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Automated Detection of Retinal Cell Nuclei in 3D Micro-CT Images of Zebrafish using Support Vector Machine Classification

Yifu Ding, Thomas Tavalara, Keith Cheng*

Jake Gittlen Laboratories for Cancer Research and Department of Pathology, Penn State College of Medicine, Hershey, PA, USA 17033

Abstract

Our group is developing a method to examine biological specimens in cellular detail using synchrotron microCT. The method can acquire 3D images of tissue at micrometer-scale resolutions, allowing for individual cell types to be visualized in the context of the entire specimen. For model organism research, this tool will enable the rapid characterization of tissue architecture and cellular morphology from every organ system. This characterization is critical for proposed and ongoing “phenome” projects that aim to phenotype whole-organism mutants and diseased tissues from different organisms including humans. With the envisioned collection of hundreds to thousands of images for a phenome project, it is important to develop quantitative image analysis tools for the automated scoring of organism phenotypes across organ systems. Here we present a first step towards that goal, demonstrating the use of support vector machines (SVM) in detecting retinal cell nuclei in 3D images of wild-type zebrafish. In addition, we apply the SVM classifier on a mutant zebrafish to examine whether SVMs can be used to capture phenotypic differences in these images. The long-term goal of this work is to allow cellular and tissue morphology to be characterized quantitatively for many organ systems, at the level of the whole-organism.

Keywords

synchrotron micro-computed tomography; support vector machines; cell nuclei detection; zebrafish

1. INTRODUCTION

With the advent of next-generation sequencing and genome editing technologies, it has become possible to study genes in a high-throughput manner. As the capacity to genotype increases, investigators have recognized a need for more precise phenotypes to describe and quantify genetic variants. There have been proposed and ongoing “phenome” projects in both humans and model organisms that aim to study phenotype in a high-throughput manner similar to genomics [1–3]. There has been research into high-throughput whole-body and

* kcheng76@gmail.com; phone: 717-531-5635.

whole-organ phenotyping of model organisms using histology, commercial small animal microCT, and magnetic resonance (MR) histology [4–6]. However, these methods are either two-dimensional (histology), or do not have an adequate resolution to see individual cell types (small animal microCT and MR histology).

Our group has been developing the use of synchrotron microCT for high-throughput phenotyping of zebrafish [7,8]. Currently, this method can achieve micrometer-scale isotropic resolution of tissue specimens. As seen in Figure 1, the 3D images capture the whole organism with an adequate resolution for visualization of individual cells. This allows cell and tissue morphology to be seen in the context of the whole organism. The inherently digital and 3D nature of the acquired image volumes enables the development of automated tools to characterize and quantify organism phenotype.

In this work, we apply a supervised learning model using support vector machine (SVM) classification to automatically detect retinal cell nuclei from wild-type zebrafish larvae. The zebrafish vary in age from 3–5 days post-fertilization. We show that an SVM classifier with good performance can be trained from a small number of sub-images. We also show that a trained SVM classifier can detect nuclei in image volumes that were not part of the training set. Finally, we apply the SVM classifier on a mutant zebrafish to demonstrate the feasibility of using this approach to automatically characterize organism phenotype.

2. METHODS

Sacrificed 3-, 4-, and 5-day post-fertilization (dpf) wild-type zebrafish samples were fixed in formalin, stained with phosphotungstic acid (PTA), dehydrated, and placed in a polymer resin. Synchrotron microCT images were acquired from Beamline 2-BM at Advanced Photon Source in Argonne National Laboratory (Lemont, IL). As seen in Figure 2, the left and right eye regions of each image were extracted to serve as the starting point of automated analysis.

SVMs [9] are a popular approach in machine learning for supervised classification of data. They have been used in a wide variety of applications including handwriting recognition and pedestrian detection [10,11]. In pathology, SVMs have been used for fibroblast cell nuclei detection and colon tissue classification [12,13]. Given a set of n training examples, $(\vec{x}_1, y_1) \dots (\vec{x}_n, y_n)$, where $\vec{x}_i \in R^p$ and $y_i \in \{-1, +1\}$, the SVM classifier aims to produce a separating hyperplane that gives the maximum margin between $\vec{x}_i$ points where $y_i = 1$ and $\vec{x}_i$ points where $y_i = -1$. The SVM classifier can be constructed to allow for incorrectly classified (soft-margin) and non-linear data (kernel trick) [14]. The decision function from a soft-margin classifier utilizing the kernel trick takes the form:

$$f(\vec{x}) = \text{sgn} \left[\sum_{i=1}^n \alpha_i y_i K(\vec{x}, \vec{x}_i) + b \right] \quad (1)$$

where, $\vec{x}$ is the point to be classified, α_i are coefficients computed through quadratic programming, b is the offset derived from the training data, and $\vec{x}_i$ and y_i are the training

vectors and corresponding class labels. Here, $K(\vec{x}, \vec{x}_i)$ represents the kernel. For this work, we use the radial basis function (RBF), $K = \exp\left(-\gamma\|\vec{x} - \vec{x}_i^T\|^2\right)$, kernel. The kernel parameter γ defines the influence of each training point. In addition, SVMs have a general C parameter that balances between misclassification and a smooth decision boundary. The grid search method was employed to find the optimal parameters in the parameter space, and *k-fold* cross-validation with $k = 5$ was used to guide parameter selection [15].

The SVM is trained by providing the classifier with positive and negative examples of cell nuclei. In our case, we initially identified 100 nuclei and 100 non-nuclei in each wild-type zebrafish eye. The identified nuclei were from retinal cells. Epithelial cell nuclei near the eye lens were classified as non-nuclei. Through an iterative process we identified an additional 100 nuclei and non-nuclei examples from false negative and false positive outputs of the SVM. For the input feature vector, $\vec{x}_i$, we were interested in 1) whether raw pixel values or gradient edge information or both would produce a better classification, and 2) how big of a window around each nucleus was necessary for the best classification.

We were also interested in how well the SVM classifier would generalize to detect nuclei on new images. We performed experiments where the SVMs were trained without training data from certain images. The withheld data were then used to test the performance of the SVM. Finally, we used a trained SVM to detect nuclei on a mutant zebrafish, *huli-hutu* (*hht*) that has deficient DNA polymerase function [16]. We compare the results of retinal nuclei detection between wild-type and *hht* zebrafish to demonstrate the feasibility of using this approach to automatically characterize eye phenotype in zebrafish mutants.

3. RESULTS

The manually classified training data composes of *x*-, *y*- and *z*-coordinates for the center of a retinal nuclei. We first looked for an optimal way to convert these coordinates into input feature vectors for training. We compared different grid sizes around the retinal nuclei coordinate. The retinal nuclei in these images range in size from 2–6 voxels (~1.5–4.5 μm) in diameter, with most nuclei falling within 3–5 voxels (~2.0–3.5 μm) in diameter. A range of cubic grid sizes was tested to determine the optimal number of voxels around the retinal nuclei center to place in the input vector. In addition, we wanted to determine whether raw voxel values, gradient edge information, or a combination of both would be optimal for the input vector. The parameters were explored as training data for the SVM. An exhaustive search of C and γ was done to find the optimal tuning parameters.

As shown in Figure 3, a grid size of 3 voxels or greater resulted in greater than 90% accuracy for the SVM classifier. In addition, the use of raw voxel values, with or without gradient information resulted in the best SVM classifier performance. For our experiments, we use a feature vector that contains both the raw voxel and gradient edge information, making the feature vector size $2m^3$, where m is the grid size. Small grid sizes were chosen (3 or 5 voxels) to decrease the size of the input vector and speed up training of the SVM.

The SVM classifier's performance was also qualitatively evaluated. First, a SVM classifier was trained using the manually classified training data (700 nuclei and 700 non-nuclei training examples spread across 6 extracted eye regions). The trained SVM was then used to detect nuclei in the left eye of the 5-dpf zebrafish. Each voxel in the image was evaluated by the SVM as either nuclei or non-nuclei. For a voxel in the 5-dpf left eye image, a cube with edge length m was created around the voxel. This cube (and the corresponding cube on the gradient image) was used as the input vector for the SVM. The original voxel in the image is given the value predicted from the SVM classifier. This process is repeated for each voxel in the image, and the original image is zero-padded so that input vectors can be computed for voxels on the edge of the image. As can be seen in Figure 4, the SVM classifier has similar performance in retinal nuclei detection with a $3 \times 3 \times 3$ voxel or a $5 \times 5 \times 5$ voxel grid size. In both the coronal and sagittal cross-section, the smaller grid size input misclassifies more of the eye lens as retinal cell nuclei.

Next, the SVM classifier trained with the $5 \times 5 \times 5$ voxel grid size in the previous example was used to detect retinal nuclei in each extracted eye image. From Figure 3, this set of inputs as the feature vector has an accuracy around 95%, after 5-fold cross-validation. In Figure 5, a sagittal cross-section of each eye, along with the nuclei detection results are displayed. An overlay of the detection results on the original cross-section is also shown.

From the results, the SVM classifier does a good job detecting retinal nuclei in the ganglion cell layer that compose the inner ring of nuclei of each image. The classifier is less successful at detecting nuclei of the amacrine and bipolar retinal cell layers that compose the large outer ring of nuclei in each image. For example, there are gaps in the detected nuclei for the outer ring in the in both 3-dpf eye images (at 7 o'clock for 3-dpf left and 11 o'clock for 3-dpf right). A possible explanation for the gaps in the images is that the cell nuclei in these images are smaller because the zebrafish are less developed. Adding more training examples of these small cell nuclei could alleviate some of the gaps.

The trained classifier also does a good job of classifying the eye lens and inner plexiform layer (large dark band) of the eye as non-nuclei. In addition, it classifies most of the photoreceptor layer (thin, outer most layer of cells) in each image as non-nuclei, although a small number of photoreceptors are still classified as nuclei in a few eye images. The photoreceptor layer was manually classified as non-nuclei in the training set, as it has oblong-shaped cells that are significantly different from the round, spherical cell nuclei found in the other layers of the retina. Overall, the SVM classifier does a good job at detecting retinal nuclei in these images, although its performance could be improved.

Next, we examined whether a trained SVM classifier could be generalized to detect retinal nuclei in images that did not contribute to the training data. For true automated detection of nuclei, a previously trained SVM classifier should be used to classify new data, without the need to manually select training examples in the new image. This also ensures that the trained SVM classifier is not overfitting the training set.

The accuracy of SVM classifiers that were trained using only a subset of the available eye images is shown in Table 1. For each image in the training set, both the extracted left and

right eye of the zebrafish were used to train the classifier. The classifier was then used to classify test cases (previously classified positive and negative examples) in new images. The results from these test cases were then evaluated for accuracy by comparing them to their manual classification. The accuracy of the SVM classifier on its own training data after parameter optimization and cross-validation is also reported (in *italics*). A $5 \times 5 \times 5$ voxel grid size was used for both SVM training and evaluation.

Overall, the SVM classifiers that were trained with examples from only one image did not perform well on detecting nuclei in the other images (accuracy $\sim 80\%$), with one exception. The classifier trained using only 4-dpf zebrafish showed very good performance at detecting nuclei in the 3-dpf zebrafish. However, the same classifier also performed poorly in detecting nuclei for the 5-dpf zebrafish. A possible explanation for this discrepancy is that the manually classified data for the 3-dpf and 4-dpf zebrafish are more similar than the data for the 5-dpf zebrafish. The size distribution of the retinal nuclei in the 5-dpf zebrafish is different than the nuclei size distribution for the 3-dpf and 4-dpf zebrafish (e.g. there are more numbers of larger and developed nuclei in the 5-dpf zebrafish). The poor performance could also be the result of variation in the image intensity introduced during specimen preparation or image acquisition. As shown in Figure 2, 5-dpf zebrafish eyes are overall darker than the 3-dpf and 4-dpf eyes (the images have the same window/level settings). Finally, it is also possible the manually classified nuclei in the 3-dpf zebrafish are not a representative sample of all the 3-dpf nuclei. In this case, more training examples will be needed to ensure a representative sample. The performance of the SVM classifier improved when retinal nuclei examples from two zebrafish images were used for training data, as the nuclei were detected with around 90% accuracy.

Next, as shown in Figure 6, we use a previously trained SVM classifier to detect retinal nuclei in both wild-type and mutant 3-dpf zebrafish. These 3-dpf eye data sets were not part of the SVM training. The mutant, *huli-hutu* (*hht*), has deficient DNA polymerase function, resulting in apoptotic cells in the eye region. When compared to the results in Figure 5, which used all available examples in the training data, the SVM classifier does a good job at detecting nuclei in the ganglion cell layer (inner ring) and a fair job at detecting nuclei in the amacrine and bipolar cell layer (outer ring) in 3-dpf wild-type zebrafish. The same classifier also detected nuclei in the 3-dpf *hht* zebrafish. A comparison between the wild-type and mutant zebrafish shows that there are considerably fewer nuclei in the mutant zebrafish eyes, and the existing nuclei in the mutant eyes are disordered. The results show the feasibility of using a trained SVM classifier to automatically detect nuclei and acquire phenotypic information of an organism.

4. DISCUSSION

The use of synchrotron microCT to image biological specimens with cellular resolution in 3D allows for changes in cell and tissue morphology to be visualized in the context of the larger sample. For model organism research, this imaging method enables the phenotyping of whole-body or whole-organ samples. However, phenotyping the hundreds to thousands of anticipated whole-organism samples needed for a phenome project requires the development of automated tools. Here, we have demonstrated the feasibility of using support vector

machine classification to detect retinal nuclei in synchrotron microCT images of zebrafish larvae.

The results of the retinal nuclei detection show that the SVM classifier does a satisfactory job in finding nuclei, especially when training examples are available from the image. We showed that using feature vectors with raw voxel value inputs (with or without edge information) and with a $3 \times 3 \times 3$ voxel or larger grid size resulted in trained SVM classifiers with high quantitative accuracy. While the quantitative accuracy for the SVM classifier is high, qualitative analysis shows that the final detection results can still be improved. Nuclei detection is more accurate for some zebrafish images than others. In addition, specific regions of the zebrafish eye are detected with higher accuracy than other regions. These results suggest that while nuclei detection using SVM classifiers is promising for synchrotron microCT images of zebrafish, additional training examples, along with increased validation (i.e. sensitivity and specificity analysis) are required to train a robust SVM classifier that detects cell nuclei with acceptable quantitative and qualitative accuracy.

Automated detection is the first step for further analysis of organism/specimen phenotype. In this case, segmentation of the cell nuclei after detection will allow for cellular morphology (cell nuclei size and shape) to be quantified and compared between zebrafish eye samples. Along with detection and segmentation, the cell nuclei can also be visualized in 3D from synchrotron microCT acquisitions. In Figure 7, we show a maximum intensity projection (MIP) of the results of retinal nuclei detection from the 5-dpf zebrafish. These visualizations can provide insight about the location and distribution of cell nuclei in the 3D image. Ongoing research will explore the use of machine learning methods to detect other cell and tissue types in synchrotron microCT images.

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Figure 1:

Example image of fixed and stained 5-day post-fertilization (dpf) zebrafish obtained from synchrotron microCT. Sagittal (a) and transverse (b) views of the **same** zebrafish are shown. Histology of 5-dpf zebrafish with the same views is provided for comparison (c-d). Due to the physical cutting of the specimen, the histology samples are from **different** zebrafish.

Figure 2:
Extracted left and right eye regions from 3-, 4-, and 5-dpf zebrafish used for automated analysis.

Figure 3:

The effect of grid size on SVM classification accuracy. Varying grid sizes were tested to determine the optimal size for the input vector. Grid sizes of 3 voxels (per side) or larger had good accuracy. In addition, the use of raw pixel values either alone or with gradient information resulted in the highest accuracy.

Figure 4:

Qualitative comparison of the effect of grid size on retinal cell nuclei detection in a 5-dpf zebrafish. SVM classifiers trained using either grid size have similar performance. However, the smaller grid size misclassifies more of the eye lens as retinal nuclei.

Figure 5:

Results of nuclei detection using the trained SVM classifier with a 5 voxel grid size for the input feature vector. The classifier does very well at detecting nuclei in the inner ring (ganglion cell layer) of all the images. In the 4-dpf and 5-dpf zebrafish, the classifier also does well in detecting the large outer ring of retinal nuclei (amacrine and bipolar cells). The classifier does not detect the some outer ring cell nuclei in the 3-dpf zebrafish. Overall, the classifier does a good job with nuclei detection, and the inclusion of more training data (especially from the 3-dpf zebrafish) should improve its performance.

Figure 6:

Results of nuclei detection using a previously-trained SVM classifier on 3-dpf wild-type and mutant (*hht*) zebrafish. The mutant has deficient DNA polymerase function, resulting in apoptotic cells in the eyes. From the detected nuclei images, the mutant zebrafish has fewer nuclei, found in a disordered arrangement.

Figure 7:
Maximum intensity projection (MIP) of detected retinal cell nuclei in wild-type 5-dpf zebrafish eyes. The inner ganglion cell layer and the outer amacrine and bipolar cell layers can be seen. The location and distribution of the retinal cell nuclei can be appreciated in 3D.

Table 1:

Effect of Different Training Sets on SVM Classifier Accuracy

Training Set	Accuracy		
	3-dpf	4-dpf	5-dpf
3-dpf	<i>0.97</i>	0.88	0.81
4-dpf	0.96	<i>0.97</i>	0.73
5-dpf	0.85	0.84	<i>0.94</i>
3-dpf + 4-dpf	<i>0.97</i>	<i>0.97</i>	0.87
3-dpf + 5-dpf	<i>0.95</i>	0.91	<i>0.95</i>
4-dpf + 5-dpf	0.96	<i>0.95</i>	<i>0.95</i>