Comparison of Several Classes of Algorithms for Cytoplasm to Nucleus Translocation

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Abstract

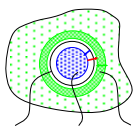
Cellular assays assessed by image analysis provide new type of information, but also present several new sources of variability compared to traditional screening. Understanding of these sources and of the behavior of image analysis algorithms under different conditions is important for obtaining reliable data from imaging assays. We have developed several new and implemented several commonly used measures of cytoplasm to nucleus translocation (CNT). The quality of these measures is analyzed with respect to user parameters, number of analyzed cells, uniformity of illumination, accuracy of focusing, and depth of field.

Algorithms for Image Analysis of Cytoplasm to Nucleus Translocation in this Study

- Algorithms based on 2D distributions of signal stain and counter stain, e.g. "Slope" algorithms[4,5]
- Algorithms based on measuring signal stain in cellular compartments: nucleus, cytoplasm, or part of cytoplasm (ring)
  - Algorithms based on measuring by segmenting the image into cellular compartments[3]
  - Algorithms based on measuring without image segmentation into cellular compartments

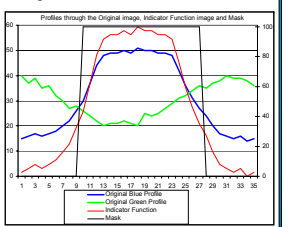
Indicator Functions and their Use for Analysis of Cytoplasm to Nucleus Translocation

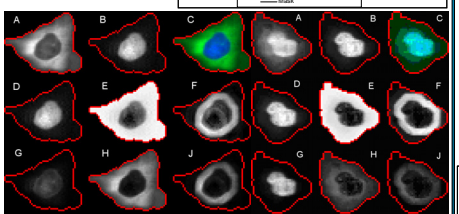
Algorithms based on segmentation[8] are widely used for analysis of cytoplasm to nucleus translocation. The approach is to use the nuclear stain image to identify the nuclear mask; then to find the mask of what is some approximation of the cytoplasm, usually a ring around the nucleus as shown in the figure. After the masks representing the nucleus and the cytoplasm are found, the average and/or total intensity of the signal stain are calculated in each mask. The measure of translocation is the ratio or the difference between these intensities.



Parameters: **A** – Erosion of the nuclear mask
B – Gap between the eroded nuclear mask and the ring mask
C – Width of the ring mask

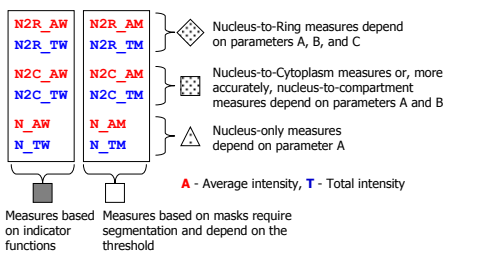
Indicator function is a generalization of the notion of mask; it indicates the probability that a pixel belongs to the object. In the context of nuclear translocation the nuclear indicator function may be produced by simply scaling the blue profile to a convenient range, e.g. [0-100%]. Integrated intensity of the signal stain (green) in the object is produced by integrating green in the whole image, using the indicator function as weight.





Indicator functions of cell images. Left panel corresponds to a negative cell where stained proteins are localized predominantly in cytoplasm. Right panel corresponds to a positive cell where stained proteins are localized predominantly in nucleus.
A – image of stained proteins, B – image of nuclear stain,
C – pseudo-color composite of A and B,
D – same as B normalized to [0-255] and used as indicator function of nucleus,
E – complement of D within the component containing the cell (shown by red contour) – used as indicator function of cytoplasm,
F – dilation of D minus D normalized to [0-255] – used as indicator function of the ring around the nucleus.
G – A weighted (multiplied) by D – the image of protein content in nucleus,
H – A weighted by E – the image of protein content in cytoplasm,
J – A weighted by F – the image of protein content in a ring around the nucleus.

A family of assay measures can be constructed as ratios of the average (A) or total (T) amounts of stained protein in the three compartments: nucleus (N), cytoplasm (C), and ring (R). For more convenient scaling we use $100*\log_2()$ of all ratio measures. In addition, a non-ratio measure of the amount of signal stain in the nucleus may be also useful. The measures may be produced by segmentation into masks of nucleus and cytoplasm (M) or without segmentation by the use of indicator functions (W). The family of measures is given by the formulas:
N2{R|C}_{A|T}{W|M} and **N_{A|T}{W|M}**.

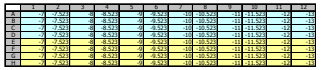


Steps of Algorithm Comparison

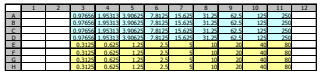
- Establish quality measure[1,2] and a representative image set
- Analyze all available algorithms with a wide range of parameters
- Choose the best algorithms/measures and a narrow range of parameters
- Analyze dependency of the assay measures and their quality on relevant factors

Plates Used in Algorithm Comparison

Vitra Plate. Cells were plated at about 10,000 cells per well in a 96 well microtiter plate (Packard ViewPlate) and incubated overnight. The cells were then treated with varying doses of TNFa for 30 minutes. This treatment results in the activation of NFKB and the translocation of the p65 subunit from the cytoplasm to the nucleus. The cells were subsequently fixed and immuno-stained for p65 and counterstained with the Hoechst nuclear dye. The plate was acquired at Vitra Bioscience on the CellCard reader - a microscope-based system. Image size 1360*1024 pixels, 8-bit per pixel.

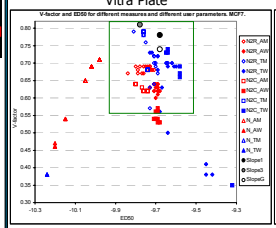


BioImage Plate. This assay quantifies the Forkhead fusion protein (FKHR-EGFP) accumulated in the nuclei of stably transfected human osteosarcoma cells, U2OS. In proliferating cells FKHR is localized in the cytoplasm. Even without stimulation, Forkhead is constantly moving into the nucleus, but is transported out by export proteins. Upon inhibition of nuclear export, FKHR accumulates in the nucleus. In this assay, export is inhibited by blocking PI3 kinase / PKB signaling by incubating cells for 1 hr with a drug as indicated in the plate map. Nuclear staining: DRAQ. The images were acquired at BioImage on the IN Cell Analyzer 3000. Image size 640*640 pixels.

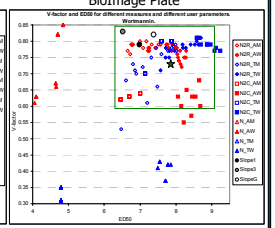


Parameter Dependency of V-factor and ED_{50}

Vitra Plate



BioImage Plate

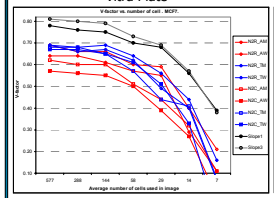


Green rectangles represent narrow range of parameters.

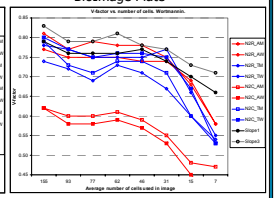
★ Log(Nuc/Cyt), where Nuc/Cyt is the InCell3000 measure

Dependency of V-factor on the Number of Cells

Vitra Plate



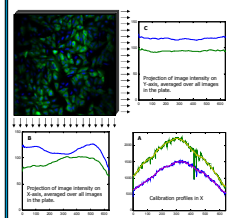
BioImage Plate



Parameters: Nuclear erosion = 1, Gap = 2, Ring = 4

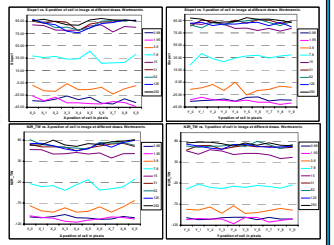
Study of the cell number dependency was done on random subsets of cells from the whole image to eliminate possible positional dependency.

Uniformity of Illumination and CNT Algorithms



Images acquired on IN Cell 3000 provide a convenient model to study the dependency of cellular measures on non-uniformity of illumination. Y-direction, which corresponds to the stage movement has uniform brightness, but X-direction, which corresponds to the scan of the laser beam has variable brightness. Calibration curves provided with the images are shown in Figure A. Actual brightness in the two channels averaged over the entire plate is shown in figures B (X-projection) and C (Y-projection). The plots show uniform intensity in Y and non-uniform intensity in X.

To assess if the brightness non-uniformity affects the cellular measures produced by the presented family of CNT algorithms, we plotted the measures against X-coordinates of cells and against Y-coordinates of cells. Two of the measures are shown below. The plots show that there is no dependency on either X or Y position of cells. Other measures, which are not shown, exhibit similar behavior.



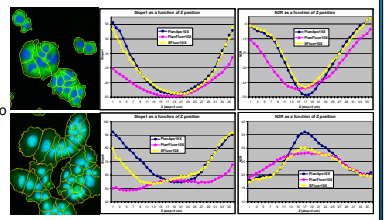
This allows us to conclude that variation in brightness alone does not affect cellular measures if the algorithm is specifically designed to compensate for it.

Dependency of CNT Measures on Focus Position

CNT measures are almost identical within ~10μm from the best focus position in 10X.

Objectives with lower NA are more tolerant to focusing inaccuracy.

Slope1 and N2R_AM show similar dependency on Z-position on negative cells and opposite dependency on positive cells.

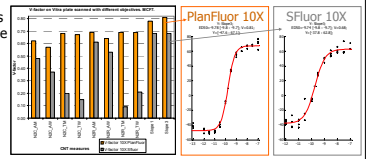


The importance of this dependency is not only in setting focusing requirements, but also in establishing the need for flat field.

Dependency of CNT Measures on the Depth of Field in a Non-confocal System

The Vitra plate was scanned with a PlanFluor10X 0.3NA objective and with a SFluor10X 0.5NA objective (Nikon). The images were processed with the same parameters: nuclear erosion=1, gap=2, ring=4. V-factor shows that PlanFluor always gives better results. Measures based on total amount of fluorescence suffer the most.

Our hypothesis is that this result is due to the relative thickness of cells and the depth of field of the two objectives. The depth of field of PlanFluor 10X is about 8.5 μm, and the depth of field of SFluor 10X is about 3.6 μm.

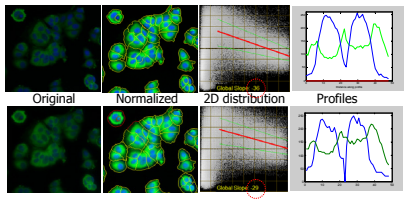


The thickness of attached cells is probably between these two values. If the cell is thicker than the depth of field of the objective it will appear fuzzy. This will make negative cells look less negative and reduce the dynamic range of the measurements. The following illustration supports this hypothesis.

Analysis of the minimal and maximal values of the fitted logistic curves indeed shows reduced dynamic range with the SFluor objective. This does not explain, in full, the decrease in V-factor. The variation among replica wells also was higher with the SFluor objective. This may be attributed to focusing artifacts due to shallow depth of field.

PlanFluor10X, 0.3NA, Depth of field = 8.5 μm

SFluor10X, 0.5NA, Depth of field = 3.6 μm



Conclusions

Algorithms and measures:

1. Measures based on assessing signal stain in nucleus alone show much higher variability than other measures, both in V-factor and in ED_{50}.
2. There is a systematic shift between ED_{50} of different measures. If the goal is comparing drugs or cell types, then a single measure should be used for the whole experiment.
3. Different measures have maximum quality at different parameter values. For a realistic comparison, the quality of different measures must be averaged over a reasonable parameter range. Therefore, measures which do not depend on parameters have a big advantage.
4. Conceptually similar measures implemented by different vendors can show significant difference in quality.

Choosing an instrument:

1. Illumination non-uniformity alone does not play an important role if the algorithms can compensate for it.
2. Flatness of field and accuracy of focusing are critical and cannot be easily compensated for algorithmically.

Experiment design:

1. Objectives in a wide-field system should be chosen based on their depth of field and cell thickness.
2. Focusing accuracy of the system must be evaluated and focusing strategy established to provide for small error (e.g., <10μm in 10X).
3. Required number of cells can be established by analyzing a fraction of images and selecting a number above which quality does not significantly improve with cell number.

References

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2. I. Ravkin "Quality Measures for Imaging-based Cellular Assays" SBS 2004 conference poster #P12024
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5. Ilya Ravkin, Vladimir Temov, "Image analysis without segmentation - A new method to measure cytoplasm to nucleus translocation", poster, High Content Analysis Conference, San Francisco 2005.