# AUTOMATED IMAGE PROCESSING TO QUANTIFY NEURITE GROWTH IN LUHMES HUMAN NEURONAL PRECURSOR CELLS

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## ABSTRACT

Chemicals that specifically inhibit human neurite outgrowth pose a hazard to the developing nervous system. Identifying such chemicals remains a major challenge in biological research. In response to the need for more efficient methods to identify potential developmental neurotoxicants, an image processing framework is presented that allows to automatically quantify neurite growth in LUHMES human neuronal precursor cells. For this purpose, a H-33342 staining is used in order to identify the outline of the nucleus of each neuronal cell. Based on this outline, a region growing approach is performed that expands the soma until an intensity threshold is reached, which allows to quantify the number of cells with neurites. The results demonstrate that our image processing framework can rapidly quantify chemical effects on neurite outgrowth. Concentration-response data for neurite outgrowth allows for the determination of the specificity of chemical effects on developing neuronal cells. Further studies will examine the utility of the approach for other cell-based assays of neurite outgrowth.

## 1. INTRODUCTION

Chemicals that inhibit human neurite outgrowth act as developmental neurotoxicants, but their identification remains a major challenge. There are thousands of chemicals commercially available, but relatively few have been adequately characterized with respect to their potential effects on human health. For example, of the 3000 high production volume chemicals (chemicals produced or imported into the United States at or above 1 million pounds per year), nearly half have no basic toxicity data available [1], and only 7% have a complete set of toxicity data, including developmental toxicity. In the absence of data, the risk of developmental neurotoxicity for these chemicals is unknown, but it is estimated to be high [2]. Accordingly, there is an increased public concern that exposure to chemicals in the environment may be partially responsible for the increased number of cases of neurological disorders in children and adults.

In this work, an image processing framework is presented to quantify the number of cells with neurites in Lund human mesencephalic (LUHMES) human neuronal precursor cells [3]. LUHMES cells are conditionally-immortalized non-transformed human fetal cells that can be differentiated to acquire a dopaminergic neuronal phenotype under appropriate growth conditions.

The compounds investigated in this work have previously shown to influence neurite outgrowth. Brefeldin A (BrefA) inhibits membrane trafficking and has shown to inhibit neurite outgrowth in human embryonic stem cell derived cells [4]. Cycloheximide (CHX) is an inhibitor of protein biosynthesis, which impairs axonal elongation [5], and Flavopiridol (Flav) inhibits Cycline dependent kinase 5, a kinase playing an important role in growth cones.

The labelling and detection methods applied to the cells in this work are all non-invasive. To allow extensive networking, the neuronal cells are grown at high density, which results in microscopy images that are more challenging to process from an image analysis point of view. During the course of the biological study, viable cells without neurites had been observed on some of the images recorded. In order to investigate at the single cell level if the compounds considered affect neurite outgrowth (independent of the triggering of overall cell death), an image processing system is needed that counts viable cells without neurites longer than the diameter of the cell body.

The image processing framework presented in this work allows to automatically identify viable cells which are assessed with respect to their neurite length. The system outputs the counts of neuronal cells without neurites below the specified threshold.

## 2. MATERIAL AND METHODS

In this section, the image acquisition and image processing methods for quantifying the number of cells with neurites in LUHMES human neuronal precursor cells are presented. The software platform KNIME is used as a basis for the image processing system, which allows for a versatile and flexible development process and quality assessment. The image processing methods utilized in this work comprise identification of viable cells in combination with a customized region growing algorithm. Finally, the cell scores for each class are presented for convenient evaluation.

### 2.1. Image data

For the experiment, Lund human mesencephalic (LUHMES) cells were used and microscopy images were generated on an Assay-Scan II High Content Screening Reader, Cellomics. For each cell image, a H-33342 staining was performed in order to clearly identify the nuclei of the neuronal cells. Imaging of the cell soma was performed using the vital dye calcein-AM, which allows to separate out dead cells that cannot transform and accumulate calcein.

Microscopy images were acquired for cell populations which had been treated with a high concentration of the potential neurotoxins, with a low concentration of the potential neurotoxins or with no substance at all (control images). Neuronal cells at the border of the microscopy image are automatically detected and labelled by the screening system, which allows to exclude them from the subsequent analysis.

### 2.2. Software framework

The image processing pipeline for quantifying the number of cells with neurites was developed using the software platform KNIME (The Konstanz Information Miner [6]), which is an open-source tool for data integration, processing, analysis and exploration. Essentially, KNIME ist designed to import, transform and visualize large data sets in a convenient and easy to use way. KNIME workflows consist of interacting nodes, which may each represent an algorithm, a single import routine or a visualization tool. The exchange of data between nodes is accomplished via data tables which are passed from one node to another by node connections. The graphical user interface makes it possible to construct workflows consisting of different nodes and their interconnection via a simple drag-and-drop mechanism. The data flow is visually represented by connections between the nodes, typically starting with a node to import the data, followed by one or more processing nodes and finally one or more output nodes. Recently, KNIME has been extended to provide basic image processing nodes such as image input/output and standard thresholding and segmentation algorithms.

The image processing workflow presented in this work consists of several custom KNIME nodes that are combined with standard image processing nodes. This concept allows to batch process large amounts of microscopy images and to automatically save the results. Additionally, due to the modular design of KNIME workflows, it is possible to assess intermediate results at every stage of the processing pipeline.

### 2.3. Quantification of neurite growth

In order to quantify the number of cells with neurites, the nuclei of the neuronal cells are extracted from the H-33342 images in a first step. For each neuronal cell, all pixels belonging to the nucleus are classified as seedpoints. The soma region is then grown from these seed points based on growing conditions, which take into account the pixel intensity and accumulated path lenght. In this way, the initial contour of the nucleus advances outwards towards the unlabelled space. For each pixel added to the expanding volume, the length of the path to the initial boundary of the nucleus is computed and stored.

Expansion of the volume terminates if either the accumulated length has reached a maximum threshold (e.g. the neurites have reached a length longer than the diameter of the cell body), or if the minimum intensity value that admits expansion (lower intensity threshold) is reached. In Figure 1, the user interface to control region growing is shown.

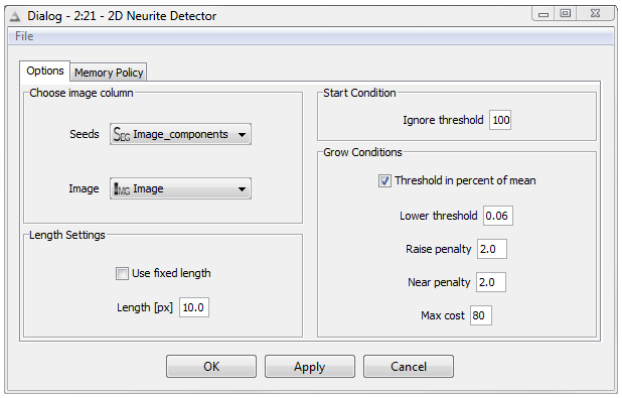


Figure 1. KNIME user interface to load the microscopy data, to set neurite length of interest, and to pre-define thresholds for region growing.

Region growing is performed for each neuronal cell on the microscopy image. Based on the path length values associated with each pixel during the growing process, it is possible to assess the neurite length for every neuronal cell. The length threshold specified in the user-interface (Figure 1) refers to the minimum length that needs to be observed in order to certify neurite growth, and is used to classify each cell.

Finally, the original image as well as the segmentation result of the nuclei and the soma are presented along with the total cell count and cell numbers for neurons showing neurite growth versus no growth. The entire KNIME processing pipeline for data analysis and visualization is presented in Figure 2.

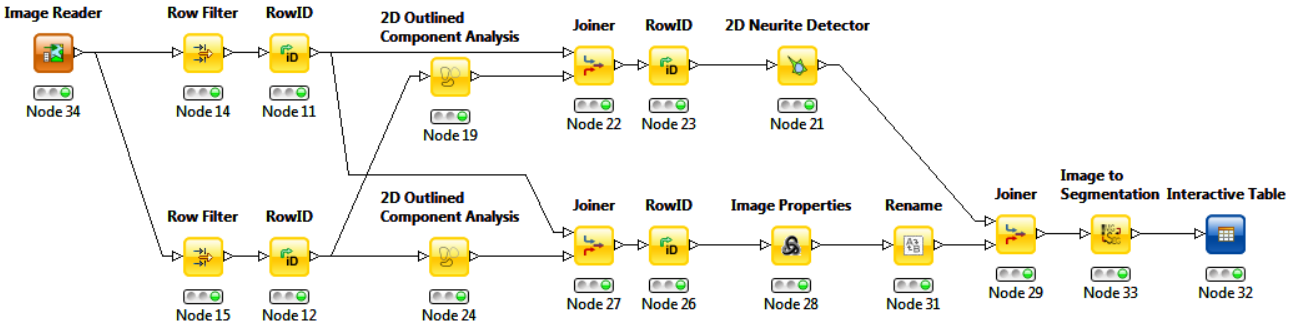


Figure 2. KNIME workflow for neurite growth data analysis. The pipeline comprises nodes for data preprocessing, detection of the outline of the nuclei, region growing for neurite growth detection, and visual representation.

## 3. RESULTS

The image processing framework was applied to 25 microscopy image pairs (512×512 pixels) of LUHMES human neuronal precursor cells treated with BrefA, CHX or Flavo. On a PC with an Intel Xeon W3540, 2.93GHz, 6 GB RAM, image analysis took 1 minute, 3 seconds.

Visual inspection of the segmentation and quantification results showed that the processing pipeline performs as expected. In Figure 3, microscopy images where a lower concentration *(upper row)* and a higher concentration *(lower row)* of CHX has been added are shown. In each row, analysis results for one particular microscopy image are displayed: The original calcein image is presented on the left, then the nuclei obtained from H-33342 staining are shown *(middle image)*, followed by the region growing result *(right image)*. The colors on the right image are randomized and delineate individual cells. The colors on the middle image indicate group membership, i.e. cells without *(light grey)* or with *(dark grey)* neurite growth. Quantitative results of cell counts are provided on the right: first of all, the total number of cells, followed by the number of viable cells with neurite growth below the specified length threshold, and the number of viable cells with neurite length above the threshold. Finally, the number of cells with a calcein intensity below a user-defined threshold is provided, which are ignored in the analysis.

| | #cells | #noGrowth | #growth | #ignored |
|---|---|---|---|---|
| $\text{BrefA}_{high}$ | 122 | 106 (97%) | 3 (3%) | 13 |
| $\text{BrefA}_{low}$ | 194 | 44 (23%) | 150 (77%) | 0 |
| Control | 132 | 16 (12%) | 115 (88%) | 1 |
| $\text{CHX}_{high}$ | 245 | 136 (56%) | 108 (44%) | 1 |
| $\text{CHX}_{low}$ | 252 | 83 (33%) | 169 (67%) | 0 |
| Control | 170 | 29 (17%) | 140 (83%) | 1 |
| $\text{Flavo}_{high}$ | 197 | 96 (49%) | 98 (51%) | 3 |
| $\text{Flavo}_{low}$ | 172 | 62 (37%) | 107 (63%) | 3 |
| Control | 254 | 32 (13%) | 220 (87%) | 2 |

Table 1. Results for BrefA, CHX and Flavo. Total number of viable cells (#cells) depending on whether the compound has been applied, and numbers of cells with (#growth) and without neurite growth (#noGrowth) (both total numbers and percentage).

## 4. DISCUSSION

The results of our automated image processing approach are very satisfactory and are in line with visual inspection. The analysis methodology presented in this work is hardly feasible with manual processing, which would be rater-dependent and error-prone due to bias introduced in the measurement of individual neurites. As shown in Table 1, the automated evaluation reveals that the LUHMES human neuronal precursor cells tolerate a low dose of BrefA, CHX or Flavo and still show neurite growth. Whether the thresholds that define neurite growth are biologically meaningful is a question beyond the scope of this work and will be investigated in future studies.

The quantitative differences of the measured growth in different microscopy images is within the expected scope and can be explained by natural variations of neurite growth, depending on the density of neurons and other environmental effects.

## 5. CONCLUSION

In this work, an automated system is presented for quantifying the number of cells with neurites in LUHMES human neuronal precursor cells. The processing pipeline comprises standard image processing algorithms in combination with a customized region growing approach and a specific scoring method to count neuronal cells without neurites. Identifying chemicals that act as developmental neurotoxicants is a major challenge in current research. Computational tools that facilitate the extraction of quantitative data from such experiments are therefore of great interest to the biology community.

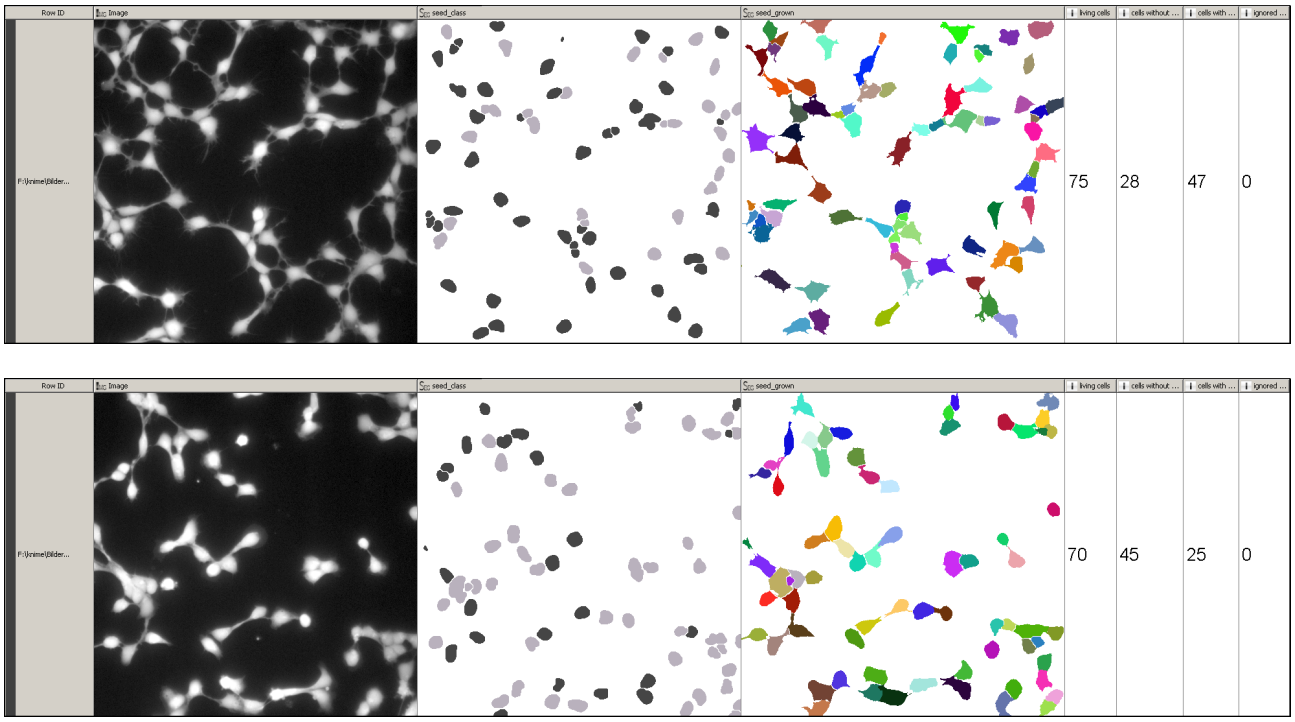


Figure 3. Low concentration of CHX *(upper row)*, high concentration of CHX *(lower row)*. In each row, the original calcein image *(left image)*, the cell nuclei based on H-33342 *(middle image)*, and the region growing result *(right image)* are shown. The numerical values on the rigth indicate the total number of cells, the number of viable cells with neurite growth below and above the specified length threshold, and the number of cells ignored in the analysis.

## 6. REFERENCES


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