

A primer on deep learning-based cellular image classification of changes in the spatial distribution of the Golgi apparatus after experimental manipulation

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Abstract

The visual classification of cell images according to differences in the spatial patterns of subcellular structure is an important methodology in cell and developmental biology. Experimental perturbation of cell function can induce changes in the spatial distribution of organelles and their associated markers or labels. Here, we demonstrate how to achieve accurate, unbiased, high-throughput image classification using an artificial intelligence (AI) algorithm. We show that a convolutional neural network (CNN) algorithm can classify distinct patterns of Golgi images after drug or siRNA treatments, and we review our methods from cell preparation to image acquisition and CNN analysis.

Key words: convolutional neural network, image classification, golgins, Golgi, microtubule

1. Introduction

Fluorescence microscopy, including immunofluorescence and live-cell labeling, is a standard approach to visualize protein localization in cell and developmental biology. The availability of a variety of fluorescent dyes or proteins facilitates the multi-color labeling of subcellular structures. Fluorescence microscopy is essential for visualizing the spatial distribution patterns of organelles such as the Golgi apparatus using the advantage of high contrast (i.e., bright signal over dark background) image acquisition. Moreover, recent advances in super-resolution microscopy have extended fluorescence methods to the nanoscale. However, images are acquired as digital data and, therefore, image processing and quantitative analyses require large computational resources.

As large digital data in bioimaging increases, artificial intelligence (AI) technologies are becoming applicable. Breakthroughs include deep learning-based image classification, segmentation, and restoration [1, 2]. At the stem of the growing number of deep learning algorithms is the convolutional neural network (CNN). The CNN architecture is simple and powerful exemplified in recent work demonstrating that CNN-based image classification and analysis can extract cell cycle phase-specific features from cell images [3]. Here we show how CNN-based applications can classify immunofluorescence images of the Golgi apparatus according to its spatial distribution pattern. The experimental conditions include 1) major perturbation of Golgi distribution by Nocodazole treatment and 2) potential subtle disruptions by Giantin depletion.

2. Materials

Obtain the following reagents and supplies from appropriate companies. Examples of company names and catalog numbers are listed. Prepare all solutions using pure water or analytical grade reagents. Prepare and store all reagents at room temperature unless indicated. An .stl file for the coverslip rack is available upon downloading at 10.18926/61943. The Python code is available via GitHub (<https://github.com/dtakao-lab/Nagao2020>).

- a. Alcian Blue Sigma A5268
- b. Round coverslips Fisherbrand #12-545-80 12CIR.-1
 Matsunami #1, 12 mm
- c. Nocodazole Sigma M1404
- d. Mowiol® 4-88 Sigma 81381
- e. HeLa cell line ATCC ® CCL-2™
- f. Anti-GM130 BDBiosciences Clone 35, 610822
- g. Anti-alpha tubulin Sigma T5168 Clone B-5-1-2
- h. Alexa fluor-conjugated secondary antibodies (Thermo)
- i. Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution
 Thermo H3570
- j. Other reagents, such as ethanol (14712-05), DMSO (Dimethyl Sulfoxide, 09659-14), Tris (Tris(hydroxymethyl)aminomethane, 35401-25), DMEM (DMEM Dulbecco's Modified Eagle Media, 08458-45), FBS (Fetal Bovine Serum), PBS (Dulbecco's phosphate-buffered saline, 14249-95), 0.5g/l-Trypsin/0.53mmol/l-EDTA Solution (35553-74), methanol (21914-45), PFA (4%-Paraformaldehyde Phosphate Buffer Solution, 09154-14), BSA (Albumin, Bovine Serum, Globulin Free, 01281-97) can be obtained from Nacalai Tesque (Kyoto, Japan)

Alcian Blue solution: Disperse Alcian Blue powder (Sigma) in 30 mL distilled water at 1% (w/v) in a 50 mL tube. Before adding 1 box or 100~200 coverslips to the tube, confirm that there is no precipitation of Alcian Blue dye in the tube. To remove the precipitation, the solution can be paper filtered.

Coverslip stands: To obtain the plastic racks by 3D printing, an .stl file is available upon downloading at 10.18926/61943. Print this by any 3D printer with a high level of performance to accurately create the narrow furrows for the coverslips. An example of a 3D printer is Partpro300 xT (XYZ PRINTING Japan, Tokyo, Japan).

Nocodazole stock solution: Dissolve 2 mg of nocodazole powder in 10 mL of DMSO to make 0.664 mM nocodazole stock solution.

Mounting solution: Mounting solution, either commercially available or prepared by dissolving in Mowiol® can be used. To make Mowiol® mounting solution, add 6 g of glycerol, 2.4 g of Mowiol® 4-88, 6 g of water, and a stirrer bar in a 50 mL tube. The 50 mL tube should be closed lid and placed on a stirrer upside down, and mixed for about 2h. After adding 12 mL of 0.2 M Tris-HCl (pH 8.5) to the 50 mL tube, the tube should be placed on a hot stirrer set at 50°C until the Mowiol® dissolves. If a hot stirrer is not available, put the tube in a 53°C water bath and occasionally mix by inversion.

Centrifuge the tube to remove any undissolved material as sediment. The supernatant should be aliquoted and stored in a freezer at -20 degrees C or below.

3. Methods

3.1 *Preparation of Alcian Blue-coated coverslips*

1. For the stable attachment of the cells to glass coverslips, round coverslips should be coated with Alcian Blue before plating cells.
2. Incubate coverslips with Alcian Blue solution overnight or longer at room temperature on a rotator. This step can be shortened using a microwave oven.
3. After collecting used Alcian Blue solution for reuse, coverslips should be washed with distilled water until the supernatant becomes clear. After discarding the supernatant, the coverslips should be stored in 70% Ethanol until use.

3.2 *Plating cells on Alcian Blue-coated coverslips*

1. A HeLa cell line should be maintained in DMEM supplemented with 10% FBS (Culture medium) at 37°C in a 5% CO₂ incubator.
2. One day before transfection or staining, cells should be plated onto the Alcian Blue-coated coverslip-containing dishes. Since ethanol affects cell viability and morphology, the Alcian Blue-coated coverslips should be placed on the cell culture dish and air-dried completely before plating (*see Note 1*).
3. RNAi of Giantin is described in the other chapter. To perform Nocodazole treatment, a Nocodazole stock solution should be applied onto the cells at 1:1000 dilution for 30 min. This should be sufficient for complete microtubule disruption as observed by alpha-tubulin staining.

3.3 *Fixation with cold methanol and rehydration*

1. Add 0.6 mL of 100% methanol to each well of a 12 well-plate on a metal plate and chill in a -20°C freezer overnight or longer.

2. Cells on coverslips for treatment with drugs or nucleic acids should be directly moved one-by-one into the cold methanol-containing wells with the cell side up.

3. After two minutes, PBS should be gently added to the top of each well and they should be incubated more than 10 min at room temperature for rehydration.

4. Alternatively, fixation can be performed using PBS containing 3.7~4% paraformaldehyde (PFA) with 15 min incubation at room temperature. Importantly, PFA fixation requires subsequent permeabilization with a 5-minute incubation with 0.1% Triton X100/PBS, whereas methanol fixation does not.

3.4 *Blocking, staining, mounting, and sealing*

1. For blocking and washing of coverslips, plastic racks shown in Figure 1B should be placed in a 6-well plate with forceps (Figure 1A and C).

2. After rehydration or permeabilization of cells on coverslips, they should be moved into the furrow of the rack in 4% BSA/PBS filled wells and incubated for 15 min at room temperature. Alternatively, cells can be blocked with 0.2% fish skin gelatin (Sigma)/PBS. It is important not to lose track of the cell side. Meanwhile, round filter paper should be placed into the lid of a 15 cm dish and wetted by water.

3. A square of parafilm should be placed on top of the filter paper and covered with a 15 cm dish. Note that the 15 cm dish should be used upside down (Figure 1D). Antibodies should be diluted in 4% BSA/PBS in advance. Primary antibodies, such as anti-GM130 for cis-Golgi, anti-Giantin [4], for Golgi, and anti-alpha tubulin for microtubules should be diluted at 1:50, 1:400, and 1:400, respectively. Five to ten micro-liters of the diluted antibodies should be placed on the parafilm in the 15 cm dish.

4. The coverslips should be removed from blocking solution by standing them vertically for 5 sec on a piece of filter paper and then placed on top of the antibody solution with the cell side down. To prevent coverslips from drying out, they should be covered by the 15 cm dish, incubated for 15 min at room temperature, then moved to the rack in a PBS-filled well (PBS1).

5. The rack should be incubated successively to PBS2, PBS3, and 4% BSA/PBS in order for one-minute intervals.

6. Alexa fluor-conjugated secondary antibodies (Thermo) were also diluted in 4% BSA/PBS at 1:100. Hoechst dye at 1:10000 for nuclei can be included with the diluted secondary antibodies and co-stained simultaneously. The fluorescent dye-conjugated to the secondary antibodies or nuclear staining dyes should be appropriate for the filter set of the microscope. Secondary antibodies can be incubated in the same way as the primary antibodies, but covered with aluminum foil or similar material to avoid bleaching the fluorescent dyes.

7. After washing with PBS three times, coverslips should be rinsed in water. The residual water on the coverslips should be removed by standing them vertically for 5 sec on a piece of filter paper.

8. For mounting coverslips on a glass slide, the mounting solution should be taken into the tip of a P20 micro-pipet set to 20 μ l and placed on a glass slide. It is important to stop the plunger in the middle and not to push the plunger all the way out to avoid pipetting air bubbles.

9. The drained coverslips should be gently placed on top of the mounting solution, carefully avoiding air bubbles. The overflow mounting solution should be wiped off by placing a filter paper on top of it and pressing lightly and then air-dried.

10. Once the coverslips are dried, to prevent further drying out, coverslips can be sealed with nail polish. Be aware that you will not be able to observe the areas covered with nail polish.

3.5 *Image acquisition*

1. Any standard microscope setup will work. Typically, a wide-field or confocal microscope equipped with a 60x oil immersion objective achieves good performance for image acquisition of fluorescently-labelled mammalian cultured cells. For the data shown here, we used a spinning disc confocal microscope (Olympus IXplore SpinSR, [5]) equipped with a 60× oil-immersion objective (PLAPON60×OSC2/NA 1.4, Olympus) and a complementary metal–oxide–semiconductor (CMOS) camera (ORCA-Flash4.0 V3, Hamamatsu Photonics).

2. To cover the cell volume, Z-series images can be acquired and then converted to 2D images by max intensity projection with ImageJ.

3. An image acquisition setup is typically with 2×2 digital binning resulting in a pixel size of $0.2167 \mu\text{m}$ with a Z-interval of $1 \mu\text{m}$. These parameters are variable depending on samples and aims.

3.6 *Deep learning*

1. A large body of work has demonstrated the power of various deep learning algorithms for image classification. Here, we implement a convolutional neural network (CNN) for cell image classification [3]. The Python code is available via GitHub (<https://github.com/dtakao-lab/Nagao2020>) and includes image preparation such as cropping that can be substituted, if preferable, by ImageJ (see *Note 2, 3*).

2. Original images should be first cropped to contain only single cells for classification. Then measure the centroids of nuclei stained with DAPI or Hoechst; such nuclei can be easily segmented by thresholding. Images should be cropped with an ROI of a fixed size (e.g., 150 x 150 pixels) around the centroids. Cells should be sufficiently sparse so that each ROI includes only one cell and cropped images containing two or more cells should be manually excluded. Alternatively, automatic segmentation can be used [6-8]. In our experience, 200–500 single-cell images for each class (category) are typically sufficient and realistic, given that one would only need tens of original images that contain tens of single-cells (e.g., 20 original images containing 20 cells each will generate 400 cropped single-cell images).

3. Optionally, cropped images can be augmented to increase the number of images; we augment images to increase the number of images by four-fold.

4. Using the resultant single-cell images, the CNN model should be trained with the Python code provided in the GitHub repository. The images can be typically split 9:1 to make training and test datasets, and a subset (typically 10%) of the training data were further split to make a validation dataset. This can be achieved with the default parameter settings in our Python code that are adjustable if needed.

5. The validation dataset is used to monitor learning quality and the test dataset allows verification of the accuracy of the model after the CNN model completes learning. It is difficult to set criteria or borderlines of accuracy for model quality in a quantitative manner (e.g., while 99% accuracy will be sufficient in most applications, 80% accuracy will result in divided opinions), however, models achieving 90% accuracy or above are generally acceptable in many cell biological applications.

3.7 *Image classification of Golgi*

1. We used CNN-based image classification on images of the Golgi according to spatial patterns, i.e., “normal” and “microtubule-depolymerized”, using Giantin as a Golgi marker in the example classification (Fig. 2A). Treatment of cells with 0.4 $\mu\text{g}/\text{mL}$ nocodazole induced microtubule depolymerization and dispersion of the Golgi distribution. After training a dataset containing 215 normal and 214 microtubule-depolymerized Golgi images (before augmentation), the CNN model successfully classified the images with an accuracy more than 92% (Fig. 2B). The accuracy is defined by a correct answer rate for the test dataset (e.g., if the model can correctly answer for 92 out of 100 cell images in the test dataset, the accuracy is 92%) (*see Note 4*). At lower concentrations of nocodazole (0.025 and 0.1 $\mu\text{g}/\text{mL}$), more cells were classified as “normal” compared to a higher concentration (0.4 $\mu\text{g}/\text{mL}$), presumably reflecting the partial disruption of microtubules (Fig 2C). These results demonstrated that the CNN can classify cellular images containing different patterns.

2. We tested whether CNN models can classify Golgi images with little to no differences in their appearance. Cells were treated with control or Giantin siRNAs and a dataset of Golgi images labelled with an antibody to GM130 was prepared (Fig. 3A). Despite multiple learning trials, no models successfully classified GM130 images of control and Giantin-depleted cells (accuracies around 50%, i.e., random classification; Fig. 3B). While AI could in principle discover subtle differences in cell images unobservable to the human eye, we did not achieve it with these particular results. Although it is difficult to estimate in advance if our AI-based approach would be effective, it is worth trying to test it in most cases, given its cost effectiveness.

3. Taken together, CNN-based image classification is a powerful tool for image classification but certain limitations may be recognized. If there are obvious differences among images and automation of image classification is the primary

purpose, CNN applications will be one of the best options. Finding subtle or hidden differences in images via CNN-based classification is more challenging but still potentially useful. Given the rapid progress of AI technologies, even more powerful tools will emerge.

4. Notes

1. To avoid artificial bias, cell preparation and image acquisition should be done in an extraordinarily careful manner. The cell density, staining protocols, and the exposure time of image acquisition, for example, should be exactly the same across experimental conditions.

2. The Python code for image preparation and CNN-based classification is described in previous work [3] and available from the following GitHub repository:

<https://github.com/dtakao-lab/Nagao2020>

3. Our Python code is designed for the Jupyter Notebook coding environment, thus runs in Google Colaboratory in which the resource of GPU is available.

4. When testing new datasets, starting with smaller datasets (e.g., 50 images in each group) would be cost-effective to assess feasibility, prior to preparing hundreds of images.

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References

1. Meijering, Erik. "A Bird's-Eye View of Deep Learning in Bioimage Analysis." *Computational and Structural Biotechnology Journal* 18 (2020): 2312–25.
<https://doi.org/10.1016/j.csbj.2020.08.003>.
2. Moen, Erick, Dylan Bannon, Takamasa Kudo, William Graf, Markus Covert, and David Van Valen. "Deep Learning for Cellular Image Analysis." *Nature Methods* 16, no. 12 (December 2019): 1233–46. <https://doi.org/10.1038/s41592-019-0403-1>.
3. Nagao, Yukiko, Mika Sakamoto, Takumi Chinen, Yasushi Okada, and Daisuke Takao. "Robust Classification of Cell Cycle Phase and Biological Feature Extraction by Image-Based Deep Learning." *Molecular Biology of the Cell* 31, no. 13 (June 15, 2020): 1346–54. <https://doi.org/10.1091/mbc.E20-03-0187>.
4. Pelletier, L., E. Jokitalo, and G. Warren. "The Effect of Golgi Depletion on Exocytic Transport." *Nature Cell Biology* 2, no. 11 (November 2000): 840–46.
<https://doi.org/10.1038/35041089>.
5. Hayashi, Shinichi, and Yasushi Okada. "Ultrafast Superresolution Fluorescence Imaging with Spinning Disk Confocal Microscope Optics." *Molecular Biology of the Cell* 26, no. 9 (May 1, 2015): 1743–51. <https://doi.org/10.1091/mbc.E14-08-1287>.

6. *R-CNN for Object Detection and Instance Segmentation on Keras and TensorFlow*. 2017.
https://github.com/matterport/Mask_RCNN.
7. Stringer, Carsen, Tim Wang, Michalis Michaelos, and Marius Pachitariu. “Cellpose: A Generalist Algorithm for Cellular Segmentation.” *Nature Methods* 18, no. 1 (January 2021): 100–106. <https://doi.org/10.1038/s41592-020-01018-x>.
8. Tokuoka, Yuta, Takahiro G. Yamada, Daisuke Mashiko, Zenki Ikeda, Noriko F. Hiroi, Tetsuya J. Kobayashi, Kazuo Yamagata, and Akira Funahashi. “3D Convolutional Neural Networks-Based Segmentation to Acquire Quantitative Criteria of the Nucleus during Mouse Embryogenesis.” *NPJ Systems Biology and Applications* 6, no. 1 (October 20, 2020): 32. <https://doi.org/10.1038/s41540-020-00152-8>.

Figure legend

Figure 1. Equipment required for immunofluorescence.

A) Six-well plate for blocking and washing coverslips. The white arrow indicates the coverslip rack shown in B. B) A coverslip rack. The blueprint of this rack for 3D printing is available at [10.18926/61943](https://doi.org/10.18926/61943). C) Forceps. D) Fifteen-cm dish for staining and a cartoon for showing the layer. The black arrows indicate the coverslips.

Figure 2. Image classification of Nocodazole-treated cells.

A) Representative fluorescence images of cells treated with DMSO or Nocodazole. Cells were stained with Hoechst to mark the positions of nuclei, and antibodies to tubulin and Giantin to mark microtubules and Golgi, respectively. Cropped and masked images of Golgi are shown in the bottom panels. Tubulin staining was done to check the effect of Nocodazole treatment and only Golgi images were used for image classification.

B) Learning curves of a representative CNN model. Accuracy (success rate) and loss (error rate) are parameters to assess the quality of model. As training progresses successfully, accuracy and loss get closer to 1 and 0, respectively. If the parameters for training and validation (test) largely diverge from each other, it is an indicator of wrong learning.

C) Fraction of Golgi images classified as “microtubule-depolymerized” or “normal”. Labels in bars are the numbers of cell images in each category.

Figure 3. Image classification of Giantin-depleted cells.

A) Representative fluorescence images of cells treated with control or Giantin RNAi. Cells were stained with Hoechst and antibodies to Giantin (not shown) and a Golgi marker GM130, respectively. Only GM130 images were used for image classification.

B) Learning curves of a representative CNN model.