Combining machine learning with stereology: The next generation of unbiased 3D stereology for cell counting

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Introduction

Stereology is a rigorous and unbiased methodology for quantifying features of biological tissues such as the size, shape, distribution, and quantity of objects. Although it is the gold-standard for quantification, wide-spread adoption of stereological analysis has been hindered because it is labor-intensive, even with modern software tools.

Cellairus dramatically accelerates stereological cell counting through the use of machine learning. Once the machine learning algorithms are trained, Cellairus identifies cells in 3D volumes throughout brain regions using the same observer criteria as a human expert.

Manual and automated stereology were performed in order to assess the performance of Cellairus across multiple imaging technologies. True positive and false positive detection rates were quantified and compared between cell counting methods.

Research Methods

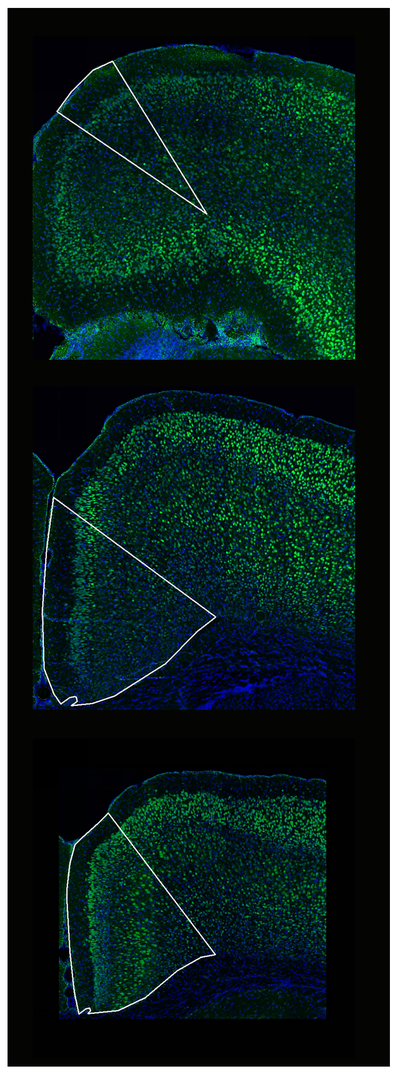
Imaging fluorescent mouse brain specimens

Tissue samples were acquired from experiments performed in accordance with guidelines for the care and use of laboratory animals by FD Neurotechnologies. 30µm thick mouse brain coronal sections were incubated with DAPI and NeuN with a Alexa Fluor 488 fluorescent secondary. A randomized start was selected for the sections containing the anterior cingulate cortex, yielding 9 sections in the dataset.

3D Whole Slide Images of the region of the hemisphere containing the region of interest were acquired using a resonant scanning confocal microscope system equipped with a 60x (1.4 NA) Zeiss oil lens. Images of the region of interest (anterior cingulate cortex) were acquired at z-step increments of 0.76 µm and saved in a 3D image file (.jpx) for further analysis.

Stereological Methods

Stereological cell counts were obtained using the Optical Fractionator design-based stereologic probe. Contours of the anterior cingulate cortex in each section were drawn manually using the Allen Mouse Brain atlas as an anatomical reference. The same grid throw and counting sites were used for both manual validation and automated detection.



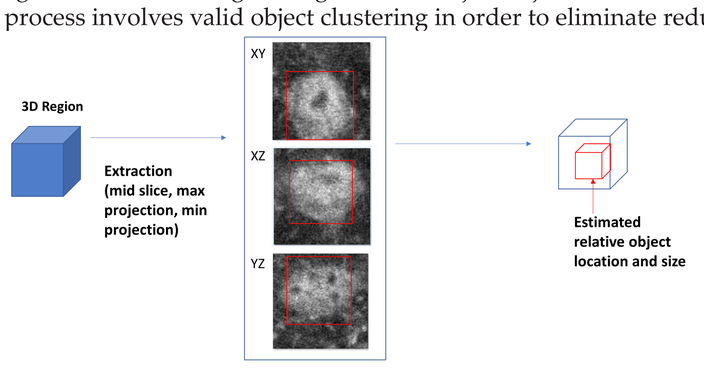
Stereological Design Parameters	
Region of Interest	Anterior Cingulate Cortex
Cut Section Thickness	30 µm
Section Sampling Fraction	1/16
Counting Frame Size	50 x 50 µm
Grid Size	200 x 200 µm
Disector Height	15 µm
Guard Zone Height	2 µm
Total Number of Sites	118
Total Number of Sections	9

Object Detection and Validation using Machine Learning

Cellairus integrates candidate object detection, machine learning, and stereologic counting rules to identify, classify, and quantify cells in 3D microscopy images. Object detection uses a multiple scale model of cell appearance to identify the locations and boundaries of candidate cells within the image.

We use multiple scale Laplacian of Gaussian (LoG) filters to perform the detection in 3D of candidate regions most likely to contain objects of interest. LoG filter scale range selection is determined by matching the size of the target objects. Filter response thresholds are set to a very low value to ensure detection of low contrast objects. The region candidate detection process is designed to detect all valid target objects even at the expense of generating large numbers of false positive region candidates.

Partial maximum intensity projections in XY, YZ, and XZ reduce the 3D regions of interest into 2D images. Our custom Convolutional Neural Network (CNN) performs the task of classifying the candidate regions into valid and invalid object categories; and bounding box regression to adjust object size and location in 3D space. The final step of the detection process involves valid object clustering in order to eliminate redundant object detection.



Stereologic counting rules are applied to the updated cell models. The combination of advanced machine learning algorithms and established stereologic counting rules enables accurate and unbiased quantification of cells in 3D microscopy images that permit validation. Benchmark goals of 90% correct detection, and less than 10% for false positive and false negative rates were set.

Acknowledgements and References

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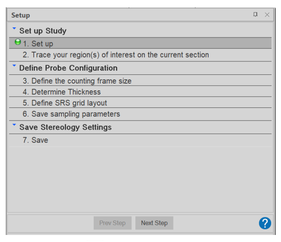
• Conflict of Interest: Content reflects use of products made by commercial employers of authors.

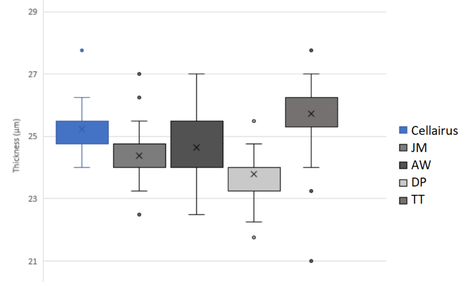
Cellairus® Workflow Operation

Stereology Parameters



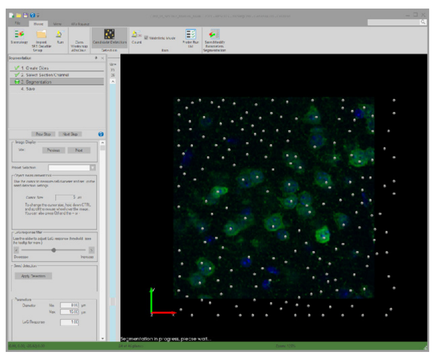
Cellairus utilizes pre-acquired 3D whole-section images or systematic random sampling (SRS) image stacks. A workflow guides the user through the process of setting up a study; delineating contours, defining the counting frame, grid spacing, and disector parameters.

Calculate Thickness



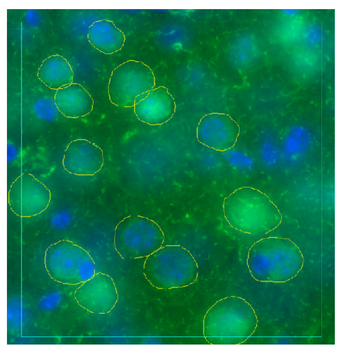
Cellairus estimates the section thickness at every site by evaluating a focus metric on all planes in the subvolume and selecting the top and bottom planes for which the focus metric rises above a stack-adapted level. Using this approach, the computed thickness variation is consistent with that obtained by manual measurement by multiple users.

Multiple Scale Laplacian of Gaussian (LoG) Region Candidate Detection



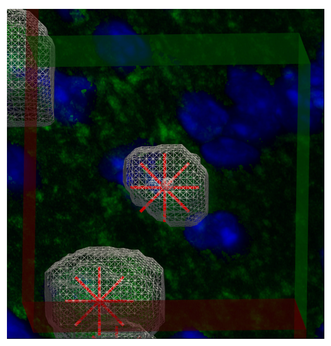
The region proposal modules that feed the classifier/regressor use multiple scale LoG filters operating on each channel. Our goal is maximum sensitivity (all valid regions must be detected) even at the expense of specificity (high number of negative object candidates).

Train Machine Learning Classifier

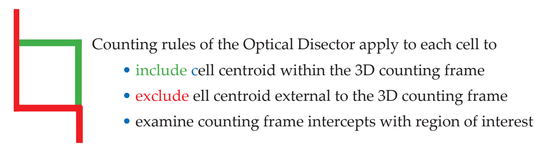


Our convolutional neural networks (CNN) are first trained by employing transfer learning. They are then fine tuned for the detection of specific cellular objects under specific tissue types, imaging conditions (confocal/widefield) and stains.

Apply Counting Rules & Calculate Population Estimates



Each counting frame site, with its automatically detected objects, is filtered to only retain the detected cells that fall within the disector.



Entire studies are rapidly processed. The nine sections of the current study were processed in ~6 minutes, while manual methods required ~6 hours.

Comparative Analysis

- For this subject, the section thicknesses measured by Cellairus were compared against section thicknesses determined manually by four trained experts. Cellairus was more consistent in section thickness measurements than manual determinations and was comparable to human determined measurements.
- Two different cellular labels, DAPI (a pan-cellular nuclear stain) and NeuN (an immunohistochemical label for neurons) highlight how differences in histological preparations can influence detection strategies & results.
- Precise marker placement by the algorithm ensures that counting rules are objectively and consistently applied throughout the study. However, manual stereology requires a subjective decision about whether an object is a cell and if it is within the counting frame. Therefore, there will be incongruencies between human and human, as well as human and machine.
- Cellairus performed well for NeuN labeled populations for both widefield and confocal images.
- Widefield images had an average True Positive Rate of 89% and a False Positive Rate of 7%.
- Confocal images had an average True Positive Rate of 89% and a False Positive Rate of 13%, however, when comparing the false positives from Cellairus to that of the manual counter, it was discovered that there were dimly labeled cells that were missed by the manual counter.

Conclusions and Future Directions

- 3D cell detection using machine learning techniques are superior to non-adaptable methods.
- Algorithms for determining section thickness align well with manual counters and enable accurate determination of the height sampling fraction.
- The use of stereology ensures that machine learning results can be validated simply and robustly. This is beneficial for testing new classifiers and running large studies alike.
- We plan to obtain ground truth data from several manual counters and compare them to data obtained by Cellairus.
- We will perform further training of classifiers for a variety of cell labelling techniques.

We are confident these strategies will produce extensible classifiers appropriate for many cell labels and regional anatomies.