APPLICATION NOTE

Volume Contrast, the analysis software of NIS-Elements

Cell Proliferation Assay and Optimization of HCA Assay, Using Label-Free Live Cell Imaging

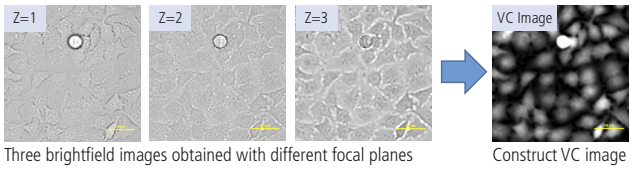
Cell count measurement is used in a wide range of research fields, including cell proliferation, cell proliferation inhibition, cytotoxicity, and cell death mechanisms. Measuring cell numbers allows analysis of the effects of drugs and components on cells. Fetal bovine serum (FBS), commonly used as a cell proliferation supplement, contains growth factors that stimulate cell proliferation. However, due to the significant variability of biological components between lots, it is necessary to perform batch testing of several lots and determine the optimum lot to obtain reproducible research results. Lot-to-lot variation can lead to assay failure and reproducibility problems.
In addition, differences in cell density also greatly affect cell proliferation and assay results and may cause affect reproducibility.
In high content analysis (HCA), which automates imaging and quantitative image analysis, it is important to perform a preliminary experiment and optimize assay conditions, such as the cell seeding numbers and drug treatment time, to match the purpose of each assay. This application note introduces a label-free cell proliferation assay using phase distribution images (Volume Contrast images) that reflect cell structures generated from brightfield images with different focal planes. This allows the HCA assay to be optimized by measuring cell numbers over time without staining living cells.

Challenges in live cell proliferation assays

Nuclear staining reagents such as Hoechst are used in short-term live cell assays. However, cell proliferation is suppressed in long-term live cell assays due to cytotoxicity by fluorescent dyes and phototoxicity by fluorescence excitation. (1)
Also, multi-well plates such as 96-well plates with small well diameters are used in HCA assays, but phase contrast observation is difficult due to the meniscus effect. Brightfield observation using transmitted light can reduce the meniscus effect but is difficult to binarize for cell observation and cell number counting due to its low contrast.

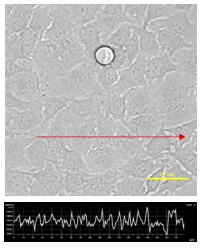
Easy binarization and segmentation can be performed by constructing VC images from brightfield images

Brightfield images have a low S/N ratio, making it difficult to binarize the target cell region. Meanwhile, in VC images, the background and cell area can be easily distinguished because pixel values are larger for areas corresponding to the thickness of cells. Therefore, segmentation is easy even in areas with high cell density, improving the accuracy of cell counts.

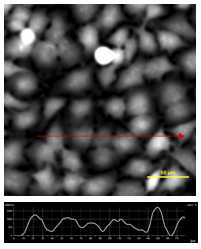


Three brightfield images obtained with different focal planes

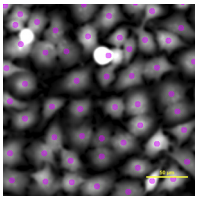
Construct VC image



Intensity profile of brightfield Image



Intensity profile of VC image

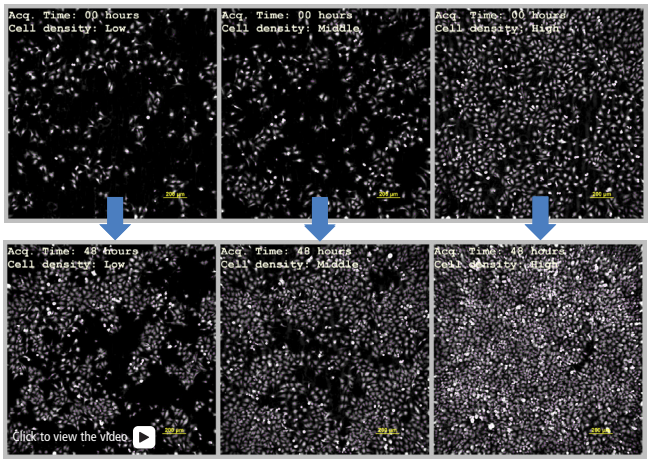


Mask image of cell count binarized from the VC image

Scale bar: 50 µm

Large field of view can reduce the effects of cell number variability

Even if the same number of cells are seeded, cell counts will vary depending on location in the well. Capturing a large field of view increases the number of cells in the field of view and provides reproducible research results.
Also, by dividing the cell counts at each time point by those in the first frame (T = 0) and normalizing, cell proliferation between wells can be compared, even if the number of cell seeds varies between them.



Sample video

Cell seeding density, left: low, center: medium, right: high
Upper row: At start of imaging, Lower row: 48 hours after start of imaging

Scale bar: 200 µm

Cell proliferation assay

Cells/Reagents/Materials

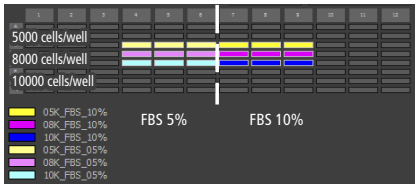
- HeLa Human cervical cancer-derived cell line (Riken Cell Bank, RCB0007)
- Minimum Essential Medium Eagle With Earle's salts (Sigma-Aldrich, M4655)
- FluoroBrite™ DMEM (Thermo Fisher Scientific, A1896701)
- Fetal Bovine Serum, qualified, USDA-approved regions (Thermo Fisher Scientific, 10437028)
- L-Glutamine (200mM) (Thermo Fisher Scientific, 25030081)
- Penicillin-Streptomycin (10,000 U/mL) (Thermo Fisher Scientific, 15140122)
- Trypsin-EDTA (0.25%), phenol red (Thermo Fisher Scientific, 25200072)
- DPBS, no calcium, no magnesium (Thermo Fisher Scientific, 14190144)
- EZVIEW® CulturePlateLB (AGCTECHNO GLASS, 5866-096)
- 96-well Cell Culture Microplate (Greiner, 655090)

Observation device/Software

- Microscope: Ti2-E (Nikon)
- Objective: CFI Plan Apo Lambda S 10X NA 0.45
- Monochrome camera: DS-Qi2
- Image analysis software: NIS-Elements (Nikon)
- Stage top incubator: STX series (TOKAI HIT)

Methods

HeLa cells were seeded into a total of 18 wells on a 96-well glass-bottom plate, 6 wells each at 3 different cell densities, 5,000 cells per well, 8,000 cells per well, and 10,000 cells per well. 5% and 10% FBS were added to 3 wells of the medium for each cell density and incubated for about 24 hours. The medium was changed before the start of image acquisition.



The cells were maintained at 37°C and 5% CO_2 in a stage-top incubator. Three brightfield Z-stack images of an area (approx. 1.76 mm x 1.76 mm) at the center of each well were captured with a 10X objective and a wide field-of-view monochrome camera supporting 25 mm FOV, every 3 hours for 2 days from about 24 hours after cell seeding.

Next, HeLa cells were seeded into a total of 9 wells on a 96-well plastic-bottom plate, 3 wells each at 3 different cell densities, 5,000 cells per well, 8,000 cells per well and 10,000 cells per well. 10% FBS was added to the medium and cultured for about 24 hours. Images were captured every 3 hours for 2 days from about 24 hours after cell seeding with the same settings as the cells in the glass-bottom plate.

The obtained brightfield images were converted into VC images using the GA3 image analysis function of NIS-Elements, and binarized to create a cell count analysis recipe. The cell count analysis recipe was executed for all images using Batch GA3 of NIS-Elements and the measured value was output in CSV format. The CSV files were imported into the Microsoft Power BI® data visualization tool to create a graph, and the measurement results were analyzed.

Results

Comparison of FBS concentrations and normalization between wells

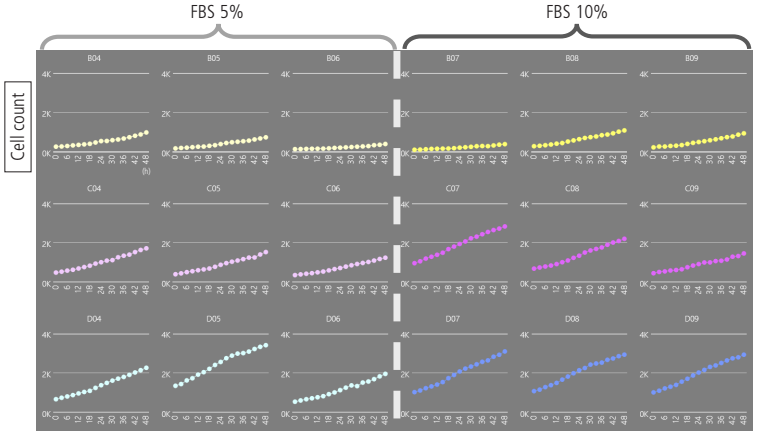


Fig. 1: Cell count measurement results from 0h to 48h in each well

Left: FBS 5%, Right: FBS 10%, Glass bottom well plate
Number of seeded cells: (upper row) 5,000 cells per well
(middle row) 8,000 cells per well
(lower row) 10,000 cells per well

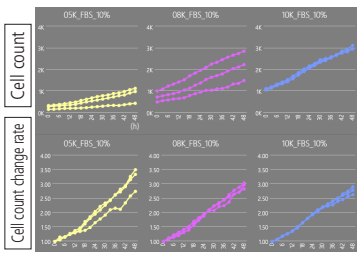


Fig. 3: Transition and change rate of cell counts

Cultured with 10% FBS, glass bottom well plate
Upper row: Measurement result of cell count
Lower row: Cell count change rate from first frame (T=0)

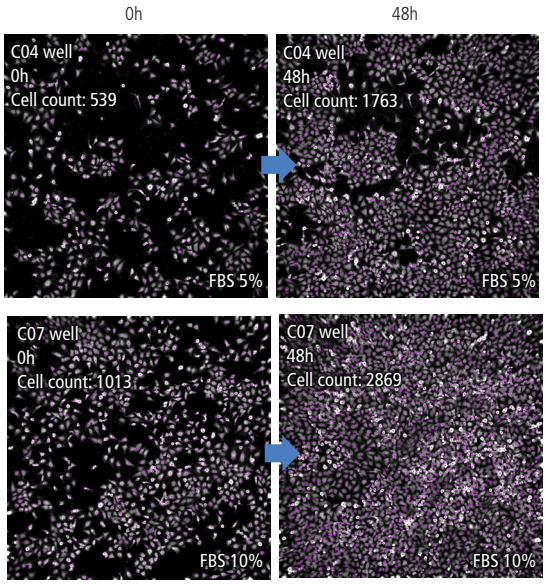


Fig. 2: Cell count mask image of each FBS concentration

Cell seeding density: medium, Glass bottom well plate
Left: At start of imaging, Right: 48hrs after start of imaging
Upper row: 5% FBS (C04 well)
Lower row: 10% FBS (C07 well)
Upper row: Rate of change in cell count from 0h to 48h: 3.27
Lower row: Rate of change in cell count from 0h to 48h: 2.83

Results

■ Comparison of cell count increase rate by cell density (cultured with 10% FBS)

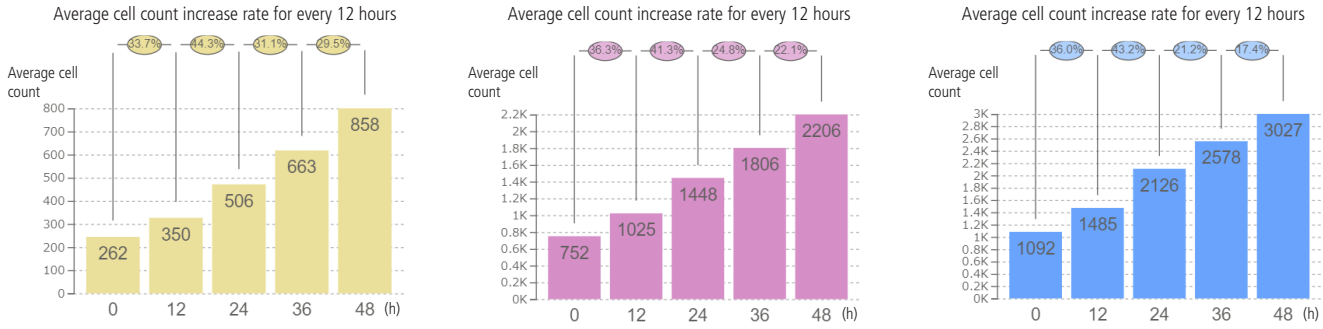


Fig. 4: Average cell count and cell count increase rate for every 12 hours

Number of seeded cells: (left) 5,000 cells per well, (center) 8,000 cells per well, (right) 10,000 cells per well

Average cell count increase rate, from 36h to 48h after start of imaging: (left) 29.5%, (center) 22.1%, (right) 17.4%

■ Average cell count change rate (cultured with 10% FBS)

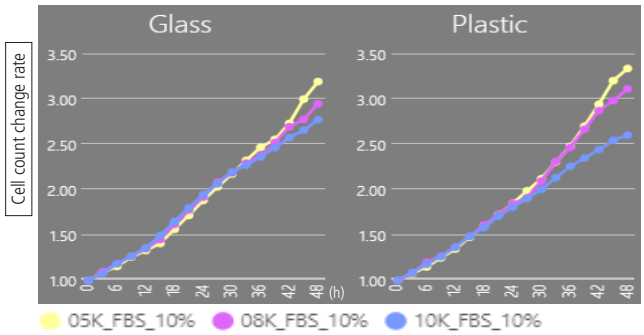


Fig. 5: Cell count change rates from first frame (T = 0)

Left: glass bottom well plate

Right: plastic bottom well plate

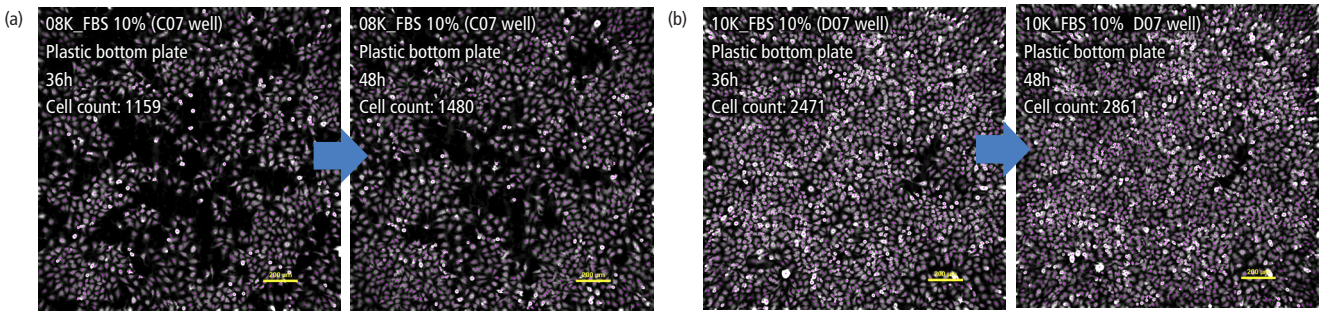


Fig. 6: Mask images of wells with medium- and high-densities of seeded cells

Scale bar: 200 µm

Cultured with 10% FBS, Plastic bottom well plate

(a) Number of seeded cells: 8,000 cells per well, Elapsed time: 36h (left), 48h (right), Cell count increase rate from 36h to 48h after start of imaging: 27% (C07 well)

(b) Number of seeded cells: 10,000 cells per well, Elapsed time: 36h (left), 48h (right), Cell count increase rate from 36h to 48h after start of imaging: 15% (C07 well)

- The cell count could be measured without labeling even in areas of high cell density, by creating a VC image from the brightfield image (Fig. 1 and Fig. 2).
- Even if the cell count in each well varies, cell proliferation can be compared between wells by dividing the cell count at each time point by the cell count in the first frame (T = 0) and normalizing it (Fig. 3).
- As the cell density increased, the cell proliferation ability was reduced (Fig. 4).
- Cell proliferation was significantly suppressed after 36 hours in wells with high cell seeding density (Fig. 5 and Fig. 6).
- The condition of seeding 8000 cells per well with 10% FBS did not reduce proliferation of the cells even at the latter stages of the assay, confirming that this is the optimal condition for performing the assay over periods of 36 hours or more in these experiments (Fig. 5).

Summary

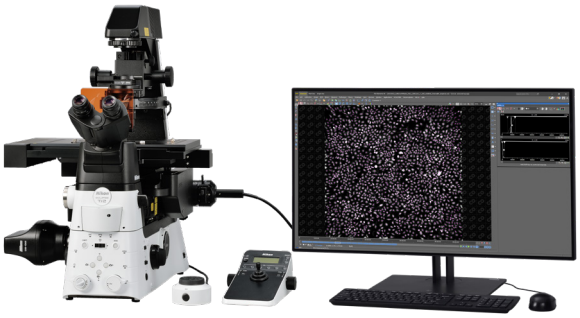
- Using the Volume Contrast (VC) function of NIS-Elements, cells can be analyzed without their proliferation being affected by the cytotoxicity or phototoxicity of fluorescent dyes.
- Cell counting using VC images can reduce the problems caused by the meniscus effect, and is a useful method for long-term live cell high content analysis on a 96-well plate.
- By capturing a large field of view in one shot with a 25 mm FOV camera and counting cells, highly reproducible results can be obtained while suppressing the bias in the number of cells in each field of view.
- The VC function can be used for assay optimization of HCA.
- It can be used for FBS batch testing, reagent toxicity testing, and the development of cell culture-related products such as FBS alternative reagents and scaffolding materials.

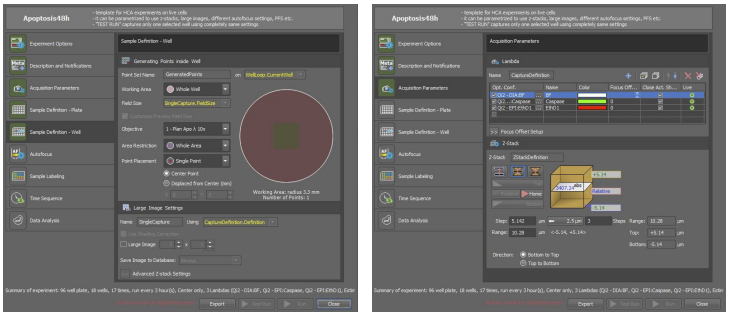
Reference

1. Highly accurate and non-invasive cell counts utilizing machine learning, Nikon Application Note: SW_app_ConSeg_e_04

High content analysis (HCA) microscope system

The High Content Analysis (HCA) option of NIS-Elements imaging software is installed in the Ti2-E microscope and camera combination. Quick and easy execution, from image acquisition to analysis, is possible with one system. In the experiment in this paper, we used the HCA Wizard to acquire images and the Volume Contrast function to create VC images for analysis.

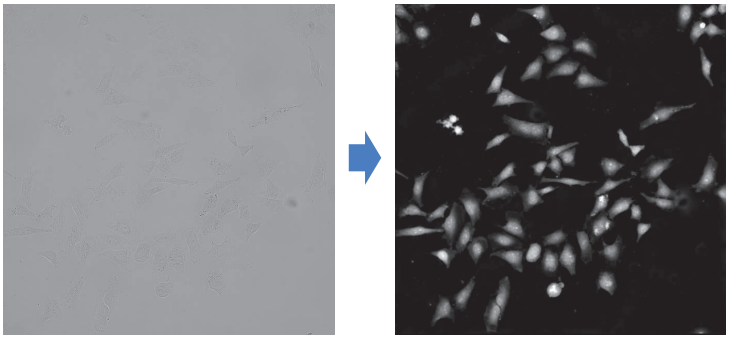




HCA Wizard

Volume Contrast analysis software of NIS-Elements

This add-on module allows NIS-Elements imaging software to construct fluorescence-like phase distribution images from brightfield images captured at multiple z-depths. Label-free quantitative phase analysis is possible without the need for special optical accessories.

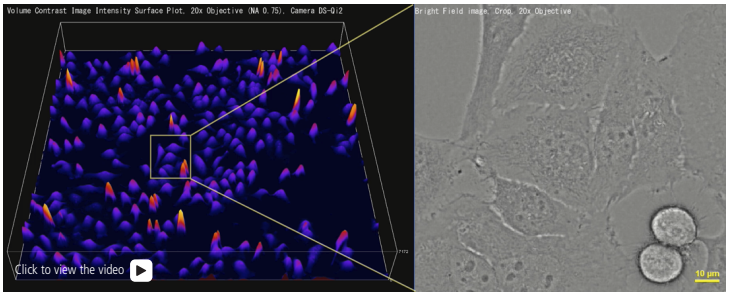


Brightfield image

Volume Contrast image

DS-Qi2 monochrome microscope camera

A wide field of view of 25mm can be captured in one shot even during cell observation and high-resolution dynamic observation using a 20X objective, in addition to cell counting using a 10X objective. Therefore, it is possible to acquire the area of interest during cell division at the appropriate timing.



Left: 3D time-lapse image of VC image with intensity surface profile displayed
Right: Time-lapse of the enlarged area of interest of EDF images converted from brightfield images
Objective: 20X, Z-stack: 7 images (Z range: 3.25µm)

Sample video