DeepNeurite™: Identification of neurites from non-specific binding of fluorescence probes through deep learning

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Abstract
The nervous system plays an important role in human health and disease, and the unique morphologies of the neurons underlie its ability to interface with tissues and organs throughout the entire body. In vitro, neurons can be grown alone or with other cell types to gain insight into how they communicate with other cell types in a more controlled experimental setup. To measure neuron growth and to study neuronal connectivity in vitro, neurite identification is an essential readout. However, non-specific binding of fluorescence probes, a fundamental issue of fluorescence imaging, impairs neurite identification through conventional mathematical morphology-based methods, especially in neuron and other cell type co-culture imaging conditions. Here, we utilized a deep learning algorithm and developed a computational tool called DeepNeurite™, to overcome this challenge. We demonstrated that DeepNeurite™ can accurately identify neurite structure in images acquired from microfluidic compartmentalized chambers where neurons were co-cultured, such as with a human prostate cancer cell line, PC3. We further validated that the model can be generalized to handle a direct co-culture in which neurons and lung cancer cells (DMS273) are grown intermingled in the same well. Using this method, we observed more neurite growth into PC3 containing chambers in microfluidic compartmentalized chambers, which could be blocked by an NGF antibody. Finally, we applied DeepNeurite™ coupled with functional calcium imaging to study the communication of primary sensory neurons and cancer cells. We showed that the cancer cells closer to neurites exhibit greater calcium activity in response to neuronal stimulation. This method opens lots of opportunities to study the effect of neurons on various other cell types. This model could further tackle the off-target labeling of the fluorescence probe in other subcellular structures or cell types.

Kai-Chih Huang is first author.

Correction added on January 24, 2022, after first Online publication: Special collection category statement has been added and the same has been updated.

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1 | INTRODUCTION

Innervation has been reported to impact tumor progression, aggressiveness, and metastasis, which can be achieved through direct neurite-cancer interaction or indirectly through other cell types such as immune cells, or both. Therefore, establishing assays to probe the interaction among neurons, cancer cells, and immune cells is essential for drug discovery and therapeutic target validation. Various co-culture assays have been developed to simulate the nerve-cancer microenvironment.

Neuron-cancer co-cultures feature neurites, which are often considered proxies for axon and dendrites produced by neurons in vivo. In vitro, neurites exhibit unique and elaborate morphologies. The length of these neurites indicates neuronal growth, which may be influenced by chemotactants and other treatments. Moreover, neurites are important even in vitro in that they can bring a neuron into close proximity or direct contact with other neurons and other cell types and permit rapid chemical or electrical communications between cells. Thus, neurite length is an important parameter in vitro and requires accurate quantification in a variety of experimental conditions, including treatment with pharmacological agents and in co-culture with non-neuronal cell types.

Several methods have been developed to segment neurites in fluorescence images by imaging canonical neuronal markers. Most of the methods are based on a series of mathematical morphological operations in image processing. Powell et al., further demonstrated using other fluorescence channels to remove autofluorescence backgrounds. These methods usually work well in neuron monocultures or in neuron/glia mixed cultures. However, it is complicated in neuron cancer co-culture as some cancer cells could be labeled by neuronal marker staining. The non-specific binding of fluorescent probes, which is a pervasive problem in co-culture fluorescence imaging, frequently impairs the quantification of neurite length in the co-culture systems. Therefore, a method that performs well even in the presence of non-specific binding of fluorescent probes is needed.

Modern computer visions and deep learning methods have dramatically improved microscopic imaging analyses in classification, segmentation, and de-noising. In particular, over the past decades, deep learning has enabled robust, rapid object segmentation, including automatic identification of cell nuclei from fluorescence images, segmentation of neuronal structures in electron microscopic images stacks, prediction of fluorescent labels from transmitted-light images, and identification of specific cell types in H&E digital slides. However, this method has yet to be fully applied to neurite quantification.

We applied computer vision methods and developed a computational pipeline called DeepNeurite™ to enable the robust quantification of neurite length. DeepNeurite™ utilizes a deep learning algorithm to tackle the fundamental issue of non-specific labeling of fluorescence probes. The model successfully suppresses the fluorescent signal contributed from the cancer cells in the co-culture conditions and at the same time, does not sacrifice sensitivity, allowing for the detection of dim neurites. This method was validated in neuron-cancer co-culture assays from both compartmentalized cultures and direct co-cultures in standard multi-well plates. Using this method, we showed more neurite growth into a PC3 containing chamber in microfluidic compartmentalized device compared with that in monoculture neurons. Finally, we studied the interaction between neurites and cancer cells by using DeepNeurite™ coupled with a functional calcium imaging. We observed that DMS273 cancer cells closer to the neurite showed more calcium activity compared with those farther away from the neurite, suggesting the communication between neurons and cancer cells occurs at short-length scales. Our deep learning model provides a useful tool for the identification of neurites and quantification of neurite outgrowth by minimizing the interference of non-specific fluorescence.

2 | MATERIALS AND METHODS

2.1 | Compartmentalized co-culture of mouse DRG and human cancer cell line

Microfluidic compartmentalized chambers (XC-450) were purchased from Xona Microfluidics. Each chip was coated with poly-D-lysine and laminin, followed by washes and media conditioning according to the manufacturer’s protocol. Mouse DRGs were dissected from adult C57/B6 mice in house. Dissected DRGs were dissociated with Dispase I and Collagenase A following a protocol modified from previous publications.22 Neurites were apparent after three days of culture. Cancer cells were grown in culture media (PC3 in F12 media, DMS273 in Weymouth’s media) and passaged at least three times before use. We seeded these cells into the right side of
the Xona chip at 20 k cells/chip 4 days after plating the DRGs. For the NGF experiment, culture media with re-combinant human beta-NGF (R&D Systems, 20 ng/ml) or anti-human beta-NGF (R&D Systems, 400 nM) was added to the right side of the wells 4 days after the initial plating of neurons during the media change, concurrent with plating cancer cells. NGF or anti-NGF was present in the Xona Chip until the end of the experiment (3 days after application). Note that passive diffusion likely lessened the concentration gradient of NGF by the endpoint of this experiment. For co-culture with cancer cells, cancer cells were suspended in 20 k/20 ul cell suspension and added to the right side of the chamber. Xona chip co-culture continued for another 3 days before final readout.

2.2 Immunofluorescence staining

For the microfluidic compartmentalized chambers neurite growth experiment, beta3-tubulin was used to label the neurites, followed by standard immunocytochemistry methods to fix and wash the samples. Primary antibodies such as Beta-tubulin (Biolegend 801202 to a final concentration 1 ug/ml) were diluted in 5% donkey serum in PBS with 0.1% Triton-X. Secondary antibodies, such as donkey anti-mouse 647 nm (Jackson Immunoresearch 715–175–150), were diluted in the same media. Fluorescent images were acquired on a GE Delta Vision microscope.

2.3 DeepNeurite™ model

Our deep learning network was trained following the U-net structure which consists of both a down-sampling and an up-sampling path. In the down-sampling path, there are four down-sampling blocks - each block contains two 3 x 3 convolutional layers followed by a rectified linear unit (ReLU) activation function, and a batch normalization layer. A dropout layer was added in between two convolutional layers with dropout rate of 0.5 to generalize the model. Zero padding was used to compensate the channel number mismatch between the input and output tensors. The spatial down sampling was achieved by a 2 x 2 max-pooling layer with a stride of 2 x 2 pixels after each down-sampling block to perform a two times down-sampling. At the bottom of the U-net structure, there is a convolutional block that connects the down-sampling block and the up-sampling block. In the up-sampling path, there are four up-sampling blocks. Each contains two 3 x 3 convolutional layers followed by a rectified linear unit (ReLU) activation function and a batch normalization layer. The spatial up sampling is achieved by an up-convolution (convolution transpose) block that up-samples the image pixels by two times. A concatenation operation was implemented to concatenate the up-sampling block input and the down-sampling block output on the same level in the U-net structure. The last layer is another convolutional layer that maps the 32 feature channels into 1 channel with sigmoid activation function to generate a monochrome grayscale image. All the tasks were performed in Python using Keras and TensorFlow. The training of DeepNeurite™ was implemented using a GTX1080Ti graphic card.

2.4 Direct co-culture and calcium imaging

Cancer cells were grown in culture media (PC3 in F12 media, DMS273 in Weymouth) and passaged three times before implementation. The DMS273-GCaMP6m stable cell line was generated by transducing DMS273 cell line with lentivirus carrying vectors expressing GCaMP6m.

For the direct co-culture experiment, 96-well plates were coated with poly-D-lysine and Matrigel (Corning). Dissociated DRGs were plated at a density of 5 K/well. After 24 h, cancer cells were plated on top of cultured DRGs. Experiments were performed 3 days afterward.

For calcium imaging, co-cultured wells were washed with imaging buffer and placed under the fluorescent microscope (GE Healthcare, DeltaVision fluorescent microscope). To stimulate cells, 100 ul of 100 mM KCl stock solution was pipetted into the well during imaging with GFP excitation, using an imaging filter. To ensure the health of cancer cells, we check that cancer cells present calcium activities after adding cell activation cocktail (PMA/Ionomycin) solution to the well. Images were collected at the acquisition rate of 1 frame/second for 100 s. Only one field of view was acquired from each well. Imaging solutions were used following the previous live imaging process to set up the baseline level of the GCaMP6m signal. DMS273-GCaMP6m only wells were used as monoculture control for DRG/DMS273 co-culture. Images were analyzed by ImageJ for cell segmentation and fluorescence intensity trace extraction. Minimal photobleaching was observed. The fluorescence time trance presented in Figure 5D was plotted from raw data without pre-processing.

3 RESULTS

3.1 Training datasets preparation and pipeline design

DeepNeurite™, the deep learning-based neurite identification pipeline is shown in Figure 1 (see Methods). To
train an end-to-end neural network to identify neurite structure without interfering from non-specific fluorescence labeling, we first prepared the training dataset by acquiring confocal images of neurons co-cultured with PC3 cancer cells. The microfluidic compartmentalized chamber (Xona chip) configuration is shown as the coculture imaging condition in Figure 2A. Neurons from mouse dorsal root ganglion (DRG) were cultured in the left chamber, whereas PC3 prostate cancer cells were cultured in the right chamber. Neurons from the left chamber extend neurites, considered a proxy for axons, through the microgroove connecting to the right chamber as shown in Figure 2B. The channels connecting the left and right chambers are very thin (approximately 8 μm) ensuring only passage of neurites. The right chamber (Figure 2C) shows Tuj1, a pan-neuronal marker, expressed in neurites but also at a lower level in the cancer cells (Figure 2E). We created ground truth masks by manually segmenting the neurite structure and erasing the non-specific fluorescence signals. We only used a small number of spatially registered image pairs for training (120 pairs). Each image pair had a size of 512 × 512 pixels corresponding to 329 × 329 microns acquired with a 10× air objective. An example of the raw image is shown in Figure 1. To improve the accuracy of the network and generalize the model, we applied several augmentations including rotations, horizontal shifts, vertical shifts, shearing, flipping, noise additions, and magnifications.

3.2 Deep learning neural network

The neural network structure of DeepNeurite™ is based on U-net model inspired by previous work (middle of Figure 1).19 The first half of the network down-sampled the input intensity image to extract features from multiple scales through two 3 × 3 convolution layers with increasing feature depth, interwoven with 2 × 2 max-pooling layers. The other half of the network performs up-sampling to restore high-resolution segmentation mapping through two 3 × 3 convolution layers with decreasing feature depth, interleaved with 2 × 2 up-convolution layers. A batch normalization layer is added after each convolution layer to standardize layer inputs and stabilize learning processes. A dropout layer is introduced to reduce over-fitting and to generalize the model. Binary cross-entropy loss is used as the loss function. The rectified linear activation function (ReLU) is used as the activation function. Codes are available (https://github.com/khCygnaI/DeepNeurite).

3.3 Model validation

We validated our model by predicting images in the testing datasets (Figure 2C). The output image is shown in Figure 2D. From the magnified images (Figure 2E,F) with the green dotted square in Figure 2C,D, we can observe strong fluorescence signals from cancer cells, as indicated
by the green arrow shown in Figure 2E, are suppressed in the neurite prediction image shown in Figure 2F. The model increases sensitivity to identify neurite with weak fluorescence signals, as indicated by the blue arrows. The model is capable of identifying only the neurite structures, as indicated by the orange arrows, even when the neurites come into contact with the cancer cells. We measured the performance by the similarity of the predicted mask and the ground truth mask in the pixel level. The area under curve reaches 0.948 (Figure 2G), indicating that the model can accurately identify neurite structures in microfluidic compartmentalized chamber co-culture conditions. We further compared the performance of DeepNeurite™ with direct thresholding from the Tuj1 raw images. As shown in Figure 2H, predicted neurite area by DeepNeurite™ better correlates with the ground truth neurite area compared with the build-in threshold algorithm in ImageJ than in Ostu thresholding and in Huang thresholding.24,25 Furthermore, DeepNeurite™ could also handle images in different signal-to-noise conditions (Figure S1) due to the artificial addition of various levels of Gaussian noise.

Next, to test the generalization of our trained model, we acquired Tuj1 fluorescence image of cells in different culture conditions. Neurons were co-cultured with the small cell lung cancer DMS273 cells,26 instead of the prostate cancer PC3 cells in the training set. All cells were cultured in the standard polypropylene multi-well tissue culture plates instead of the microfluidic compartmentalized chambers in the training data. As shown in Figure 3A, DMS273 cancer cells express a non-specific Tuj1 fluorescence signal, indicated by the yellow arrow. Our model identifies the neurite structure and generates the neurite mask as shown in Figure 3B, which, after application to the original Tuj1 image, the cancer cells and soma of the neurons are largely suppressed, as shown in Figure 3C.

3.4 | Neurite growth quantification

Neurite outgrowth is essential for the establishment of precise neural wiring.27 Here, we applied DeepNeurite™ to study the factors affecting neurite outgrowth. We first acquired confocal images of neurons labeled in microfluidic
compartimentalized chambers with various culture conditions. Figure 4A shows representative Tuj1 staining of neurons co-cultured with PC3 cancer cells. We then utilized DeepNeurite™ to generate the neurite structure mask as shown in Figure 4B to quantify neurite length (right side of the chamber). Next, we validated DeepNeurite™ using a positive control with application of nerve growth factor (NGF). As shown in Figure 4C, we observed an increase in neurite length after NGF treatment. This result is consistent with our expectations because NGF promotes neurite outgrowth through binding TrkA, leading to the subsequent activation of PI3K/Akt/GSK3β pathways.28-30 Next, we tested whether the presence of cancer cells promotes neurite growth. By comparing the neurite length quantified by DeepNeurite™, we observed that neurite growth is promoted in PC3 co-culture conditions compared to the neurons in monoculture conditions (Figure 4C). This increase is reflected in published observations showing that prostate cancer secretes a precursor for NGF, proNGF, which can be converted to NGF and recruits axon fibers to the tumor.31 As a negative control, we treated neurons with 400 nM anti-NGF in PC3 co-cultured conditions and observed the neurite length decreased compared with neurons in direct co-culture. This result suggests that in our assay, PC3 cancer cells may secrete NGF or proNGF to promote neurite growth.

3.5 | Application to examine neurons-cancer cell crosstalk

We applied our presented algorithm to explore the possibility of communication between neurons and cancer cells. As the most abundant second messenger, calcium plays an essential role in regulating proliferation, migration, and maturation.32,33 Routinely used in studies of neuronal activation, calcium indicators can be small molecules34 or generically encoded35 and both have been extensively used to characterize neuronal activity. Therefore, we decided to use calcium imaging as a functional readout to explore the possibility of neuronal activation of cancer cells in vitro. Neurons were co-cultured with cancer cells expressing GCaMP6m, a genetically encoded calcium indicator.23,36 KCl, which only stimulated neurons, was also added to the culture medium. Simultaneous calcium imaging revealed increased calcium responses in cancer cells closer in proximity to the neurites (Figure 5A). This suggests that neurons may communicate with cancer cells in a distance-dependent manner (Figure 5A). A standard deviation image is generated to indicate the calcium activities of each cell (Figure 5B); whereas a mean intensity image from a time series of calcium fluorescence intensity is generated to indicate the cancer cell location (Figure 5C). We plotted the calcium time series of two represented responding and silent cells indicated by the yellow and white arrows, respectively, in Figure 5B,C. As shown in Figure 5D, cells indicated by the yellow arrow showed active calcium activities with a higher standard deviation, whereas cells indicated by the white arrow show no calcium activities with a low standard deviation. We noted that the silent cell still obtains baseline calcium signal, which is higher than the background region (Figure 5D). The neurite mask is generated by our deep learning model with the input of PGP9.5 images as shown in Figure 5E. The soma structures in the PGP9.5 image are suppressed after applying the neurite mask (Figure 5F). A composite image of the neurite image, mean intensity
image of cancer, and standard deviation image of cancer cell is shown in Figure 5G. We then used this neurite mask to calculate the distance of cancer cell center to the nearest neurite structure. As shown in Figure 5H, we observed cancer cells closer to the neurite, showing a higher standard deviation of calcium activities compared to cancer cells further away from the neurite. The observation confirms the result that silent cells, such as cells indicated by the white arrows in Figure 5B,C, are further away from the neurite (Figure 5G), demonstrating that neurons communicate with DMS273 cancer cells through the close contacts of neurites and further induce downstream calcium activities.

4 | DISCUSSION

Our deep learning approach enables neurite identification by confocal fluorescence imaging without the interference of non-specific binding of fluorescent antibodies signal. In addition to identifying neurites stained by TuJ1 from PC3 cancer cells in microfluidic compartmentalized chamber co-culture condition where the model was trained, we demonstrated that our model is generally able to handle different imaging conditions, including different cancer cell lines (DMS273 cancer cell line), different neural markers (PGP9.5), different culture conditions (in petri dish), and images in different signal-to-noise ratios. Our model also successfully identifies the neurites from the soma of neurons even though the soma structure is not presented in the original training dataset. We noticed that image augmentation is essential to generalize the model, given that our model was trained in a relatively small dataset with a total of 120 images. Transfer learning could also be applied to further improve accuracy in different imaging conditions.

For the first application of our pipeline, we utilized our model to quantify the neurite growth in microfluidic compartmentalized chambers. As shown in Figure 2, DeepNeurite™ obtains high sensitivity and accuracy to identify the neurite structure. However, we observed large variability between the repeat experiments in Figure 4C. The variability may come from some biological factors, that is, the initial seeding condition of DRG neurons.
For the second application, we analyzed data from live calcium imaging experiments to see whether our tool could help uncover how neurons may interact with cancer cells in vitro. We used the genetically encoded calcium indicator, GCaMP6m, to report changes in free calcium inside cancer cells. It is well documented that neuronal activation can cause calcium influx in other neurons and in glial cells, as well as in muscle cells at the neuromuscular junction and in cardiomyocytes, and that genetically encoded calcium indicators can report on these transient changes in free calcium ions. We observed intracellular calcium responses of cancer cells while stimulating neurons (Figure 5). Using DeepNeurite™, we identified and segmented neurites from all images and observed that the proximity of a cancer cell to the closest neighboring neurite was correlated with the likelihood of calcium influx in response to neuronal activation. A picture that accurately represented the location of neurites in the system was critical to the study. Our result suggests that neurites communicate with cancer cells by a distance-dependent mechanism. There are reports of synaptic activation in cancers present in central nervous system, in part discovered through neuronal activation of calcium influx into glioma cells.3,4 The ability of neurons to directly cause calcium influx in peripheral tumors has so far been largely unknown. Unlike the chemical synapses described in glioma, here we see evidence of medium-range communication. We hypothesize that this phenomenon could be based on the release of cytokines or chemokines from neurites as well as local diffusion and detection by receptors on the cancer cells. Further study is needed to test this hypothesis.

The method we presented in this study opens a variety of opportunities to study exoneural biology, including accurate quantification of neurite growth as well as the study of crosstalk between neurites and other cell types. Since our model is general and open source, the algorithm can potentially be applied in clinopathological analyses to identify nerves from immunofluorescence images. In addition, we demonstrated a method to overcome the off-target and non-specific binding of fluorescence signals, which is a pervasive and common problem in fluorescence microscopy. We trained our model to identify neurites as a testing bed; in the future, the presented method could be extended to the other subcellular structures or cell types to improve feature identification despite the inherent off-target labeling of fluorescence probes.
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Conflict of interest

This work is funded and achieved by Cygnal Therapeutics, a Flagship Pioneering company.

Authors' Contributions

K-C. Huang and H. Dai designed the research; K-C. Huang analyzed the data; K-C. Huang designed the deep learning model; S. Lou and A. Fink performed the experiments on microfluidic compartmentalized chambers; C-C. Wang and J. Turner performed the experiments on calcium imaging. M. S. Thanawala performed the co-culture experiments in petri dishes. K-C. Huang, S. Lou, L. Ji, and M. S. Thanawala wrote the paper; M. Sadaghiani, P. Huang, and H. Dai supervised the project.

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