APPLICATION NOTE

***NIS.ai* AI module for microscopes (Convert.ai/Segment.ai)**

Highly accurate and non-invasive cell counts utilizing machine learning

Although quantification of cell numbers is an important method in biological and medical research, measurement of fluorescent stained cell nuclei etc. involves many problems in terms of measurement accuracy and phototoxicity. The NIS.ai functions built into the NIS-Elements software can utilize machine learning to measure cells. In this application note, we demonstrate that the number of cells can be measured with high inference accuracy from diascopic phase contrast images using the NIS.ai function, avoiding the effects of fluorescent reagents and excitation light irradiation.

Accurately quantifying cell numbers from phase contrast images of unstained cells

Currently, hemocytometers, measurement of cell confluency in diascopic images, and image processing of stained cell nuclei, etc. are commonly used for cell count measurements. However, these methods have the following problems:

- Adherent cells need to be detached.
- Achieving high accuracy is difficult because the cell area during culturing changes according to cell confluency.
- Since conventional image processing requires cell staining, color reduction due to cell division, and photobleaching and phototoxicity due to excitation light, etc. often cause problems.
- The toxic effect on cells due to dye staining must also be considered.

On the other hand, in addition to solving the above problems, cell counting by the NIS.ai function utilizing machine learning has the following advantages.

- Highly accurate inference can be achieved with just a few training images.
- More advanced analyses can be conveniently performed by using the NIS.ai function in combination with the existing image analysis functions of NIS-Elements.

Digital staining (using Convert.ai) and segmentation (using Segment.ai) of cell nuclei were performed using a phase contrast image, and the accuracy of a cell count based on the result was compared with that based on a result using a fluorescent image.

Materials and Methods

- Cell: BS-C-1 cells (RFP-laminB1 constitutive expression cell line), HeLa cells
- Reagent: Hoechst 33342 (Thermo Fisher Scientific), NucSpotLive 488 Nuclear Stain (Biotium Inc.)
- Microscope: Ti-E inverted microscope (Nikon)
- Objective: CFI S Plan Fluor ELWD ADM 20XC NA0.45 (Nikon)
- Measurement conditions: Using a microscope equipped with the above objective, time-lapse images of a sample were captured every 2 hours in a stage top incubator (Tokai Hit) with a 37˚C and 5% CO_2 environment. An EMCCD camera (iXon3, Andor) was used for image acquisition. Fixed cells were also photographed under the same conditions.

After image acquisition, an estimation of cell nuclei was obtained using either Convert.ai or Segment.ai of the NIS.ai function, and quantified using the nuclear counting function of the NIS-Elements software.

Training Conditions

		Convert.ai		Segment.ai
Cell type used		BS-C-1 cells	HeLa cells	HeLa cells
No. of training images		70	75	24
Training image	Input	Phase contrast image		
	Output	RFP fluorescent image (expressing laminB1)	Hoechst fluorescent image (nuclear fluorescence staining)	Visually segmented mask
Training frequency (iterations)		1000		

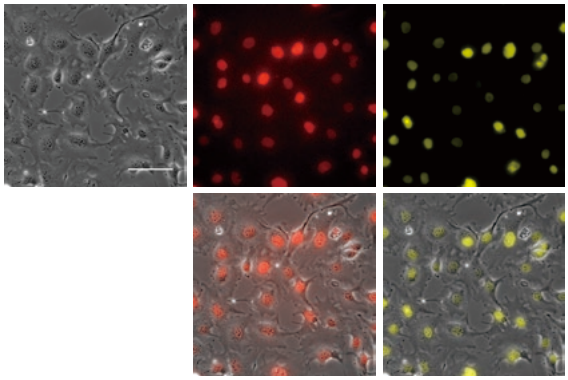
Results

Since it is possible to estimate the position and number of cell nuclei and dead cells using phase contrast images, consideration of the performance of fluorescent dyes and the effects of fluorescent dyes on cells is not required. For this reason, using the NIS.ai function is very effective for repeated assays and analyses of cells that are sensitive to fluorescent dye staining.

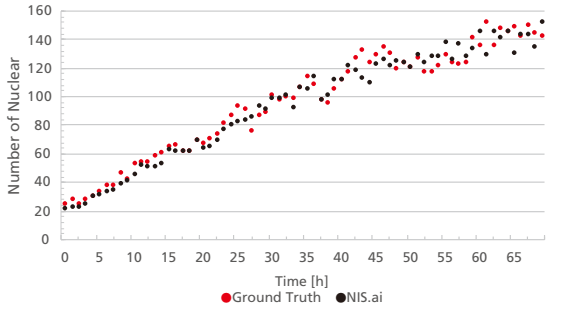
The NIS.ai function enables learning and inference with a small number of training images. Furthermore, it can be applied to various analyses when combined with other existing functions of the NIS-Elements software.

1. Cell nucleus estimation (using Convert.ai)

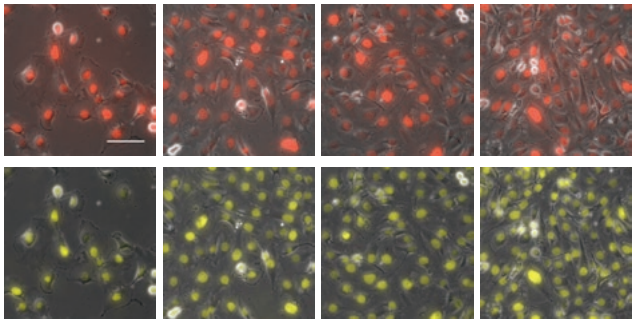
(a)



(b)



(c)



(d)

Confluency (%)	Number of Nuclear					F-score
	Ground Truth	NIS.ai	True Positive	False Negative	False Positive	
30	24	22	22	2	0	0.96
50	65	63	62	3	1	0.97
80	73	69	67	6	2	0.94
100	101	99	89	12	10	0.89

(e)

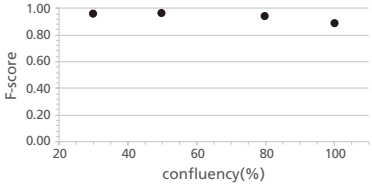


Fig. 1: Estimating the cell nucleus using NIS.ai (Convert.ai)

(a) From left to right: (phase contrast) image input to NIS.ai, fluorescent image of RFP-laminB1 as ground truth, and output image by NIS.ai. The lower row shows images merged with the phase contrast image. Scale bar: 100μm

(b) Cell proliferation scatter plot

(c) Inferred cell images with different densities (from the left, 30/50/80/100%). The upper row shows the ground truth of fluorescence of nuclei (RFP-laminB1) and the lower row shows the images output by NIS.ai (Convert.ai). Scale bar: 100μm

(d, e) Quantitative evaluation of accuracy. Detection (true positive), false detection (false negative), and over-detection (false positive) at each cell density were determined using the coordinates of the center of gravity of the nuclei in ground truth and the nuclei estimated by Convert.ai. F-scores were also calculated.

Creating a growth curve

Verification of cell proliferation using microscopic images is often performed using the number of cell nuclei counted by such methods as fluorescent protein expressing cell usage and fluorescent dye staining, as well as cell confluency in diascopic images.

However, the process of generating a stable cell line that expresses fluorescent proteins is complicated, and cells that transiently express fluorescent proteins have such risks as unstable gene transfer efficiency and an overexpression effect on cell function.

In addition, when using cell confluency in the field of view as an index, it is difficult to obtain accurate measurements because the cell area varies from cell to cell or by confluency. For example, even if confluency exceeds 100%, the cells continue to divide for a while, but the confluency remains unchanged.

Accordingly, it was verified whether the cell nucleus could be accurately estimated from a phase contrast image using NIS.ai (Convert.ai).

Methods

- A BS-C-1 cell line which consistently expresses RFP-laminB1 localized in the nuclear envelope was photographed every 2 hours and verified (Fig. 1a).
- 70 training images (phase-contrast images and RFP-laminB1 fluorescent images) with different cell confluencies (20%, 50%, 80%, 100%, 120%) were captured and prepared to create a learning data set in advance.
- A growth curve was created by plotting the number of cell nuclei at each time point (Fig. 1b).
- The accuracy of the estimation results was verified using the F-score that is a general evaluation index for machine learning (Fig. 1 d, e). The closer the F-score is to 1, the higher the accuracy.
- Each value was calculated based on an evaluation standard of whether the center of gravity of the nucleus in the ground truth image and that of the nucleus estimated by Convert.ai are within 10 pixels of each other.

Results

- Nucleus localization in the image estimated by Convert.ai was confirmed as being substantially the same as localization of the ground truth of RFP-laminB1 (Fig. 1a).
- The proliferation curve created based on the image inferred by Convert.ai was extremely close to that of the fluorescent image (Fig. 1b).
- It was shown that estimation accuracy is maintained even when cell confluency changes (Fig. 1c).
- In the verifications using F-scores, Convert.ai maintained sufficient estimation accuracy for all cell confluences. It was also possible to estimate cell nuclei even when the number of cells increased and the distance between cells decreased (Fig. 1d, e).
- There was a tendency for estimation accuracy to increase with lower cell confluences.

Summary

- Nuclear digital stain using NIS.ai (Convert.ai) is possible for highly accurate estimation without the need for staining.
- NIS.ai (Convert.ai) is an effective tool for users because it has such merits as preventing phototoxicity due to fluorescence excitation and reducing experimental costs and work such as staining.

2. Live cell count (using Convert.ai and Segment.ai)

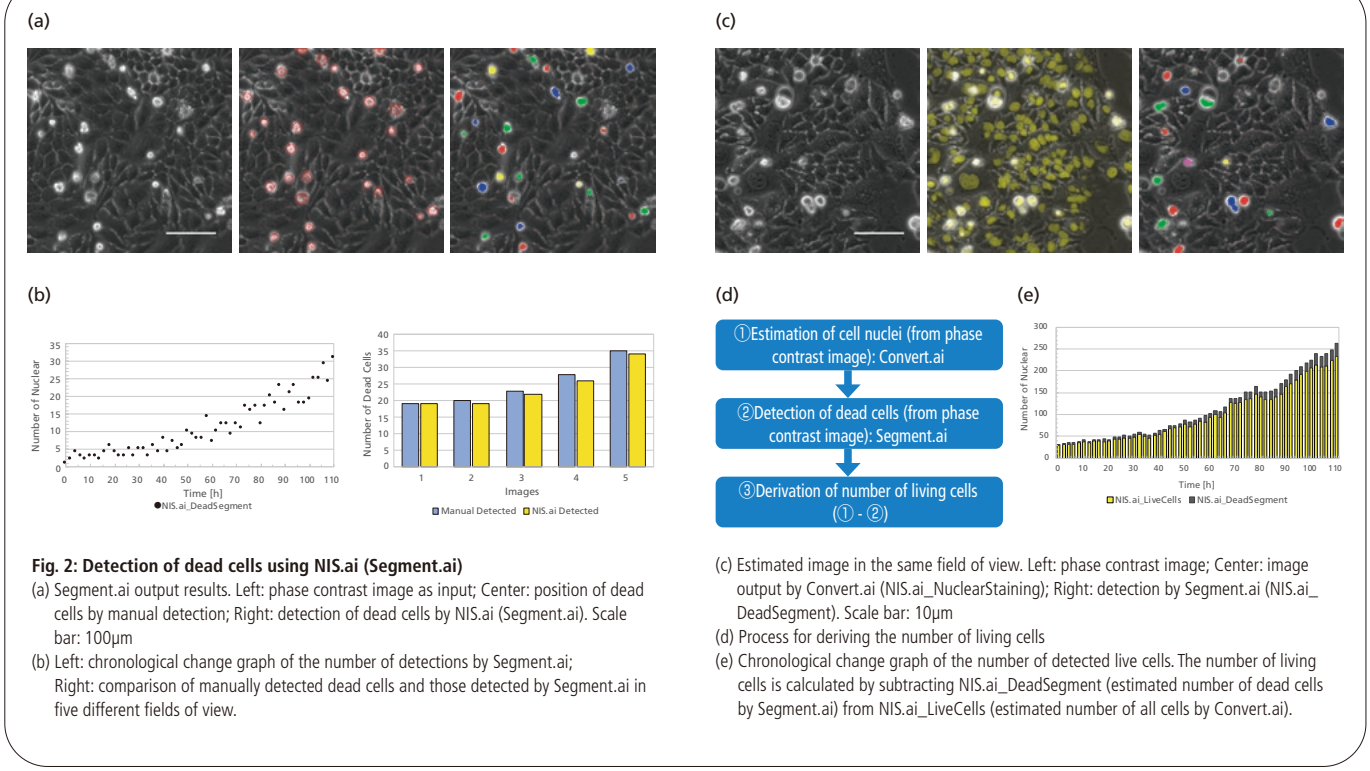


Fig. 2: Detection of dead cells using NIS.ai (Segment.ai)

(a) Segment.ai output results. Left: phase contrast image as input; Center: position of dead cells by manual detection; Right: detection of dead cells by NIS.ai (Segment.ai). Scale bar: 100µm

(b) Left: chronological change graph of the number of detections by Segment.ai; Right: comparison of manually detected dead cells and those detected by Segment.ai in five different fields of view.

(c) Estimated image in the same field of view. Left: phase contrast image; Center: image output by Convert.ai (NIS.ai_NuclearStaining); Right: detection by Segment.ai (NIS.ai_DeadSegment). Scale bar: 10µm

(d) Process for deriving the number of living cells

(e) Chronological change graph of the number of detected live cells. The number of living cells is calculated by subtracting NIS.ai_DeadSegment (estimated number of dead cells by Segment.ai) from NIS.ai_LiveCells (estimated number of all cells by Convert.ai).

Detection of dead cells

Since the number of dead cells increases as confluency increases in proliferating cells, it affects accurate live cell counts. On the other hand, since easy-to-use stain reagents such as Hoechst also stain dead cells, this makes it difficult to distinguish only living cells. Although dead cells can be removed by washing, it is complicated since it requires working on the microscope stage during long-term fixed-point time-lapse imaging. Therefore, whether efficient dead cell estimation of HeLa cells is possible was investigated using NIS.ai (Segment.ai).

Methods

- As training images, 24 images of dead cell masks were created visually and manually with the degree of chromatin aggregation, cell morphology, and halo intensity of phase contrast images as indices.
- In order to estimate the total cell nuclei, 75 training images (phase contrast images and Hoechst fluorescent stained images) with different cell confluences (20%, 50%, 80%, 100%, 120%) were taken in advance and utilized in Convert.ai.

Results

- It became clear that Segment.ai can estimate only dead cell regions from phase contrast images (Fig. 2a).
- The result shows that the inference area of dead cells by Segment.ai increases with the passage of time (Fig. 2b).
- This result is consistent with previous findings that cell death occurs and increases as the culture period increases and confluency increases.
- To verify accuracy, Segment.ai output was compared with visual detection, and approximate values were obtained for each of the five different fields of view (Fig. 2b).

Next, the possibility of counting only the number of living cells was examined.

- The number of living cells was obtained by subtracting the number of dead cells estimated by Segment.ai from the number of whole cells estimated by Convert.ai (Fig. 2c, d).
- The percentage of dead cells increases with the passage of culture time and about 20% of dead cells were mixed in overconfluent states (Fig. 2e).

Summary

- In the past, multiple stainings had to be performed to count the total number of cells and the number of dead cells, but these numbers can now be obtained by NIS.ai using only diascopic (phase contrast) images.
- Since counting from diascopic images does not require fluorescent staining, fluorescent staining can be used for other detection purposes.
- It is suggested that when cell morphology is used as an index for training models, as in this investigation, it can be used to detect not only dead cells, but also cells showing unique morphologies in a cell population.
- The NIS.ai function can be an effective tool for screening assays that require large amounts of image processing and assays that require multiple simultaneous stainings.

3. Avoiding the effects of fluorescent dye photobleaching (using Convert.ai)

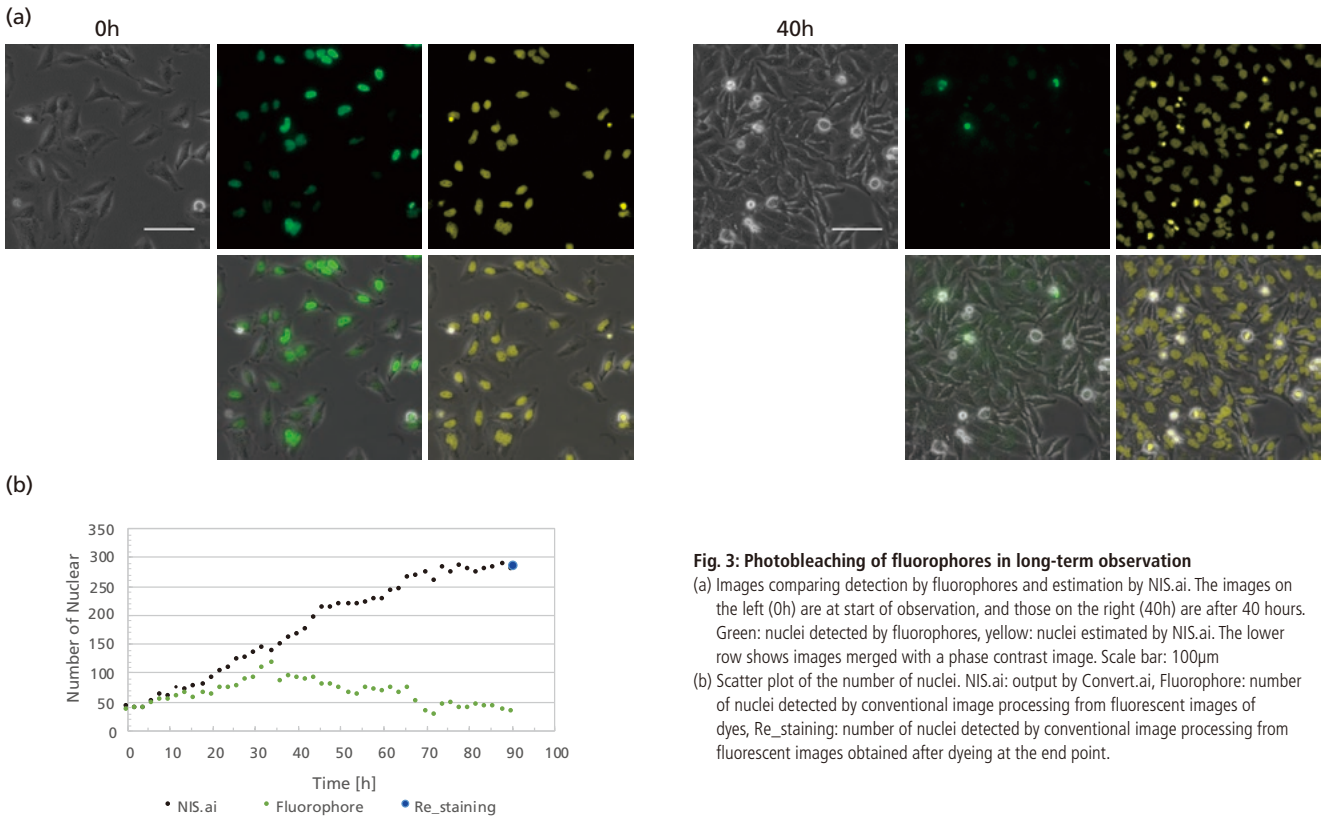


Fig. 3: Photobleaching of fluorophores in long-term observation
(a) Images comparing detection by fluorophores and estimation by NIS.ai. The images on the left (0h) are at start of observation, and those on the right (40h) are after 40 hours. Green: nuclei detected by fluorophores, yellow: nuclei estimated by NIS.ai. The lower row shows images merged with a phase contrast image. Scale bar: 100µm
(b) Scatter plot of the number of nuclei. NIS.ai: output by Convert.ai, Fluorophore: number of nuclei detected by conventional image processing from fluorescent images of dyes, Re_staining: number of nuclei detected by conventional image processing from fluorescent images obtained after dyeing at the end point.

Creating a growth curve

Generally, a nuclear count using microscopic images is performed by staining cell nuclei with a fluorescent dye. However, since the fluorescent dye is not synthesized inside the cell, the amount of dye per cell decreases with division, resulting in color reduction. Also, the potential effect of fluorescent dye toxicity on cell dynamics, in addition to fluorescent photobleaching and phototoxicity caused by repeated irradiation of excitation light, cannot be ignored. Moreover, depending on cell type, there are some cells to which a fluorescent dye cannot be applied, and some cells that dye unevenly.
We therefore investigated whether using NIS.ai (Convert.ai) can solve the above problems.

Methods

- Counted fluorescent dye-stained HeLa cell nuclei using conventional image processing.
- The above cell number was compared with the number of cell nuclei estimated from a phase contrast image by Convert.ai.
- The training model created and used in "2. Live cell count" was used for the estimation of cell nuclei.

Results

- In conventional image processing, which performs nuclear detection using a fluorescent dye, detection becomes difficult from the early stages as the dye decays, and deviation from the result estimated by Convert.ai increased with time (Fig. 3a, b).
- To confirm accuracy, the cell nuclei were stained at the end point and compared with the estimation by Convert.ai. The result confirmed that the estimation by Convert.ai accurately represented the number of cells.

Summary

The following advantages of NIS.ai (Convert.ai) were recognized.

- Stable estimation of cell numbers is possible
- Reducing the cost and workload of staining and the effect of phototoxicity due to excitation light is possible while maintaining high nuclear count accuracy.
- There is no need to consider optimum dyeing conditions.
- Since phototoxicity can be minimized, NIS.ai (Convert.ai) is very effective for assays that require long-term observation and assays with short imaging intervals.

4. Avoiding the effects of fluorescent dye staining on cells (using Convert.ai)

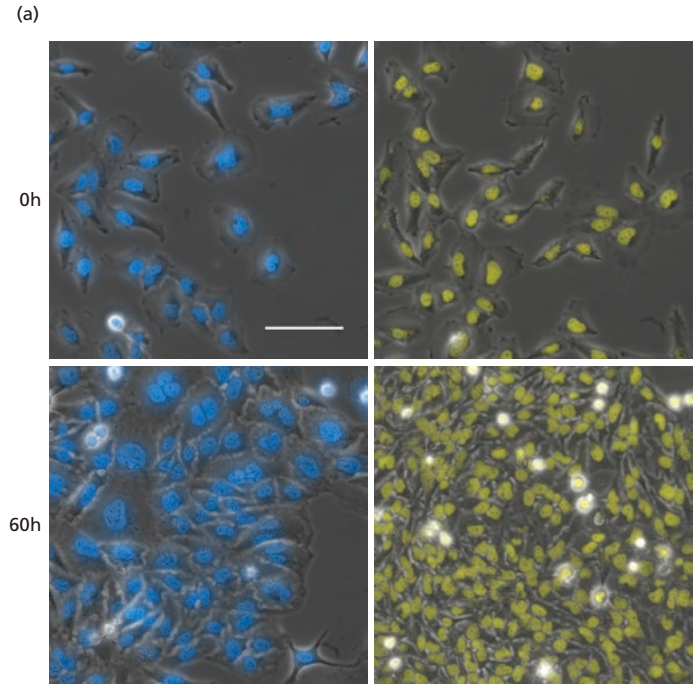


Fig. 4: Toxic effect on cells by fluorophores

(a) The images on the left show a Hoechst experimental group, while those on the right are images output by Convert.ai of a non-Hoechst experimental group. The upper row (0h) were taken at the start of observation and the lower row (60h) after 60 hours. Scale bar: 100μm

(b) A scatter plot, where the average number of nuclei in each of the following four fields of view was plotted (average ± SD).

- Con.Es: output by Convert.ai (non-Hoechst experimental group)
- Con.Dye: number of stained nuclei (non-Hoechst experimental group)
  * Stained/measured only at the end point to avoid the effects of dyes
- Hoechst.Es: output by Convert.ai (Hoechst experimental group),
- Hoechst.Dye: number of stained nuclei (Hoechst experimental group)

Detecting the effects of dyes

Caution was required, since dyes commonly used for cell nuclei, such as Hoechst, affect cells depending on conditions.
Therefore, NIS.ai (Convert.ai) was used to evaluate the effect of Hoechst dyes on cell behavior.

Methods

- HeLa cell nuclei were stained with Hoechst and counted by conventional image processing (Hoechst experimental group).
- NIS.ai (Convert.ai) estimation was also performed on the images of the Hoechst experimental group.
- As a control experiment, the same number of HeLa cells was seeded in another well, and their cell nuclei estimated by Convert.ai (non-Hoechst experimental group).
- To confirm the estimation accuracy of the non-Hoechst experimental group, staining with fluorescent dye was performed at the end point, and the number of cells counted by conventional image processing.
- A growth curve was created under each condition to evaluate the effect of stain dye on cell growth.
- The training model created and used in "2. Live cell count" was used for the estimation of cell nuclei.

Results

- Regarding the number of cell nuclei in the Hoechst experimental group, the results of the cell nucleus count by conventional image processing and the cell nucleus count by Convert.ai were in agreement (Fig. 4b).
- The growth curve (Fig. 4b) confirmed that in the Hoechst experimental group, there was a tendency for proliferation to be suppressed compared to the non-Hoechst experimental group (not because of increased cell death). This is clear from the images (Fig. 4a). This phenomenon can be an obstacle to accurately grasping the behavior of cells.

Summary

By using NIS.ai (Convert.ai and Segment.ai), it is possible to observe and understand the correct behavior of cells without the above concerns. Label-free cell nucleus detection has advantages for users.

Product Information

AI module for microscopes (Convert.ai)

Enables a network to be trained to generate fluorescent images from unstained cell images in phase contrast, differential interference contrast, and other types of images. As this makes long-term time-lapse imaging possible without fluorescent staining, non-invasive analysis that does not damage cells due to excitation light can be realized.

AI module for microscopes (Segment.ai)

Enables a network to be trained to generate images in which only the target cells are identified from images that contain a variety of cells. As conventional binarization cannot classify cells of a specific shape or size, manual classification is necessary; however, Segment.ai enables automatic learning-based classification.