AN ADVANCED IMAGE PROCESSING APPROACH BASED ON PARALLEL GROWTH AND OVERLAP HANDLING TO QUANTIFY NEURITE GROWTH

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ABSTRACT

Methods to assess neurite growth in populations of neuronal cells are required for many applications in biological image analysis. In response to the need for efficient methods to assess neurite growth, we have previously proposed an image processing framework to quantify the number of viable cells and the extent of neurite growth [1]. The approach is based on region growing and uses cost penalties and an upper cost limit to ensure that neurite outgrowth is not overestimated. However, these thresholds need to be defined manually and are set to a fixed value for the entire image. Also, the approach is not able to account for overlapping neurites in dense cell populations. For this reason, we propose two extensions to overcome the aforementioned shortcomings: By growing all regions originating from individual cell nuclei simultaneously, the approach adapts to the underlying microscopy image and doesn't require manually defined cost limits. An overlap handling is introduced, which is particularly valuable in dense cell populations with overlapping neurites. The results demonstrate that our advanced image processing approach generates results which are even closer to the manual ground truth.

1. INTRODUCTION

The quantitative and rapid analysis of large numbers of image data is one major bottleneck of high-throughput fluorescence microscopy assays. Existing tools provided by the manufacturers of automated microscopes and of integrated high content imaging systems usually do not deliver satisfying results due to the large variability of the tested biological systems. For this reason, there is a need for custom tools optimized for the analysis of specific microscopy endpoints.

Neurite outgrowth is a hallmark phenotype for de- and regeneration in the nervous system. Due to its robustness and sensitivity it is an endpoint of choice for the in vitro testing of toxic chemicals, in particular those suspected to affect neuronal development. For example, of the 3000 high production volume chemicals (chemicals produced or imported into the United States at or above one million pounds per year), nearly half have no basic toxicity data available [2], 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 [3]. 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.

Lund human mesencephalic (LUHMES) cells are a fetal human mesencephalic cell line which has been established as a general human neuronal cell model [4]. They are easy to handle and differentiate in vitro into highly homogenous cultures of mature dopaminergic neurons. LUHMES cells are thus particularly well suited for large scale testing of neurotoxicants in single-cell based assays.

In previous work, we have established a high-throughput live cell imaging system for identifying neurotoxic agents [5], which enables quantifying the overall neurite mass as well as cell viability in differentiated LUHMES cultures. We presented an image processing framework to compute the number of viable cells with and without neurite growth in these images [1]. This approach is based on region growing performed for one cell at a time. In order to make sure that neurite outgrowth is not overestimated as shown in Figure 1 *(left)*, cost penalties and an upper cost limit are employed to ensure that the region growing maintains a minimum distance to other cell nuclei. However, these thresholds need to be defined manually and are set to a fixed value for the entire image. Also, the approach was not able to account for overlapping neurites.

In this work, we propose an advanced approach which comprises two extensions to overcome the aforementioned shortcomings. By growing all regions originating from individual cell nuclei simultaneously, as shown in Figure 1 *(right)*, the approach adapts to the underlying microscopy image and doesn't require manually defined cost limits.

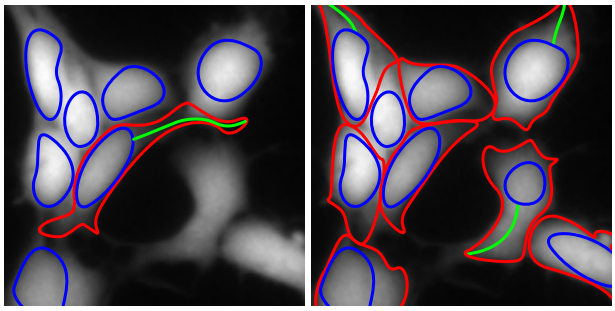

Figure 1. *Red:* Grown region. *Green*: Path with maximum length. *Left:* Neurite growth according to [1] grows one region at a time. Cost penalties stop growth when approaching other nuclei. *Right:* Proposed algorithm, grows all nuclei simultaneously, no cost penalties needed.

Furthermore, an overlap handling is introduced, which is particularly valuable in dense cell populations with overlapping neurites.

The microscopy images used in this work were acquired in a toxicity study of U0126, which has previously been shown to influence neurite outgrowth: U0126 is a potent inhibitor of the mitogen-activated protein kinase (MAPK) pathway, which is involved in regulating neurite growth. MAPKs have been implicated in a variety of cellular functions, including neuronal differentiation [6].

In this work, only non-invasive labelling and detection methods are applied. The neuronal cells are grown at high density to allow extensive networking, which results in microscopy images that are challenging to process from an image analysis point of view. In order to investigate if the considered compound affects neurite outgrowth at the single cell level, an image processing system is needed that counts viable cells with and without neurite growth.

The presented image processing framework allows quantifying the number of cells with extensions longer than one cell diameter (defined here as neurites) and outputs the counts of neuronal cells with and without neurites. Compared to [1], this advanced approach doesn't require manually defined cost limits for region growing and is also able to account for cells with overlapping neurites, which is particularly important for cell populations grown at high density. Results show that the proposed advanced approach follows the manual ground-truth more closely and is therefore preferred by the biologists.

2. MATERIAL AND METHODS

2.1. Image data

Microscopy images of LUHMES cells were acquired on an Assay-Scan II High Content Screening (HCS) System, Cellomics. In order to clearly identify the nuclei, neuronal cells were stained with the DNA dye H-33342. Imaging of the cell shape region (cytoplasm), including the cell body (soma) and its extensions (neurites), was performed using the vital dye calcein-AM. Since dead cells cannot accumulate and retain this dye in their cytoplasm, the calcein channel is also used to exclude dead cells from further analysis.

Microscopy images were acquired for cell populations which had been treated with control medium or increasing concentrations of the test compound. Partly visible neuronal cells at the border of the microscopy image are automatically detected and labelled by the screening system and excluded from subsequent analysis.

2.2. Software framework

The presented approach was developed using the software platform KNIME (The Konstanz Information Miner [7]), which is an open-source tool for data integration, processing, analysis and exploration. Essentially, KNIME workflows consist of interacting nodes which exchange data via data tables which are passed from one node to another according to their 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 advanced image processing workflow presented in this work consists of several custom KNIME nodes that extend the workflow presented in [1].

2.3. Quantification of neurite growth

The approach for quantifying the number of cells with neurites presented in [1] as well as the advanced method introduced in this work comprise two general steps: First, the nuclei of the neuronal cells are segmented from the H-33342 images, and for each neuronal cell all pixels belonging to the nucleus are classified as seedpoints. Secondly, the cytoplasm region of viable cells is then grown from these seed points to expand the initial contour of the nucleus outwards. For each pixel added to the expanding volume, the length of the path to the initial boundary of the nucleus is computed and stored.

The approach presented in this work has two major extensions, which are outlined in the following:

Parallel growth: The approach operates in the style of the Dijsktra algorithm [8], which is commonly used in computer science for different types of search problems. It builds up a graph with nodes (corresponding to pixels) and edges, where the edges are assigned a local cost corresponding to the Euclidean distance between pixel centers plus the normalized inverted intensity. For performing the search, the algorithm maintains two lists, an open list comprising all pixels currently under consideration and a closed list containing pixels that have already been visited. In the beginning, the open list comprises all pixels at the border of every cell nucleus and the closed list is empty. Each node c stores the accumulated cost required to travel along the path to the respective node, the accumulated path length, the previous node and the nucleus from which the node was reached. In each iteration, the algorithm selects the node with lowest accumulated cost from the open list, adds all neighbor pixels n with an intensity value above the cytoplasm intensity threshold t_{cyto} to the open list and moves the selected node to the closed list. If

the path length exceeds the length threshold $l_{\min}$, the corresponding nucleus is marked as grown. These processing steps continue until the open list is empty.

Overlap handling: Although parallel growth eliminates the need of a cost penalty, it is too restrictive in case of cell clusters with overlapping neurites. The proposed overlap handling softens the region border and introduces a permeable area were regions from multiple nuclei can overlap. Algorithm 1 shows the pseudocode of the overlap handling. The algorithm maintains a global open list O. Each individual node c on the open list keeps track of the accumulated distance, cost and which nuclei reached and closed the node. In each iteration, the algorithm selects the node with lowest accumulated cost from the open list, adds all neighbor pixels n with an intensity value above t_{cyto} to the open list and marks the current node as partly closed. If a node is only partly closed it can be reopened for another nucleus. The permeable area is updated by backtracking and completely closing all nodes with a distance to the open list greater than l_{offset}. This permeable area makes it possible for neurites to overlap to a certain, user-defined extent.

These extensions narrow down the parameter set to the neurite length threshold $l_{\min}$, the intensity threshold t_{cyto} and the permeable area offset l_{offset}.

while $O \neq \emptyset$ **do**
  $c \leftarrow$ `removeBest(`O`)`
  if $c.dist > l_{\min}$ **then** $State[c.nuc] \leftarrow$ "grown"
  foreach $n \in$ `neighbors(`c`)` **do**
    if $c.nuc \in n.Closed$ **then** `continue`
    if $n.intensity < t_{\text{cyto}}$ **then**
      `backtrackCloseCompletely(`$c, 0$`)`
      `continue`
    if `isClosedCompletely(`$n.nuc$`)` **then**
      `continue`
    $cost \leftarrow c.cost + |\overrightarrow{c,n}| + \gamma(c.intensity)$
    $dist \leftarrow c.dist + |\overrightarrow{c,n}|$
    if $n.Closed \neq \emptyset$ **then**
      if $dist + n.dist > l_{\min}$ **then**
        $State[c.nuc] \leftarrow$ "grown"
        $State[n.nuc] \leftarrow$ "grown"
    if $n \in O$ **then**
      if $n.cost > cost$ **then**
        $n.cost, n.dist \leftarrow cost, dist$
        $n.nuc, n.Path[c.nuc] \leftarrow c.nuc, c$
    else
      $n.cost, n.dist \leftarrow cost, dist$
      $n.nuc, n.Path[c.nuc] \leftarrow c.nuc, c$
    $O \leftarrow O \cup \{n\}$
  `backtrackCloseCompletely(`c, l_{offset}`)`
  $c.Closed \leftarrow c.Closed \cup \{c.nuc\}$

Algorithm 1: A node c holds intensity $c.intensity$, nucleus $c.nuc$, cost $c.cost$, distance $c.dist$, parent nodes $c.Path$. The function γ computes the normalized inverted intensity and l_{offset} controls the local offset of the permeable area. Lower case variables hold scalar values, upper case variables refer to lists.

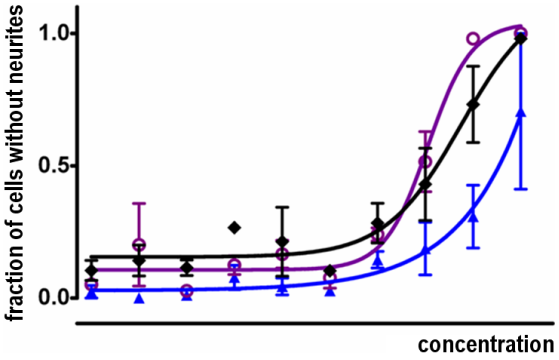

Figure 2. Decreasing neurite outgrowth for U0126. *Purple:* Manual ground truth. *Blue:* Automated method [1]. *Black:* Proposed advanced method.

3. RESULTS

The image processing framework was applied to microscopy images (two-channel images, 512×512 pixels per channel) of LUHMES human neuronal precursor cells treated with U0126. On a PC with an Intel Xeon W5130, 2 GHz, 4 GB RAM, image analysis took 2 minutes, 27 seconds for ten concentration levels and three microscopy images per level.

In Figure 2, a direct comparison of the automated approach proposed in [1] *(blue)* and the advanced method presented in this work *(black)* is provided. Both approaches are compared against a manual groud truth *(purple)*, for the chemical U0126. The x-axis denotes an increasing concentration of the chemical, the y-axis the decrease of neurite outgrowth. The curve obtained from the advanced method follows the manual ground truth more closely. This is also reflected by computing the difference between manual evaluation and either method for the measured concentrations for each of the ten concentration levels. The difference between mean values of the manual evaluation and the automated analysis [1] amounts to an average value of 0.185, compared to 0.081 in case of the proposed advanced approach versus manual ground truth, respectively.

In Figure 3, a visual result of the implemented method is provided. Red nuclei indicate dead cells according to the calcein staining. Green corresponds to cell nuclei with neurite growth, and grey to nuclei without neurite growth according to the analysis. For each cell with neurite growth, the maximum path length between the border of the nucleus and the border of the cytoplasm is indicated by a green line. Pixels that have been visited by the algorithm during front propagation are denoted by dark green. The maximum extension of each region grown from the outline of its nucleus is outlined by a red rim, which may disappear in case of overlapping neurites, though.

4. DISCUSSION

The development of automated methods to assess the effects of treatment with toxic substances is challenging, particularly at low concentrations which defines the sen-

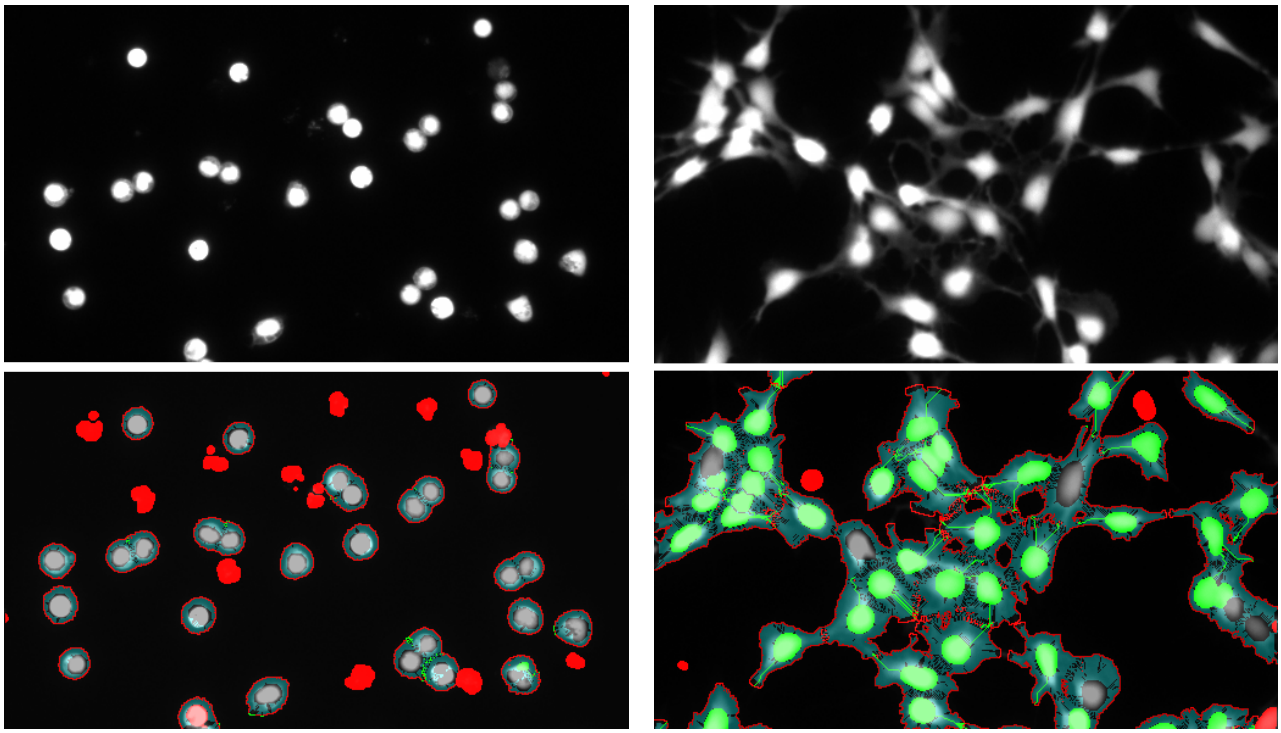

Figure 3. Neuronal cells without *(left)* and with *(right)* neurite growth. Original images *(upper row)* and image analysis result *(lower row)*. Red nuclei correspond to dead cells, green nuclei to cells with neurite growth, and grey nuclei to viable cells without neurite growth. In case of neurite growth, the maximum distance between nucleus and boundary of the cytoplasm is indicated by a green line. Pixels that have been visited by the algorithm are denoted by dark green. The red rim delineates the boundary of the maximally dilated regions after growing from individual nuclei. In case of overlapping neurites this boundary disappears.

sitivity of the toxicity test. The neurons naturally tend to form clusters, which makes it difficult to detect the length of single neurites.

The result obtained by the proposed algorithm is a binary decision whether a cell exhibits neurite growth or not. According to Figure 2, the result is very close to the human ground truth, even for low concentrations of the neurotoxicant, and also reaches the same absolute extent of neurite growth inhibition for the maximum concentration of U0126.

5. CONCLUSION

In this work, an advanced system is presented that outputs the counts of neuronal cells with and without neurites in LUHMES human neuronal precursor cells. The results demonstrate that our advanced image processing approach can reliably quantify chemical effects on initial neurite outgrowth. Compared to the manual ground truth, improved results were obtained with the advanced approach presented in this work. Identifying chemicals that act as developmental neurotoxicants is a major challenge in current research. Computational tools that facilitate the extraction of quantitative data from respective experiments are therefore of great interest to the biology community.

6. REFERENCES

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