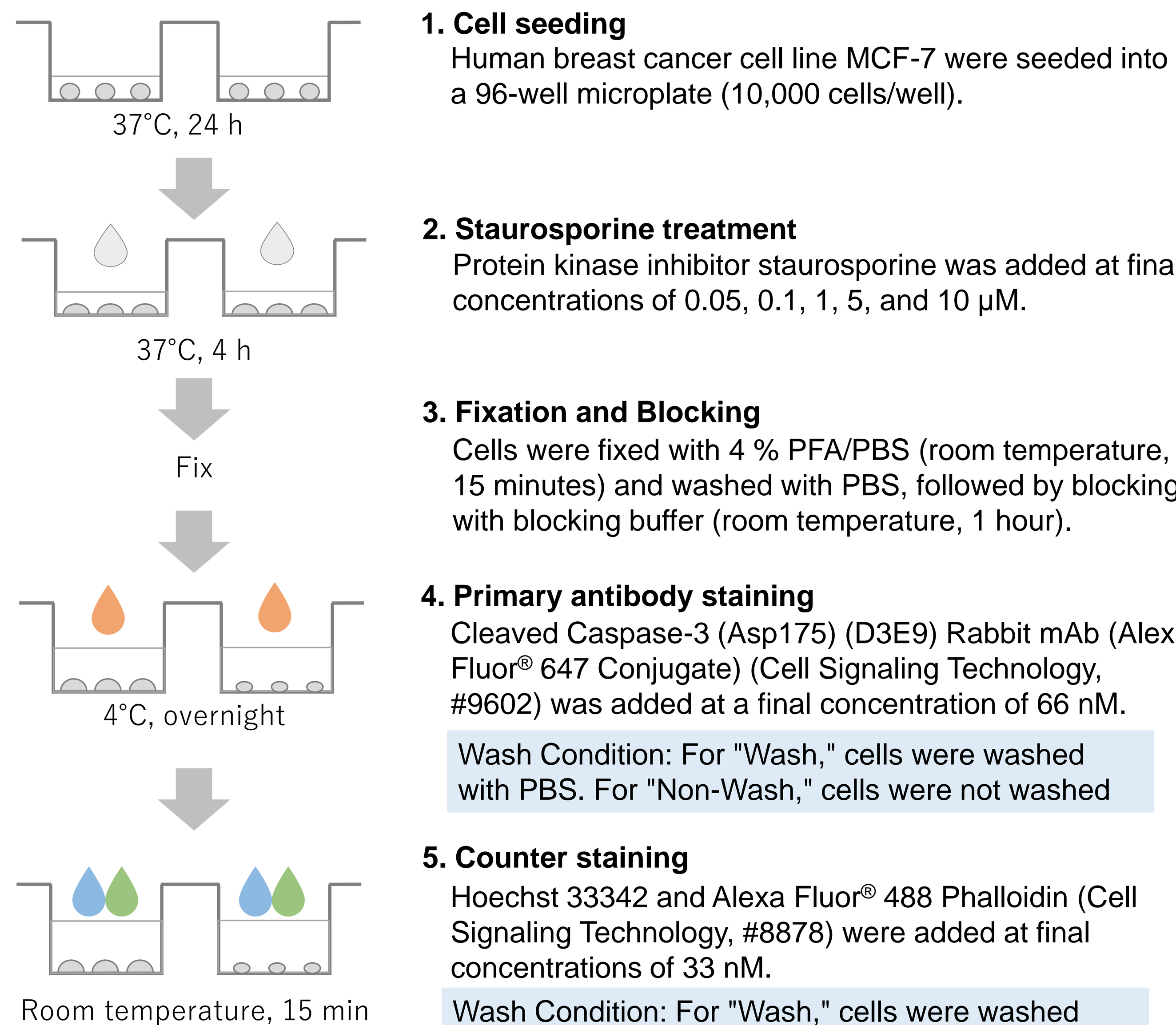
Zyncscan®, a patented technology by Hamamatsu Photonics, combines light-sheet optics and image processing to enable advanced fluorescence imaging in microplate assays.

It emits a sheet of excitation light from directly below the sample and captures XZ tomographic fluorescence images from an oblique downward direction.

By continuously acquiring these images while moving the microplate, Zyncscan® constructs 3D fluorescence images within minutes.

This technology also features real-time background fluorescence removal, achieved by three-dimensionally separating background fluorescence from sample fluorescence during image acquisition.

Materials and Methods



1. Cell seeding
Human breast cancer cell line MCF-7 were seeded into a 96-well microplate (10,000 cells/well).

2. Staurosporine treatment
Protein kinase inhibitor staurosporine was added at final concentrations of 0.05, 0.1, 1, 5, and 10 μM.

3. Fixation and Blocking
Cells were fixed with 4 % PFA/PBS (room temperature, 15 minutes) and washed with PBS, followed by blocking with blocking buffer (room temperature, 1 hour).

4. Primary antibody staining
Cleaved Caspase-3 (Asp175) (D3E9) Rabbit mAb (Alexa Fluor® 647 Conjugate) (Cell Signaling Technology, #9602) was added at a final concentration of 66 nM.

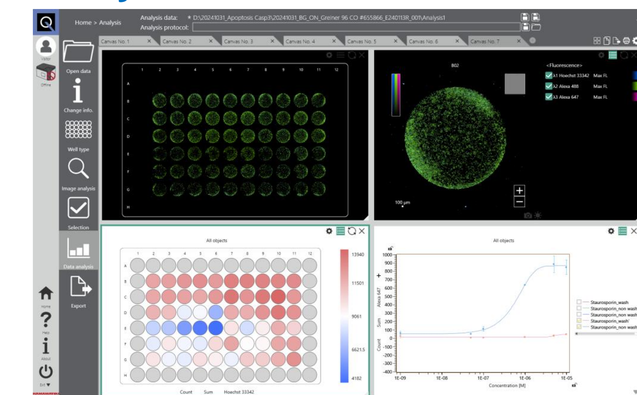
Wash Condition: For "Wash," cells were washed with PBS. For "Non-Wash," cells were not washed

5. Counter staining
Hoechst 33342 and Alexa Fluor® 488 Phalloidin (Cell Signaling Technology, #8878) were added at final concentrations of 33 nM.

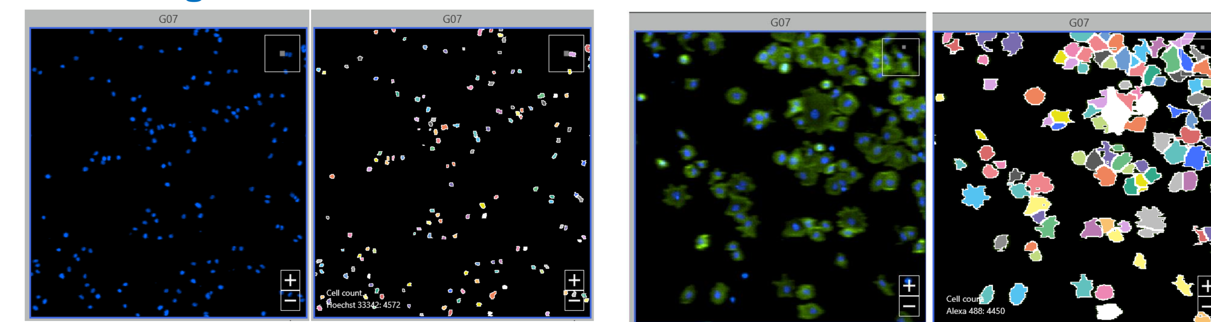
Wash Condition: For "Wash," cells were washed with PBS. For "Non-Wash," cells were not washed

6. Measurement and analysis using CYTOQUBE®

Analysis screen of CYTOQUBE®



Cell segmentation



Hoechst 33342 fluorescence was used as a counterstain to identify individual cells, and phalloidin fluorescence was used to confirm that individual cells were correctly identified.

Results

Non-Wash Antibody-Based Apoptosis Detection with CYTOQUBE® Technology

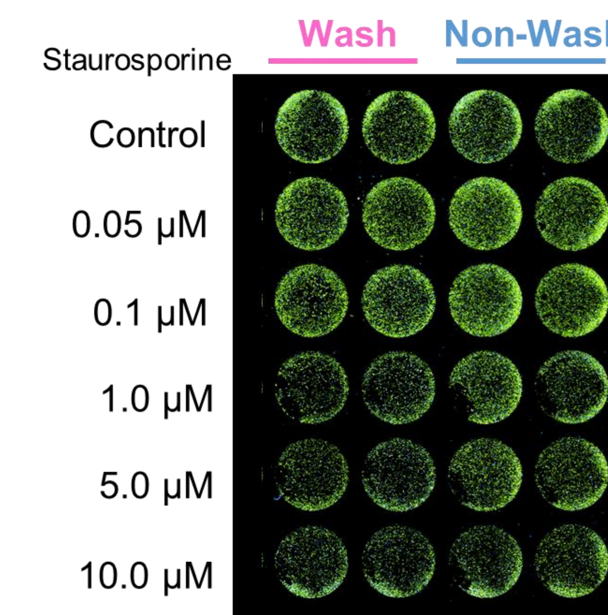


Fig.1 Fluorescence image of a 96 well microplate

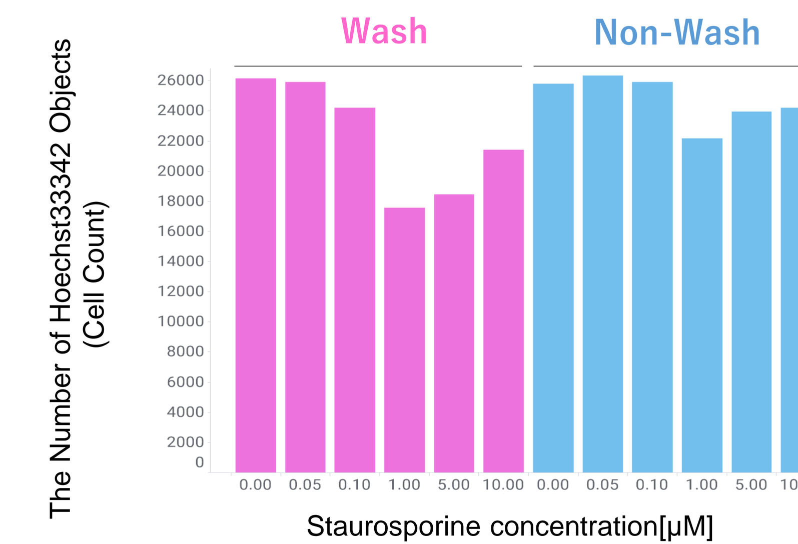


Fig. 2 Comparison of cell counts between Wash and Non-Wash conditions

Fig.2 illustrates the total number of Hoechst33342-positive objects (indicative of cell count) under Wash and Non-Wash conditions across various concentrations of Staurosporine (0 to 10 μM).

In the Wash condition, a dose-dependent decrease in cell count is observed, with a significant reduction at higher Staurosporine concentrations, particularly at 10 μM.

In the Non-Wash condition, the cell count remains relatively consistent across all Staurosporine concentrations, showing no significant reduction.

This data suggests that washing steps during the assay lead to cell detachment, particularly at higher drug concentrations, highlighting the advantage of avoiding wash steps for maintaining accurate cell counts.

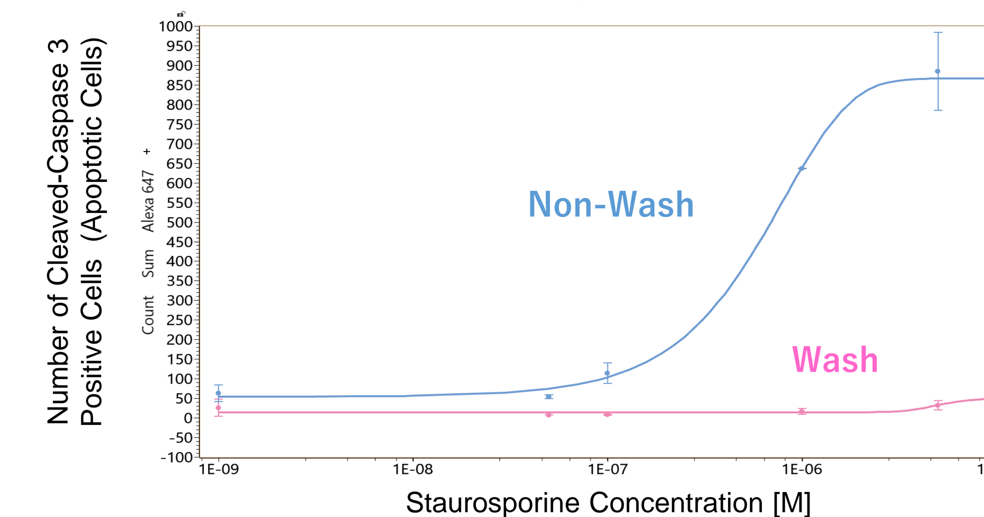


Fig. 3 Dose-Response Curve of Staurosporine-Induced Apoptosis

Fig.3 shows the dose-response curve for Cleaved Caspase-3 positive cells, comparing Wash and Non-Wash conditions. The fitting method used was a 5-parameter logistic model. The x-axis indicates the staurosporine concentration (log scale), while the y-axis represents the number of Cleaved Caspase-3 positive cells (Alexa 647 fluorescence).

In the Non-Wash condition, the dose-dependent response is clearly observed, with a sharp increase in Cleaved Caspase-3 positive cells as the staurosporine concentration rises, yielding an EC50 value of 609.5 nM. In contrast, the Wash condition shows minimal response, with a significantly higher EC50 value of 54.7 μM.

This result highlights the impact of the washing step on assay sensitivity and emphasizes the benefits of minimizing washing in detecting apoptotic signals accurately. The results underline the utility of CYTOQUBE® for achieving both higher sensitivity and more reproducible data in apoptosis detection.

Reliable Measurement of Mitochondrial Membrane Potential Using TMRE in Wash-Free Assays

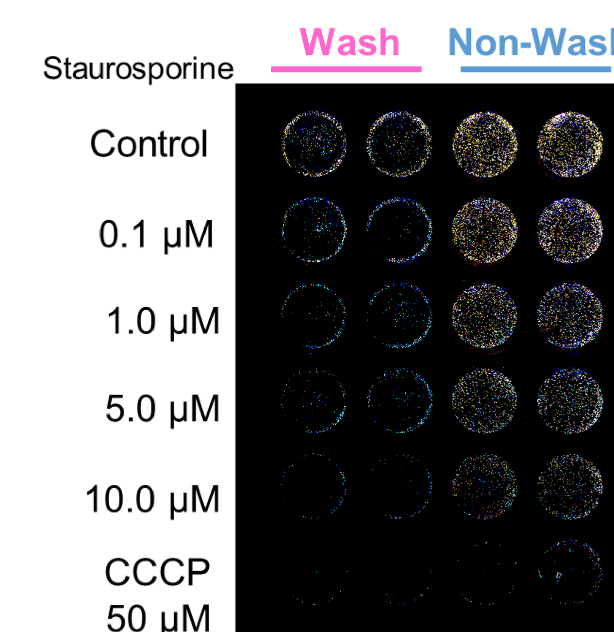


Fig.4 Fluorescence image of a 96 well microplate

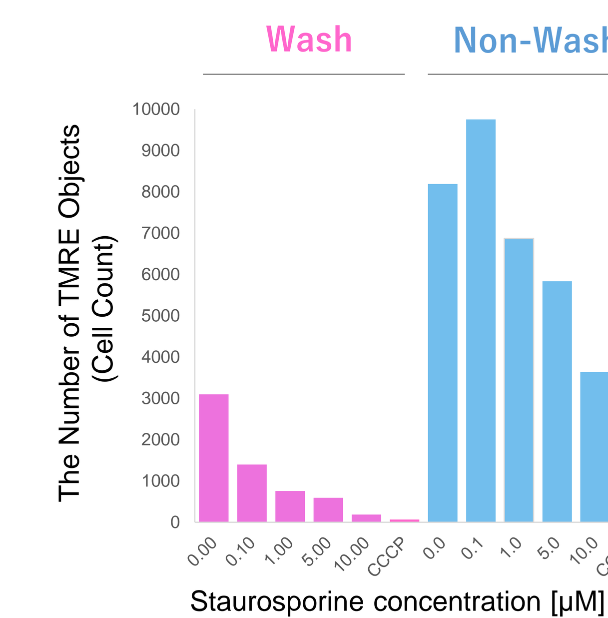


Fig. 5 Comparison of cell counts of TMRE-positive cells between Wash and Non-Wash conditions

To further evaluate the applicability of CYTOQUBE® for other functional assays, mitochondrial membrane potential was measured using TMRE staining. TMRE selectively accumulates in intact, polarized mitochondria, providing a reliable indicator of mitochondrial health.

Mitochondrial Membrane Potential Assay Kit (II) (Cell Signaling Technology, #13296)
Cells were treated with staurosporine (4 hours) for apoptosis induction or CCCP (15 minutes) as a positive control for mitochondrial depolarization. TMRE was added at a concentration of 2 μM, and cells were incubated for 20 minutes.
Wash Condition: For "Wash," cells were washed with PBS. For "Non-Wash," cells were not washed

As in antibody-based apoptosis assays, the Wash condition caused cell detachment, leading to decreased signal intensity and increased variability. In contrast, the Non-Wash condition maintained cell integrity, enabling precise and reliable measurements of mitochondrial membrane potential.

These findings suggest that CYTOQUBE® is also suitable for functional assays such as mitochondrial membrane potential measurement, further broadening its applicability in drug discovery and cell biology research.

Summary

Accurate and efficient fluorescence assays are essential for drug discovery and screening. We demonstrated the advantages of wash-free antibody-based apoptosis detection using Cleaved Caspase-3 as a marker. By eliminating washing steps, we preserved cell counts and achieved precise dose-response curve generation, highlighting the sensitivity and reliability of this approach. Additionally, we applied this wash-free method to mitochondrial membrane potential measurement using TMRE, successfully obtaining consistent and reliable results. These outcomes were made possible by CYTOQUBE's advanced background fluorescence removal function. This innovative technology paves the way for broader applications in high-throughput screening, toxicity evaluation, and other fluorescence-based research areas, offering a robust solution for drug discovery.

We would like to extend our heartfelt gratitude to Ms. Mahomi Suzuki of Cell Signaling Technology Japan, Inc., for providing invaluable advice in conducting this experiment.

For further information, questions, and comments, please contact YWang@hamamatsu.com