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ImagePAD, a Novel Counting Application for the Apple iPad®, Used to Quantify Axons in the Mouse Optic Nerve

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Abstract

The present article introduces a new and easy to use counting application for the Apple iPad. The application “ImagePAD” takes advantage of the advanced user interface features offered by the Apple iOS® platform, simplifying the rather tedious task of quantifying features in anatomical studies. For example, the image under analysis can be easily panned and zoomed using iOS-supported multi-touch gestures without losing the spatial context of the counting task, which is extremely important for ensuring count accuracy. This application allows one to quantify up to 5 different types of objects in a single field and output the data in a tab-delimited format for subsequent analysis. We describe two examples of the use of the application: quantifying axons in the optic nerve of the C57BL/6J mouse and determining the percentage of cells labeled with NeuN or ChAT in the retinal ganglion cell layer. For the optic nerve, contiguous images at 60× magnification were taken and transferred onto an Apple iPad®. Axons were counted by tapping on the touch-sensitive screen using ImagePAD. Nine optic nerves were sampled and the number of axons in the nerves ranged from 38872 axons to 50196 axons with an average of 44846 axons per nerve (SD = 3980 axons).

Keywords

nerve counting; optic nerve; axon loss; iPad; method; IOP; quantification; application; retina

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1. Introduction

Quantifying the number and type of cells within a structure is a basic part of life sciences research and has been for over 60 years (Abercrombie, 1946). Since that time, many discussions about the validity and reliability of different counting approaches have taken place, with some being rather contentious (Abercrombie, 1946; Benes and Lange, 2001; Bjugn and Gundersen, 1993; Coggeshall and Lekan, 1996; Geuna, 2000; Guillery and Herrup, 1997; West, 1999; West and Slomianka, 1998). As new imaging techniques have arisen, additional requirements have been imposed on cell counting procedures in order to ensure that accurate and repeatable results are obtained. For example, what started as counting whole cells in two dimensions, has been complicated by adding a z-axis or estimating densities in order to determine which cells would be appropriate to count. The goal of developing the application described in this article is not to redefine cell counting or to propose a new fundamental cell-counting strategy, but rather to provide a tool that can assist scientists performing cell-counting tasks to manage the competing requirements of efficiency and accuracy.

There are many research and commercial software programs currently available which support cell counting, usually as a part of a broader image analysis feature set. Specific examples include ImageJ (National Institutes of Health, Bethesda, MD, USA, imagej.nih.gov), STEPanizer (Institute of Anatomy, University of Bern, Switzerland, www.stepanizer.com), and Stereologer (Stereology Resource Center, Chester MD, USA, www.disector.com). One common characteristic of these applications is that they are designed to operate on a traditional desktop or laptop computer, with a standard user interface consisting of a keyboard and pointing device (mouse, trackball, touchpad, etc.). This user interface configuration is not optimal for performing manual cell counting tasks due to the need to track progress of the counting task spatially across the image to avoid counting errors. In the authors' experience, a touchscreen provides a more natural interface for manual cell counting because it permits the user to maintain spatial progress by means of the stylus position. Even though metrics have not been formally established, this advantage is expected to yield a tangible benefit in terms of counting efficiency and the reduction of both omission and re-count errors during manual cell counting tasks. Although it is certainly possible to utilize one of the many available counting applications on a laptop computer equipped with a touchscreen, it was envisioned by the authors that a counting application based on the Apple iOS® platform could provide a very flexible and intuitive means for executing cell counting tasks. Toward that end, it was decided to implement a core set of cell counting features in an iOS®-based application in support of ongoing research activities. This direction offered two advantages over the use of a traditional application in conjunction with a hardware touchscreen. First, such an application is able to take advantage of the now-common multi-touch gestures supported for image viewing on iOS® (e.g. pinch-to-zoom). This offers an efficiency improvement to the cell-counting user; instead of having to press a physical button in order to scroll or zoom an image, these operations can be carried out more intuitively. Second, the ability to perform this task on a mobile device means that a large cell counting task can be broken up and performed in small blocks of otherwise "wasted" time; e.g. while waiting for an appointment, etc. The custom application that was designed in

support of the present work is called ImagePAD, and is currently available for the Apple iPad®.

To illustrate our process in a lab-oriented approach, we chose two different counting strategies: the first was to count axons in sections of optic nerves and the second was to determine the percentage of immunolabeled cells in the ganglion cell layer of a mouse retina.

2. Materials and Supplies

2.1 Mice

Nine animals were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were kept on a 12 hours on, 12 hours off light cycle with food and water *ad libitum*. One female and 6 male mice were used in the optic nerve study and two male mice were used for the analysis of the neurons in the retinal ganglion cell layer. In previous studies, no significant difference between female and male animals in regards to their axon density was found (Williams et al., 1996). Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center and the IACUC at Emory University approved all protocols involving the use of mice.

2.2 Optic Nerve Processing and Imaging

The mice were anesthetized with a mixture of 13 mg/kg of xylazine and 87 mg/kg of ketamine and were perfused transcardially with saline followed by 2% Glutaraldehyde and 2% Paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Optic nerves were post-fixed in equal parts of 2% glutaraldehyde and 0.1M phosphate buffered saline, subsequently rinsed and post-fixed in osmium tetroxide. After dehydration in increasing concentrations of ethanol, the specimen were embedded in Spurr's resin. 0.8µm thick sections were cut on an ultramicrotome (Reichert Ultracut E Ultramicrotome), stained with p-phenylenediamine (PPD) for 30 minutes and mounted with DPX. Then, an image in 10× magnification was taken with a digital camera (Olympus U-CMAD-2, Olympus, Tokyo, Japan) mounted onto a light microscope (Olympus BX-51, Olympus, Tokyo, Japan). Multiple contiguous photomicrographs were taken in 60× magnification to provide a continuous representation across the optic nerve.

2.3 Image Processing

In order to discern smaller and lightly myelinated axons, we used the CLAHE plugin (Contrast Limited Adapted Histogram Equalization) for ImageJ. CLAHE enhances local contrast significantly by computing multiple adaptive histograms corresponding to unique sections of the image and then amplifying contrast in the vicinity of a given pixel by the slope of the transformation function (Zuiderfeld, 1994). Parameters for CLAHE were set to a blocksize of 127, 256 histogram bins and a maximum slope of 3.00. Enhanced images were used for counting and measuring the cross-sectional area of the optic nerve.

2.4 Measuring the Cross-Sectional Area of the Optic Nerve

Before beginning the measurement of the area of the optic nerve, the scale was globally set in ImageJ using a scale photographed at 10× magnification. Once the global scale was set, a photomicrograph of the optic nerve (at 10× magnification) was loaded into imageJ and the threshold was set using Otsu's method to define the area of the optic nerve containing axons and to eliminate the surrounding connective tissue sheath. The magic wand tool was then used to select the highlighted mask and the area was measured in μm^2 .

2.5 Use of the Application for Counting

The ImagePad application was purchased from CoDesign Engineering Services LLC, (Tuscaloosa, AL; www.codesign-es.com) and was installed on an Apple iPad. This application was used to perform manual cell counting of images that conform to the standard file formats supported on the iOS® platform (see iPad manual). In order to be accessible to the ImagePAD application, the images must first be loaded into the iPad's photo library. This can be accomplished by one of several methods, such as setting up a 'sync folder', which is synchronized using iTunes. Other supported options for transferring images include the use of file sharing services and extracting images from email; however, for preservation of original image resolution, it has been found that synchronization using iTunes is the most reliable method. Additional methods for automating bulk image transfer are being explored for future versions of ImagePAD.

3. Detailed Methods

3.1 Detailed Application Options

The following options are available from inside the ImagePAD application:

Main Window Options—Opening the program on the iPad reveals the main window (Figure 1). There are two rows of buttons on the screen, one in white and one in black. The first series of buttons are labeled with the prefix "Type". This series of single-select buttons identifies the "active" category for counting; this allows the user to count up to five separate features in the current image. Each feature is labeled with a unique color marker on the screen and is identified in a separate column in the output file. The next button ("Select CBox") enables the counting box, which then can be placed on a single grid within the field. The counting box is implemented in the manner originally proposed by Williams and Rakic (Williams and Rakic, 1988): two sides of the grid box are highlighted red and two sides are highlighted green, identifying objects to be excluded from the count (touching the red line) and objects to be included in the count (touching the green line). The "Remove" button will delete previously placed markers in reverse order. Tapping the "Reset" button will reset all the markers and counts to zero. To avoid resetting by mistake, a confirmation prompt is presented to the user before the counts are cleared.

Across the bottom of the screen, a series of six buttons are presented (Figure 1).

1. The first is "Open Image". This button is used to select and open the image to be analyzed.

2. The second button is “Close Image”. This allows the user to remove the currently displayed image. Again, a conformation prompt will pop up after tapping this button. A new image cannot be loaded unless the previous image has first been closed.
3. The third button is “Grid Setup”. Tapping this will open a new dialog box presenting options to configure the grid (see Figure 2). These options include changing the line size of the marker, the diameter of the marker, the grid line thickness, grid size by square pixels, and the color of the grid lines. The iPad’s “pinch to zoom” feature is enabled by default; however it should be noted that as the image is zoomed in or out, the grid remains fixed on the image and the size of the grid bars in 193 or decreases, respectively. A marker size suitable to the cells being counted should be chosen so as to not obscure neighboring areas. The counting box can also be disabled and enabled from this screen.
4. The “Write Output” button stores the count values in a tab-formatted text file along with a user-specified textual description. Once counting has been finished, tapping this button will present a dialog box asking for a description of the completed counting task. Although any combination of characters, symbols or numbers can be typed in this field, it is advisable to utilize a consistent naming convention, such as that used for the original image’s file name.
5. The “Clear Output” button removes all data from the output file. After tapping this, a warning prompt will appear asking the user to confirm that all data will be removed.
6. The “View Output” button allows the user to view a summary of the existing count data for all categories (one column per category). The tab-delimited data format utilized by the current version of ImagePAD consists of seven columns. From the left to the right, the seven columns displayed include: date and time, description, and the counts of the five different feature categories (marker types/colors). A count value of “0” is recorded for any feature category that has not been used. The “View Output” dialog also provides an option for attaching the existing count data file to an email for transfer to another user or system. Alternatively, the output file can be exported via iTunes as a raw text file for further analysis. To copy the file from the iPad to a computer, it should be connected via USB and iTunes should be opened on the computer. Under the “Apps” tab in iTunes, the text file (named “ImagePadOutput.txt”) will be listed in the section “File Sharing”.

3.2 Examples of Usage

Example 1: Counting Axons in the Mouse Optic Nerve—There are several sampling methods one could use in estimating the numbers of axons in the optic nerve of the mouse. We have chosen to use a Systematic Quadrant Sampling method similar to the method used by Williams et al. (Williams et al., 1996) and Jeon et al. (Jeon et al., 1998) in their analysis of the optic nerve. The method sets up a grid across the section and includes every 5th quadrant in each of 4 rows with each row being separated vertically by 5 quadrants of the grid. Consequently, this approach spreads our sampling fields over the optic

nerve in a systematic manner, minimizing a bias that could occur if we sampled selected quadrants or randomly chosen quadrants. This type of non-biased systematic counting is not only good for the normal optic nerve, it is absolutely necessary when sampling optic nerves from animals with induced loss of ganglion cell axons such as in mouse glaucoma models, for the loss of retinal ganglion cells can be sectorial in the retina and potentially in the optic nerve.

Before beginning the process of estimating the number of axons within an optic nerve, it is necessary to define the sample sized needed to achieve the desired margin of error. To estimate the sample size (n) necessary for a margin of error (E) of 500 axons per nerve we used the equation:

$$n = \left(\frac{\left(\frac{Z_{\alpha}}{2} \right) \sigma}{E} \right)^2$$

where $Z_{\alpha}/2$ comes from a Z distribution with a 95% confidence interval and is 1.96, and where the variance of the population (σ) comes from the Standard Deviation (SD) reported by Jeon and colleagues (Jeon et al., 1998), which is 3608 axons for the C57BL/6 mouse. Solving for n reveals that we would need to count 790 axons per nerve to have a 95% confidence interval with a margin of error of ± 250 . In the present study, we sampled on average 890 axons per normal optic nerve, which is equivalent to roughly 2% of the axons in the nerve.

Step 1: Start by tapping the “Open Image” button to load the first image in 60× magnification. Verify that the following grid options are set: Grid size to 10,000 square pixels, grid line size to 1, marker size to 8 and marker line size to 2. (Note: In general, the grid size should be set to a value large enough for the counting box to contain at least a few axons. Otherwise, the effective axon density calculation performed in Step 8 will produce an aberrant result that is highly dependent on the particular grid boxes selected for counting. Selecting a grid size that is too small for the image under consideration is analogous to under-sampling the data set and should be avoided in order to ensure accuracy and repeatability.)

Step 2: Tap the “Select CBox” button and choose the first appropriate grid square by tapping it.

Step 3: As the counting box appears, its borders change to either a red or green color. Axons that are completely inside the counting box frame or that are intersecting the green border are included in the counts. Axons that are in contact with the red border are excluded from the counts. To include an axon in the active category count, touch the iPad screen and a colored mark will appear over the feature.

Step 4: When all the axons within one box have been counted, tap the button labeled “Write Output”. An input box will appear asking for a description to associate with the current counts. By convention, the description used is the file name followed by an index number for reference. Counts will then be saved to the output file and a dialog box will appear asking if counts should be reset, which should be accepted.

Step 5: Move to the next square in the grid and repeat Steps 2 through 4 with a new counting box selected. Generally, the next grid square should be selected in a systematic manner across the grid and at an appropriate distance from the initial counting box by alternating the direction in the x- and y-axis. In our study, we chose a spacing of 5 grid boxes between samples in both the vertical and horizontal direction. This approach resulted in a relatively even sampling across the cross section of the optic nerve.

Step 6: Close the image by tapping the “Close Image” button. Then, the next picture in the series taken from the same nerve at 60× magnification can be loaded and counting can be continued as described in steps 1–5.

Step 7: After all the pictures of one nerve have been counted, transfer the output file to a computer by either emailing it or copying it over through iTunes. Then, copy the data into an excel spreadsheet. Since the data is in a tab-limited format, it will be automatically inserted into separate rows and columns. Calculate the average number of axons per counting box by using the “=AVERAGE” formula within the column corresponding to the appropriate marker (if the default marker 1 has been chosen within ImagePAD, this would be the 3rd column).

Step 8: The number calculated in Step 7 (average counts of axon per counting box) is then divided by a factor to establish the effective axon density (axons per μm^2). This factor can be obtained by taking a picture of a grid replica in the same magnification settings used for the optic nerve imaging, measuring a known grid length in an image processing application like ImageJ (National Institutes of Health, Bethesda, MD, USA), converting this pixel value to the known corresponding length in μm and squaring it.

Step 9: Use an image processing application such as ImageJ to calculate the area of the optic nerve in 10× magnification by auto-thresholding or by manually tracing its circumference (see section 2.4.). This gives a value in square pixels, which can then be converted to a value per μm^2 in the same manner previously described in step 8.

Step 10: Finally, multiply the average axon count per μm^2 with the area of the optic nerve in μm^2 to acquire the number of axons per optic nerve cross section. In our counts of the C57BL/6J optic nerve, the average number of axons per optic nerve was 44846 (n=9, SD=3980).

Example 2: Quantifying Multiple Features in an Immunostained Flat-Mounted Retina—Using ImagePAD, it is also possible to count up to five different features within an image. With this approach, cell ratios can be easily determined. As an example, we provide Figure 3, which shows a flat-mounted retina that has been triple-stained with TO-PRO-3 to label all nuclei, NeuN (Neuronal Nuclei) to label neurons in the ganglion cell layer and ChAT (choline acetyltransferase) to label cholinergic amacrine cells. The counting procedure is similar to the one described in example 1, with the exception that different types/ colors of markers can be used here to distinguish between differently stained cells. In the ganglion cell layer, 40% (151) of the cells were labeled with NeuN and 17% (66) of the cells were cholinergic amacrine cells.

4. Potential Pitfalls and Trouble Shooting

The present paper describes the use of an iPad application for quantifying objects on microscopic images. The application “ImagePAD” takes advantage of the advanced user interface features offered by the Apple iOS® platform, simplifying the rather tedious task of quantifying features in anatomical studies. For example, the image under analysis can be easily panned and zoomed using iOS-supported multi-touch gestures without losing the spatial context of the counting task, which is extremely important for ensuring count accuracy. This application allows one to quantify up to 5 different types of objects in a single field and output the data in a tab-delimited format for subsequent analysis.

It has been stated that there are many equally good methods of counting, but they all depend on the level of accuracy of the counter as well as the material being studied. One specific problem that arises when counting nearly two-dimensionally is the “double count”: Certain objects may simply be counted twice in a given area when part of an object is overlapping into the next counting segment. This may result in an overcount (Guillery, 2002). However, this was just considered a theoretical problem in our approach, where we chose to only account for axons in every fifth counting box across the x- and y-axis of any given image. Using a counting box with a predefined intersector that either allows (green corner) or disallows (red corner) inclusion of overlapping objects further alleviated this problem.

Another common source of error seems to arise when addressing the influence of size, shape, orientation and spatial distribution of the objects to count. Proponents of the optic disector method, where objects are counted by creating continuous focal planes with a thin depth-of-field through a rather thick section, believe this to be a bias in standard assumption-based methods (Hatton and von Bartheld, 1999; Williams and Rakic, 1988). However, opponents of this method deem this approach cumbersome and claim that this error could be diminished by including a correction formula, such as the one postulated by Abercrombie (Hedreen, 1998).

Most of these problematic scenarios deal with fragments of cells or nuclei and the important question of whether or not to include them into the count. However, because we were interested in quantifying axons, which are direct projections of the retinal ganglion cell and thus have their cell body (with nucleus and other compartments that would suffer from shedding) in the retina, we did not find it necessary to factor in a correction formula in our first example.

Choosing Appropriate Quadrant Size

One caveat is that if a very small grid size were to be selected, such that many “zero” counts are produced during a given counting task, the resulting effective axon density would not be reliable unless nearly all of the grid boxes were selected for counting. Otherwise, the effective axon density calculation would be overly dependent on the particular grid boxes selected for counting. This scenario is analogous to the mistake of simply under-sampling the data set (e.g. selecting too few grid boxes for establishing the average for the entire population). On the other hand, if a larger grid box size is selected, such that all grid boxes contain at least several axons, then the arithmetic mean of the counts contained in these

boxes, normalized by the size of the grid box in μm^2 , is an accurate representation of the effective axon density. This approach is valid across a wide range of grid box sizes, bounded on the lower end by the size at which the number of “zero-count” boxes begins to increase rapidly. In the current work, the grid box size was selected according to the above guidance, such that there were very few if any zero counts.

Manual Counting versus Automated Counting

There are distinct advantages to automated counting. If the feature to be counted can be clearly defined, then counting programs like Cellprofiler or counting algorithms such as the “Analyze particles” function in ImageJ can make quantifying the number of a specific feature fast and easy. The automated approach potentially makes the quantification of a sample more accurate and also speeds up the process, as compared to manual counting. However, for some samples it is not easy to clearly define the feature to be quantified.

In our manual counts using ImagePAD, we were able to include counts from both large, heavily myelinated axons and small, lightly myelinated axons, while easily excluding blood vessels, glial cell bodies and glial processes. Our manual counts were surprisingly similar to the number of axons in the optic nerve reported by Jeon et al. (Jeon et al., 1998). They found that the optic nerve of the C57BL/6 mouse had 44,857 (SD=3,608) axons per nerve and in the present study we found that the C57BL/6J mouse had on average 44,846 (SD=3980) axons per nerve.

To provide an evaluation of automated counting systems, we analyzed two nerves with two different methods each. The first method was similar to that described by Lipstreu (Lipstreu, 2007) and involved enhancing the picture in MATLAB and then using different thresholding methods to outline axons. Using the “Analyze Particles” function in ImageJ we could count the number of highlighted features. The results of this strategy were very inconsistent with numbers ranging from ~12,000 to 37,000 axons per nerve using the same sample image in multiple runs. Part of the problem with this approach was the variety of cell types in the optic nerve and the dramatic differences in size of the axons that we are attempting to count. Our nerves are also stained with PPD and this creates a considerable amount of variability in the density and thickness of the myelin rings, with some axons having well outlined profiles and other, lightly myelinated axons showing only a faint stain around a very small center. All of these factors make automated counts in the optic nerve challenging. Preliminary post-processing using filters that enhance contrast or try to pre-segment images into cell types alleviated some of these problems, but did not completely solve them.

The second approach used Cellprofiler (Carpenter et al., 2006). This software package provides a more sophisticated system for segmenting features and thus appears to generate a more consistent count. Nonetheless recounting the same sample with Cellprofiler resulted in a considerable variability relative to manual counting (a four-fold increase in standard deviation). We were unable to find a threshold/segmentation value that would only identify axons and would also include both large, heavily myelinated axons as well as small, lightly myelinated axons. There is no question that manual counting using the ImagePAD application requires more time than automated counting. In our opinion however, it is more

accurate and the additional effort involved in manual counting is minimal relative to the time spent on the animal experiment and tissue processing.

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Roles in the project: J.P. Templeton assisted in the design and beta testing of the application collecting all of the data presented. F.L. Struebing wrote the majority of the final draft of the manuscript and was involved in data analysis. Andrew Lemmon designed and wrote the code for the iPad Application. E.E. Geisert had the original idea to design a counting application and was involved in all aspects of the project.

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References

- Abercrombie M. Estimation of nuclear population from microtome sections. *Anat Rec.* 1946; 94:239–247. [PubMed: 21015608]
- Benes FM, Lange N. Two-dimensional versus three-dimensional cell counting: a practical perspective. *Trends Neurosci.* 2001; 24:11–17. [PubMed: 11163882]
- Bjugn R, Gundersen HJ. Estimate of the total number of neurons and glial and endothelial cells in the rat spinal cord by means of the optical disector. *J Comp Neurol.* 1993; 328:406–414. [PubMed: 8440788]
- Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 2006; 7:R100. [PubMed: 17076895]
- Coggeshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Comp Neurol.* 1996; 364:6–15. [PubMed: 8789272]
- Geuna S. Appreciating the difference between design-based and model-based sampling strategies in quantitative morphology of the nervous system. *J Comp Neurol.* 2000; 427:333–339. [PubMed: 11054696]
- Guillery RW. On counting and counting errors. *J Comp Neurol.* 2002; 447:1–7. [PubMed: 11967890]
- Guillery RW, Herrup K. Quantification without pontification: choosing a method for counting objects in sectioned tissues. *J Comp Neurol.* 1997; 386:2–7. [PubMed: 9303520]
- Hatton WJ, von Bartheld CS. Analysis of cell death in the trochlear nucleus of the chick embryo: calibration of the optical disector counting method reveals systematic bias. *J Comp Neurol.* 1999; 409:169–186. [PubMed: 10379913]
- Hedreen JC. What was wrong with the Abercrombie and empirical cell counting methods? A review. *Anat Rec.* 1998; 250:373–380. [PubMed: 9517854]
- Jeon CJ, Strettoi E, Masland RH. The major cell populations of the mouse retina. *J Neurosci.* 1998; 18:8936–8946. [PubMed: 9786999]
- Lipstreu, WF. Automated Axon Counting in Normal Optic Nerves: Using Image Morphology and Area Based Segmentation. Department of Electrical Engineering and Computer Science Case Western Reserve University; Cleveland, OH, USA: 2007. http://enr.case.edu/merat_francis/eecs490f07/StudentPapersF07/EECS490_Lipstreu.pdf [last accessed 08/25/14]
- West MJ. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *Trends Neurosci.* 1999; 22:51–61. [PubMed: 10092043]
- West MJ, Slomianka L. Total number of neurons in the layers of the human entorhinal cortex. *Hippocampus.* 1998; 8:69–82. [PubMed: 9519888]
- Williams RW, Rakic P. Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material. *J Comp Neurol.* 1988; 278:344–352. [PubMed: 3216047]
- Williams RW, Strom RC, Rice DS, Goldowitz D. Genetic and environmental control of variation in retinal ganglion cell number in mice. *J Neurosci.* 1996; 16:7193–7205. [PubMed: 8929428]
- Zuiderfeld, K. Graphic gems IV. Academic Press Professional, Inc; San Diego, CA, USA: 1994. Contrast limited adaptive histogram equalization; p. 474–485.

Figure 1.

Light-microscopic picture of a normal optic nerve, stained with p-phenylenediamine and imaged at 60 $\times$ magnification. In the middle of the screen, the counting box as it appears on the iPad after tapping the “Select CBox” button can be seen. Note the two different colors of the corners: Every axon that touches the green line would be included into the counts, whereas any axon that touches the red line would be left out.

Figure 2. Grid setup options in ImagePAD. The grid size has been set to 100×100 pixels, or 10,000 square pixels. To achieve consistent results, the grid size should be kept constant in a given experiment between counting sessions; otherwise the density scaling constant has to be re-computed each time this value is changed (see steps 8–10 in example 1).

Figure 3. Picture of a flat-mounted retina triple-stained with TO-PRO-3, NeuN and ChAT before counting (A) and within the ImagePAD application after counting has been finished (B). Note the different types/colors of markers on the right side to distinguish between differently stained cells: yellow represents TO-PRO_3 stained cells, red represents ChAT labeled cells and orange marks NeuN labeled cells.