# The next generation of unbiased 3D stereology for cell counting: Automatic cell counts generated by Cellairus's artificial intelligence technology are analogous to manual cell counts

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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.

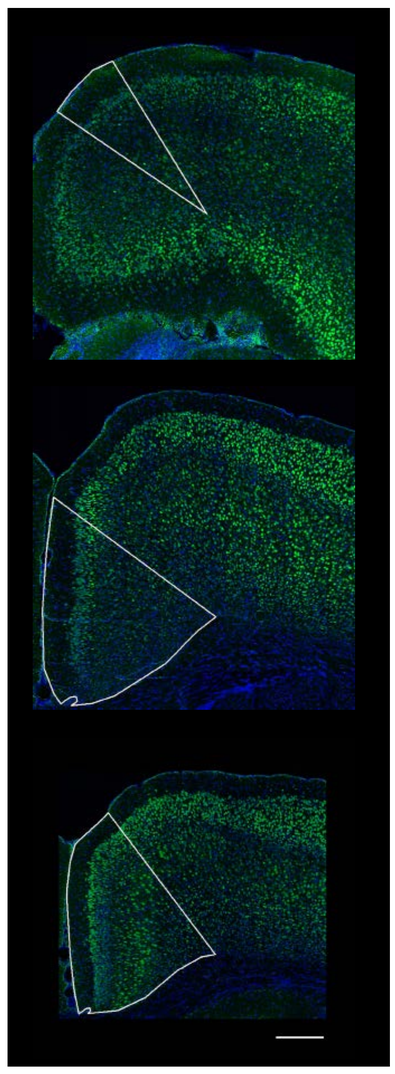
Manual and automated stereology were performed in order to assess the performance of Cellairus across multiple imaging technologies. True positive and false positive rates were quantified and compared between cell counting methods.

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



### Stereological methods

Cell number estimations were obtained using the Optical Fractionator design-based stereological 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 counting sites were used for both manual validation and automated detection.

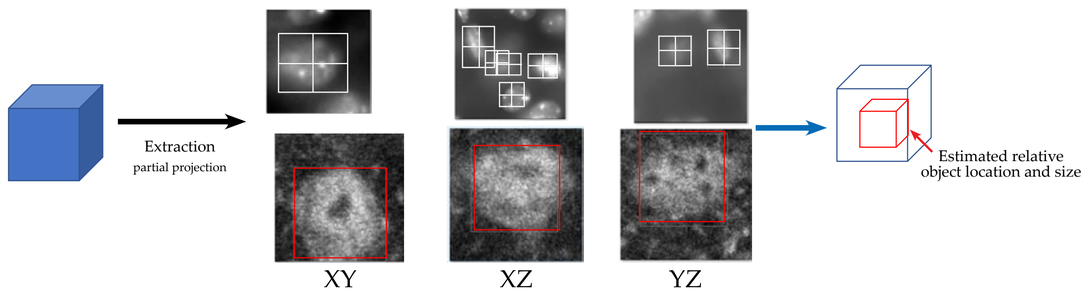
| Stereological Design Parameters | |
|---|---|
| Region of Interest | Anterior Cingulate Cortex |
| Cut Section Thickness | 30 µm |
| Section Sampling Fraction | 1/16 |
| Counting Frame Size | 50 x 50 µm |
| Grid Size | 200 x 200 µm |
| Disector Height | 15 µm |
| Guard Zone Height | 2 µm |
| Total 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. The Cellairus ML engine is based on a custom Convolutional Neural Network (CNN) architecture inspired from the FRCNN design [1]. This network can both detect and accurately locate multiple objects of various sizes within a field of view. The system operates on partial intensity projections in XY, YZ and XZ planes and combines output into 3D volumes.

Our Deep Learning engine performs the task of (i) candidate region proposal detection, (ii) classification of candidate regions into object categories (cell, artifact, etc…); (iii) bounding box regression to adjust object size and location in 3D space, (iv) object clustering using non-maximum suppression to eliminate redundant object detection.

The Deep Learning engine supports flexibility in establishing candidate regions of interest: it can use its CNNs to scan an entire 3D volume for cells of a specified size range or leverage external sources such as a multiple scale Laplacian of Gaussian (LoG) filters. LoG filter scales are tuned to match the size of the target objects and response threshold is set to a very low value to ensure detection of low contrast objects.




Stereological counting rules are applied to the updated cell models. The combination of advanced machine learning algorithms and established stereological 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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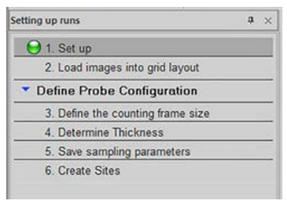
• Conflict of Interest: Content reflects use of products made by commercial employers of authors.

[1] **Faster R-CNN: Towards Real-Time Object Detection with Region Proposal Networks**; Ren, S., He, K., Girshick R., Sun, J. (2016) *arXiv* (ePrint 1506.01497)

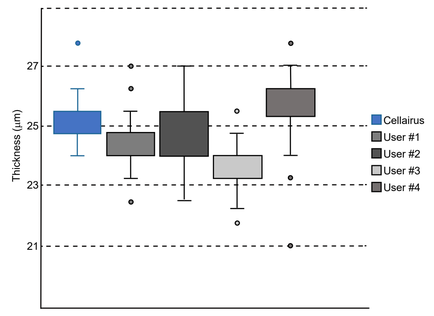
## Cellairus® Workflow Operation

### Stereology parameters



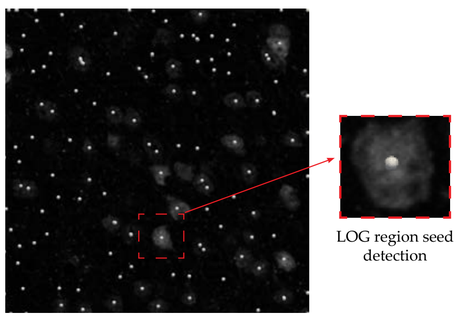

Cellairus utilizes pre-acquired 3D whole-section images or systematic random sampling (SRS) image stacks. A workflow guides the user through the process of setting up a study; delineating contours, defining the counting frame, grid spacing, and disector parameters.

### Calculate thickness



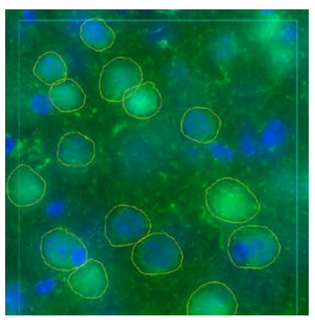

Cellairus estimates the section thickness at every site by evaluating a focus metric on all planes in the sub volume and selecting the top and bottom planes for which the focus metric rises above a stack-adapted level. Using this approach, the computed thickness variation is consistent with that obtained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

### Candidate region detection




The candidate region proposal modules use specialized CNNs or multiple scale LoG filters operating on each channel. The goal is maximum sensitivity (all valid regions must be detected) even at the expose of specificity (high number of negative candidates). The results of candidate region detection are fed to classifier and regression networks for further analysis.

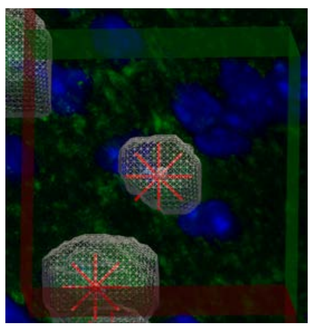
### Train machine learning classifier



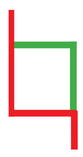
MBF Bioscience maintains and curates an extensive annotated volumetric dataset of various tissues with a variety of bio-labels and imaging modalities. This extensive data is leveraged to constantly improve a family of Deep Learning classification and regression networks, from the most generic cell detector to networks optimized for specific tissue preparation, labeling, and imaging modalities.

| Classifiers | |
|---|---|
| Cellairus R20_85 | General classifier |
| Cellairus R20_80_DAPI | DAPI classifier |
| Cellairus R20_80_NeuN | NeuN classifier |

### 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.



Counting rules of the Optical Disector apply to each cell to
- include markers completely within the 3D counting frame
- exclude markers completely external to the 3D counting frame
- examine counting frame intercepts with region of interest

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 comparable 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 DAPI and NeuN labeled populations for both widefield and confocal images.
- The images had an average True Positive Rate of 89% and a False Positive Rate of 7%.

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