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Computer aided classification of cell nuclei in the gastrointestinal tract by volume and principal axis

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Abstract

Normal function of the gastrointestinal tract involves the coordinated activity of several cell types. Human disorders of motor function of the gastrointestinal tract are often associated with changes in the number of these cells. For example, in diabetic patients, abnormalities in gastrointestinal transit are associated with changes in nerves and interstitial cells of Cajal (ICC), two key cells that generate and regulate motility. ICC are cells of mesenchymal origin that function as pacemakers and amplify neuronal signals in the gastrointestinal tract. Quantifying the changes in number of specific cell types in tissues from patients with motility disorders is challenging and requires immunolabeling for specific antigens. The shape of nuclei differs between the cell types in the wall of the gastrointestinal tract. Therefore the objective of this study was to determine whether cell nuclei can be classified by analyzing the 3D morphology of the nuclei. Furthermore, the orientation of the long axis of nuclei changes within and between the muscle layers. These features can be used to classify and differentially label the nuclei in confocal volume images of the tissue by computing the principal axis of the coordinates of the set of voxels forming each nucleus and thereby to identify cells by their nuclear morphology. Using this approach, we were able to separate and quantify nuclei in the smooth muscle layers of the tissue. Therefore we conclude that computer-aided classification of cell nuclei can be used to identify changes in the cell types expressed in gastrointestinal smooth muscle.

Keywords

feature extraction; confocal microscopy; cell differentiation; principal component analysis

1. Introduction

The breakdown, mixing and movement of content through the gastrointestinal tract is due to the complex motor behavior of the muscle wall of this organ. This motor behavior is referred to as gastrointestinal motility and is due to motor patterns that are mostly generated by cells intrinsic to the muscle wall including enteric neurons, enteric glia, smooth muscle cells and interstitial cells of Cajal (ICC). Pacemaker potentials generated in ICC and programmed reflexes in enteric neurons are the primary generators of the motor patterns in the gastrointestinal tract. Smooth muscle cells are both the target for these signals and

function as sensors of stretch and movement¹. Damage or loss of any of the cell types in the gastrointestinal tract will disturb motility. For example, abnormalities in gastrointestinal transit is a complication of diabetes in humans² and is associated with loss of inhibitory enteric neurotransmission as well as ICC³. The same loss of nitric oxide synthase-containing inhibitory nerves and ICC is also observed in diabetic mice that have changes in gastrointestinal transit^{4, 5}.

Quantifying changes in the distribution and number of different cell types in a complex tissue such as the muscle wall of the gastrointestinal tract is difficult and time consuming. Previously, we have developed methods to quantify changes in number and volume of cells in networks of neurons and ICC by multiple threshold segmentation of 3D volume rendered images⁶. The objective of the present study is to quantify changes in cell number by applying digital filtering algorithms, inhomogeneity correction and 3D object counting functions to 3D images generated from laser scanning confocal microscope scans of fluorescence-labeled mouse small intestine using the image analysis software, Analyze™.

2. Methods

2.1. Animals

Mice were handled according to procedures approved by the Mayo Clinic Institutional Animal Care and Use Committee and were killed by CO₂ inhalation in accordance with American Veterinary Medical Association guidelines. The mice used in this study expressed a gene targeted mutation in the receptor tyrosine kinase, c-Kit. This mutation does not alter the distribution or expression of c-Kit and has no effect on the cell types expressed in the tissues under study⁷. The images studied were collected from full thickness preparations of the muscularis propria of the jejunum of mouse small intestine, which is a tissue that contains two layers of smooth muscle cells separated by a (myenteric) plexus of enteric neurons, glia and ICC. A second plexus of ICC referred to as the deep muscular plexus of ICC is found close to the mucosal side of internal circular smooth muscle layer. Thus, this tissue has most of the cell types that might be affected by gastrointestinal injury.

2.2. Labeling

The morphology and distribution of cellular nuclei was determined in preparations from the full thickness of the muscle wall of the mouse jejunum by a modification of an immunohistochemical procedure that has been previously described in detail⁶. Briefly, tissues were prepared from 1 cm long pieces of the jejunum tissue, taken approximately 7 cm distal to the pylorus. The muscle layers were peeled from the mucosa and loosely pinned out on a new piece of Sylgard (Dow Corning, Midland, MI). The whole mount was then placed in cold 0.1M phosphate buffered saline (PBS) and rinsed three times for two minutes. The tissue was then fixed with freezing acetone at 4°C for 10 minutes, rinsed with PBS six times for 30 minutes, and placed in 10% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA), PBS, 0.3% Triton X-100 (Pierce, Rockford, IL) to block for 18 hours.

Nuclei in the tissues were labeled with the DNA binding dye, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Inc., Eugene, OR). The tissues were first washed with PBS 5 times for 25 minutes followed by a wash with deionized water once for 5 minutes. The tissues were then incubated with DAPI for 30 minutes at a concentration of 300nM, diluted in deionized water. The tissues were laid flat on a slide and mounted in SlowFade Gold anti-fade reagent (Molecular Probes, Eugene OR).

ICC were identified by immunoreactivity for c-Kit using a selective monoclonal antibody (ACK-2, eBiosciences, San Diego, CA). The primary antibody was incubated with the tissue overnight at 4°C diluted in 5%NDS/1X PBS/0.3% Triton X-100 to a final concentration of 3.33µg/mL. The tissue was then washed 6 times for 30 minutes and a fluorescein conjugated donkey anti-rat secondary antibody diluted to 1.3 µg/mL in 2.5%NDS/1X PBS/0.3% Triton X-100 was incubated with the tissue overnight at 4°C.

2.3. Confocal Data Collection

Images of the labeled tissue were collected on a laser scanning confocal microscope using a 40×, 1.2 numerical aperture, water-immersion objective. The excitation and emission wavelengths were chosen to optimally separate the signal from the fluorescein-labeled ICC and DAPI-labeled nuclei. The pinhole settings on the excitation laser and z step were chosen to give the optimal resolution for detecting fluorescein labeled objects and to match the thicknesses of the optical slices for both the fluorescein and DAPI labeled structures (typically 0.49 µm). Images were 512 × 512 pixels with a pixel dimension of 0.62 × 0.62 µm. The detector sensitivity and laser intensities were adjusted so that about 5% of the pixels saturated the 8 bit detector and the background signal was more than 5% higher than the minimum sensitivity of the detector. Image stacks were 60 to 70 µm (120 to 150 sections) thick.

2.4. Image Processing and Analysis

We used the Analyze^{TM8} object counter to separate nuclei and clusters of nuclei from the confocal image volume. This program applies a histogram-derived threshold to the entire volume image and performs a connectivity analysis that identifies and measures the volume of all connected regions of the volume above the threshold. The volume histogram of these objects (Fig 1) revealed a periodic multimodality typical of clusters of an integral number of similarly sized particles. Using the mean and standard deviation of the smallest mode, the single nuclei were separated from the clusters and subjected to further analysis (Fig 2).

2.4.1. Principal Component Analysis—Statisticians regularly address covarying measures by means of principal component analysis (PCA)⁹. If covarying measures have been recorded, they may be combined into a single derived measurement by finding the projection of the measurement space that shows the greatest total variance, and recalculating the measurement along that axis. This direction is called the first principal component, and multiple measures may often be combined into a single parameter with greater discriminatory power than any of the original measurements by projecting them onto the first PC. A set of N measures will have N principal components. The principal components

are mutually orthogonal and ordered and scaled by variance, so that the second PC is the axis at right angles to the first PC that shows the greatest remaining variance, and so on.

Principal components are derived from the variance and covariance of the original measurements. A set of i measures M has a mean $\bar{M}$ and a variance s^2 equal to the mean of the square of the difference of each measurement from the mean.

$$s^2 = \frac{\sum_i (M_i - \bar{M})^2}{i} \quad (1)$$

A set of multiple measures M_{xyz} has a multivariate mean or centroid $\overline{M_{xyz}}$. Each individual measure has a variance, and every pair of measures has a covariance. Covariance is the product of the difference from the mean of one variable and the difference from the mean in a different variable.

$$\text{cov}(x, y) = \frac{\sum_i (M_{x_i} - \bar{M}_x)(M_{y_i} - \bar{M}_y)}{i} \quad (2)$$

$$\text{cov}(x, z) = \frac{\sum_i (M_{x_i} - \bar{M}_x)(M_{z_i} - \bar{M}_z)}{i} \quad (3)$$

$$\text{cov}(z, y) = \frac{\sum_i (M_{z_i} - \bar{M}_z)(M_{y_i} - \bar{M}_y)}{i} \quad (4)$$

The variances and covariances are combined into a covariance matrix, a square matrix that has an eigensystem solution.

$$\begin{bmatrix} s_x^2 & \text{cov}(x, y) & \text{cov}(x, z) \\ \text{cov}(x, y) & s_y^2 & \text{cov}(z, y) \\ \text{cov}(x, z) & \text{cov}(z, y) & s_z^2 \end{bmatrix} \quad (5)$$

A matrix having an eigensystem is a linear transformation matrix. An input vector may be transformed by multiplication with the matrix to produce an output vector. An eigenvector is a vector that is simply scaled by this transformation, and its eigenvalue is the scalar factor. An $N \times N$ matrix has N eigenvectors (of number N) and N eigenvalues. Eigenvectors are generally expressed as unit vectors, but any scalar multiple of the unit eigenvector is also an eigenvector. The eigenvectors of a covariance matrix are the principal components of the original measurements and the eigenvalues are the variances in those directions.

We wrote custom code to derive a 3×3 covariance matrix from the 3D spatial coordinates of the voxels that made up each single nucleus. Code to solve the eigensystem for a 3×3 covariance matrices is in the public domain as part of the ITK¹¹ image processing toolkit (National Library of Medicine). We used an implementation of this code accessed through

the AVW¹⁰ subroutine library. We used the eigensystem calculated for each covariance matrix to create two new color 3D image volumes in which the voxels that made up each nucleus were color coded to represent the eigenvalues and first eigenvector of that nucleus.

The eigenvalues are a shape-descriptive measurement vector relevant to a variety of meaningful nuclear shape variants. Nearly equal 3D eigenvalues indicate spherical morphometry, while disk shapes are revealed by a third eigenvalue significantly smaller than the other two. The spindle-shaped nuclei of muscle cells in this application have a dominant first eigenvalue. Fairly subtle shape variations between these extremes may occupy separable regions of the eigenvalue space, and may or may not be physiologically meaningful. In this color representation, spherical objects are colored white, and spindles a pinkish red, while disks are colored a shade in between these two.

The normalized eigenvector set captures the orientation of non-spherical shapes. The first eigenvector of spindle shaped objects is aligned with the long axis of the spindle. The two muscle layers in this tissue are oriented at right angles to one another, so they are easily differentiated and the exact angle between them can be computed from the mean eigenvectors of the groups. The first eigenvector of disk shaped objects is likely to be completely random, but the third eigenvector of these objects will be normal to the planar orientation of the disk. In this color representation, the first eigenvector of muscle cells in different layers are of a color very similar to others in the layer, and significantly different than those in the other layer.

2.5. Statistical classification

The color image volumes were then submitted to statistical classification individually. An unsupervised “isodata”¹² classifier was used to identify classes that were significantly different from one another. The eigenvalues were first used to separate the muscle nuclei from the other nuclei, then the first eigenvector of the muscle nuclei was used to separate them into oriented groups. The non-muscle cell nuclei were reanalyzed using the eigenvalues in combination with the first eigenvector to detect subtle shape variations.

3. Results

The tissues prepared from adult mouse jejunum for this analysis were typical of the kind of preparations that have been studied by many investigators in the past, including our group⁶. The tissue contained multiple, closely associated cell types, as identified by DAPI-labeling of the nuclei, and shown as a maximum intensity projection of the image stack (Fig 2, upper left).

Several groups of nuclear morphologies were observed that were restricted to distinct regions of the preparation. The thickest layer of cells in this preparation was the circular muscle layer. The nuclei of muscle cells in the circular muscle layer were spindle shaped and were oriented perpendicular to the long axis of the intestinal lumen (green objects, Fig 3 bottom left panel). These nuclei were clearly separated from the nuclei of longitudinal smooth muscle cells, which had the same morphology but were orientated parallel to the long axis of the gut; that is perpendicular to the circular smooth muscle cells (red objects,

Fig 3 bottom left). The non-spindle shaped nuclei were distributed in different areas of the tissue suggesting that these might be the nuclei of specific cell types. Cells with spherical nuclei could be identified on the submucosal surface, between the muscle layers, in the myenteric plexus region, and on the serosal surface of the preparation.

In order to obtain images that could be analyzed in this fashion, it was necessary to amend the conditions for labeling the nuclei by omitting phosphate from the incubation buffer to minimize background signal in the cells. Further improvements were achieved by testing a variety of settings on the imaging device to collect the images and using the success of the histogram-based thresholding in the object counter of Analyze™ as the basis for collecting further data. In the dataset shown here, 648 single, defined nuclei were identified with a volume of $188 \pm 30 \mu\text{m}^3$ (mean $\pm$ SD). A further 207 objects appeared to be multiples of the unitary volumes. Doublets had a mean volume of $383 \pm 41 \mu\text{m}^3$ each; triplets had a mean volume of $596 \pm 55 \mu\text{m}^3$ each. Analysis of the objects separated by principal component analysis revealed that both spindle and non-spindle objects had the same mean volume (Spindle; $185 \pm 15 \mu\text{m}^3$, Non-spindle; $185 \pm 21 \mu\text{m}^3$). However, as indicated by the larger variance in the measurements, the non-spindle shaped nuclei had a broader distribution of sizes consistent with heterogeneity in these objects. Two classes had mean volumes significantly different from the group mean. Class 4 objects (yellow, Fig 3 lower row) were smaller ($174 \pm 20 \mu\text{m}^3$, $P < 0.05$) than the population mean and appeared to be located primarily in the myenteric plexus region of the tissue. Class 2 objects (pink, Fig 3 lower row) were larger ($199 \pm 17 \mu\text{m}^3$, $P < 0.05$) than the population mean and located mainly on the serosal surface of the tissue. This analysis did not determine whether these classes represented nuclei of a specific cell type.

ICC were identified by immunoreactivity for the receptor tyrosine kinase, c-Kit (Fig 3 center middle panel) in the myenteric plexus region and to the deep muscular plexus. Analysis of images collected by dual channel confocal microscopy was used to determine if any of the nuclei of ICC belonged to any of the classes of DAPI-labeled nuclei identified by principal component analysis. In the deep muscular plexus, the nuclei of ICC had no consistent morphology. In the myenteric plexus region, none of the spindle shaped nuclei co-localized with the c-Kit positive ICC. However 20 of the non-spindle shaped nuclei were clearly the nuclei of ICC (Fig 3, center middle - arrowheads). The nuclei that were restricted to ICC did not belong to any specific sub-class of non-spindle shaped nuclei.

In summary the combination of image analysis algorithms and rapid feedback between image collection and analysis makes it possible to generate high quality images suitable for quantitative analysis from complex fluorescence labeled tissue structures. This presentation will demonstrate how these high resolution images can be used to obtain quantitative information on the distribution of specific cell types in the gastrointestinal tract such as smooth muscle cells and ICC.

4. Conclusions

There are many areas of biology that involve cell counting and classification. This study demonstrates that nuclear morphology can be used to separate and quantify populations of

cells using object classification techniques. The results represent a proof of concept that can be applied broadly to quantifying stained or immunolabeled objects in histochemical preparations. Nuclei of cells in the smooth muscle layers of the mouse jejunum can be segmented from the nuclei of cells in the other layers of this anatomically complex tissue based on their shape and the orientation of their primary axis. These nuclei can be quantified making it possible to easily determine whether differences in the thickness of muscle layers reflects increased cell numbers (hyperplasia) or increased cell size (hypertrophy). This question is relevant to understanding the mechanisms of gastrointestinal muscle changes in response to functional or physical obstruction and injury in diseases such as scleroderma or myopathic intestinal pseudobstruction.

The techniques that are currently used to quantify cell numbers in gastrointestinal tissue involve manual counting techniques on small samples derived from much larger pieces of tissue. Obtaining enough observations to collect a representative sample size is time consuming. In this study, the majority of the cells in the image stack could be quickly quantified and classified thus reducing possible sampling errors by enabling a larger sample size. Successful separation of objects resulted from an iterative process of optimizing each part of the process. For the labeling technique, it was determined that thoroughly washing out the phosphate buffer before labeling with DAPI improved the ability of the image processing techniques to separate single nuclei even though there was little apparent improvement in the quality of the staining when viewed on a conventional epifluorescence microscope. Similarly, small changes in the settings on the imaging device to ensure that the background intensity was above the detection limit and that the full dynamic range of the detector was used, significantly improved the success of the image analysis. By coordinating sample preparation and image analysis, we were able to successfully demonstrate that established methods in object classification can be applied to analysis of nuclear morphology in a complex tissue.

Other methods have calculated the total volume of labeled structures in diseased or injured gastrointestinal tissue to assess the depletion of specific types of cells including ICC and neurons^{3,13}. Such studies use cell surface antigens, making it difficult to accurately measure cell number particularly for cells like ICC, neurons and glia that have complex branching morphologies. However, it is unlikely that nuclear morphology alone will be sufficient to identify cell types as there was considerable overlap between nuclei from different cell types. Nevertheless, the analysis techniques developed using DAPI as a nuclear marker can also be applied to counting and classifying cells labeled with cell specific antigens that are restricted to nuclei. Markers specific for glial¹⁴ and neuronal¹⁵ nuclei have been identified and have been used to demonstrate the effects of gastrointestinal injury on the enteric nervous system. This is a significant technical challenge because glia and neurons are intimately associated and they cannot be easily separated. The techniques developed for this study make confocal imaging, image conditioning and object classification a practical method for assessing changes in neurons and glia in complex tissues such as the wall of the gastrointestinal tract. At present no nucleus-restricted antigen is available for identifying ICC. However there are mouse strains that have been genetically modified to express marker proteins restricted to the nuclei of ICC^{16,17}. Analysis of changes in the number and

morphology of the nuclei in ICC in response to disease and injury in these strains of animals will be facilitated by the techniques described in this paper.

The use of high quality confocal images, minimum error thresholding, inhomogeneity correction and multivariate component analysis allows detection of changes in tissue morphology that could not be achieved by object selection from manually rendered image bitmaps. This will allow for better detection of changes in cell volume and number in biopsies of human tissue from patients with motility disorders and from tissue from animal models of motility disorders.

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Figure 1.
Cumulative distribution plot of the sizes of the DAPI labeled nuclei determined using the object counter in Analyze™.

Figure 2.

Upper Left - Projection image of DAPI-labeled nuclei imaged by confocal microscopy in a $230 \times 230 \times 70$ micrometer sample of mouse jejunum. Upper Center - thresholded and volume rendered image with edge-contacting nuclei removed. Upper Right - Segmented single nuclei. Lower Left - Segmented objects of volume twice (red), three times (green) and more (white) than that of a single nucleus. Lower Center - Individual double-volume objects in different colors. Lower Right - Individual triple-volume objects in different colors. As can be seen these likely represent adjacent nuclei that could not be temporally separated by light microscopy.

Figure 3.

Upper Left - Color representation of eigenvalues, used to separate muscle cell nuclei from other nuclei. Upper Right - Color representation of first eigenvector, used to separate muscle nuclei in different orientations. Center Left - Unsupervised statistical classification of eigenvalue image discriminates spindle-shaped muscle cell nuclei (blue) from other nuclei (green). Center - Image generated from merging of the image of identified non-spindle shaped nuclei in the myenteric plexus region with the image of Kit-immunoreactive ICC (red) showing colocalization of nuclei (green) with nuclear region of the ICC (arrowheads).

Center Right - Six-channel unsupervised statistical classification of the non-muscle cell nuclei using both the eigenvalues and the first eigenvector detects 5 oriented shapes. Lower Left – Unsupervised statistical classification of the first eigenvector image discriminates muscle cell nuclei in different layers based on their orientation. Lower Center – Two orientations of muscle cell nuclei and five classes of non-muscle cell nuclei. Lower Right – View of classified nuclei at right angles to the acquisition direction, showing the layering of muscle and non-muscle nuclei. LM, longitudinal muscle, CM, circular muscle, MP, myenteric plexus, DMP, deep muscular plexus.

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