An AI-Extended Solution for Performing Integrated 3D Neuro Microstructure and Nanostructure Analysis of Expansion Microscopy Data

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Introduction

Expansion microscopy (ExM) is a groundbreaking technique to visualize biological structures at nanoscale resolution. By isotropically expanding biological tissues more than ten-fold their physical size, researchers can overcome limitations of diffraction, and image messenger RNAs (mRNAs), proteins, and other biomolecules and sub-cellular structures with a variety of traditional and contemporary light microscopy techniques. More than just a method to circumvent electron microscopy for ultrastructural analysis, ExM has a myriad of advantages – including the ability for repeated hybridization, repeated antibody staining, and repeated multi-channel imaging.

To enable thorough investigation of tissues processed and imaged with ExM methods, we're presenting new software tools in Neurolucida 360 that can be used to perform comprehensive and complex 3D neuromorphological reconstruction and analysis of the spatial distribution of nanoscale populations (mRNAs, proteins, etc.) and their proximity to neuronal and vascular structures (somas, axons/dendrites, spines, synapses, vessels) across dozens of imaging channels from repeated imaging rounds. The new toolset will include automatic alignment and assembly of repeated ExM images into a single 3D registered dataset. This is a necessary and computationally-intensive step in analyzing ExM data – a task that our software will do without requiring the user to have any programming experience. Robust quantification of the size, proximity, and distribution of sub-cellular structures visible in ExM data is made easy in Neurolucida 360 with puncta detection tools that utilize machine-learning (ML) algorithms. Further software advancements include dendritic spine modelling enhancements, new ML-based axon/dendrite tracing algorithms, and integrated methods to model and quantify interactions between neurons, puncta, and the cerebral microvasculature.

Methods

Expanded Tissue Processing and Image Data Collection

In brief, the workflow of ExM consists of the following steps: (i) Biomolecules in fixed cells or tissues are functionalized with chemical handles that enable them to be covalently anchored to a polymer mesh that is evenly and densely synthesized throughout the specimen. (ii) The sample is mechanically homogenized by treatment with heat, detergent and/or proteases. (iii) Water is added that initiates polymer expansion, resulting in biomolecules being pulled apart from each other in an even, isotropic fashion.[1] Samples that are prepared with expansion techniques can then be imaged with light microscopy methods such as confocal.

Our scientific collaborators in the Edward Boyden lab at MIT conducted the expansion protocols and provided the image data of expanded tissues that were used to develop and evaluate several of the artificial intelligence (AI) extensions in Neurolucida 360 and prototype software from MBF Bioscience. The 3D microscopy image data used to highlight certain Neurolucida 360 functions (Figures 3,4,6, 7) in this presentation were processed with Expansion Revealing (ExR)[2] and Fluorescence in Situ Hybridization (FISH) technologies to visualize the morphology of GFP-labeled dendrites and dendritic spines (green) with Arc 28S RNAs (red) within the hippocampus of the mouse brain. The expansion factor using these techniques was approximately 12x. Images were acquired with a custom-built spinning disk confocal microscope.

3D Image Registration of Multiple Labeling/Imaging/Delabeling Rounds

With ExM techniques, many biomolecular markers within neuronal sub-ceullular structures can be resolved and therefore analyzed. As there is much variability in the neuronal transcriptome, comprehensive analysis may require multiple rounds of labeling mRNAs of interest with specific fluorophores. The process of labeling, imaging, and delabeling (L/I/D) a given sample is a key advantage of using ExM but it also generates complex, multi-dimensional 3D image data that existing image analysis technologies weren't designed to handle. To assess the spatial transcriptome or proteome after multiple L/I/D rounds, one needs to register each image stack from a single field of view (FOV) to each other with submicron precision.

Building off the pilot MATLAB-based software (Boyden) and our existing image montaging and registration technologies (MBF Bioscience), we have developed new, innovative techniques to seamlessly align multi-channel 3D-ExM images generated by multiple L/I/D rounds.

Implementation of 3D Convolutional Neural Network for Segmentation of 3D Images of Expanded Tissues

Our algorithms for 3D detection of neuronal cell bodies, axons, dendrites, and dendritic spines were augmented to improve the accuracy of automatic detection – as well as to heighten the efficiency of data collection by introducing fewer errors and therefore reducing the user's need to correct erroneous detections. This advancement was achieved through the implementation of a 3D convolutional neural network (CNN) built in U-Net architecture.[3]

Machine-Learning Based Puncta Detection for 3D Spatial Analysis of Subcellular Biomolecules

To enable morphological analysis of punctate objects in fixed biological tissues (e.g., mRNAs and proteins in ExM samples), we implemented an automatic puncta detection feature in Neurolucida 360. Using a variety of immunofluorescent training data from widefield and confocal microscopes, we added a machine learning based option that refines the automatic detection output through a series of validations based on shape and size.

Results

3D Image Data Registration of Multiple Imaging Rounds

- Register ExM image data from multiple color-channels in a single, high-powered FOV using rigid feature-based registration
- Register multiple multi-channel 3D FOV ExM image files into large montage image using image-based registration for optimal pair-wise alignment of overlapping stacks
- Register and compile multi-channel single-FOV stacks from subsequent L/I/D rounds into the large area montages using computationally-efficient affine registration methods

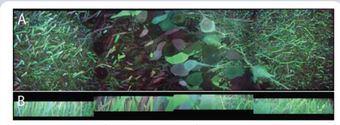

Figure 1. Example of registering FOVs of 3D ExM images labeled by BrainBow. Maximum image projections of four overlapping tiles in XY (A) and XZ (B) views. Note how the registration algorithms accomodate Z displacements during stitching assembly.

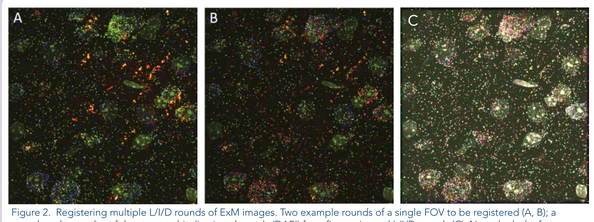

Figure 2. Registering multiple L/I/D rounds of ExM images. Two example rounds of a single FOV to be registered (A, B); a pseudo-color overlay of the structural-indicating channels (DAPI) from five registered L/I/D rounds (C). Note the lack of shadowing in C, indicating successful registration.

Dendritic Morphology Detection with Convolutional Neural Network

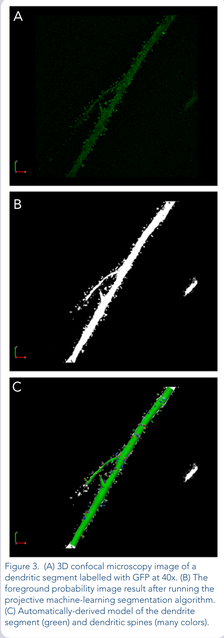

Figure 3. (A) 3D confocal microscopy image of a dendritic segment labelled with GFP at 40x. (B) The foreground probability image result after running the projective machine-learning segmentation algorithm. (C) Automatically-derived model of the dendrite segment (green) and dendritic spines (many colors).

In confocal microscopy image stacks, noise filtering and/or deconvolution may be used to improve image quality to ensure neurons appear as solidly labeled structures that can be segmented using locally-adaptive intensity thresholding techniques. ExM image stacks, however, are characterized by a much more granular appearance, with fluorophores distributed inside cell processes at varying densities, and this limits the ability of local techniques to distinguish between foreground and background. Machine learning-based segmentation is able to better classify image voxels by incorporating data-driven models that incorporate a broader spatial window of surrounding voxels. We have employed a U-Net architecture[1] to assign a foreground probability to each voxel. The network is trained using a set of manually labeled image patches.

The computed foreground probability is used to create the segmented image stack that can be readily used with our existing algorithms[4] to digitally reconstruct neuronal morphology at the increased effective resolution afforded by Expansion Microscopy.

Figure 4. An example of Neurolucida 360's automatic dendritic spine modeling and editing functions. The volumetric surface of the spine models in the field of view are visible. One of the spines has been selected in the software's "Edit" model to visualize the five points and centerline that make up the spine "backbone" structure.

The neuromorphological models generated by the automatic functions can be further refined with point editing tools. The information about each spine model in the image is stored in the MBF Neuromorphological File Format, an .xml specification that is both standardized and openly documented online.[5] Each dendritic spine model is embedded on a dendritic tree segment and has five internal points (e.g., the spine head and neck) and corresponding morphological value properties associated with it. To better elucidate the morphological differences between spines in high-resolution expanded tissues, these five points allow researchers to extract more specific measurement such as backbone length, rather than just total extent (surface of dendrite to head).

Machine-Learning Based Puncta Detection for 3D Spatial Analysis of Subcellular Biomolecules

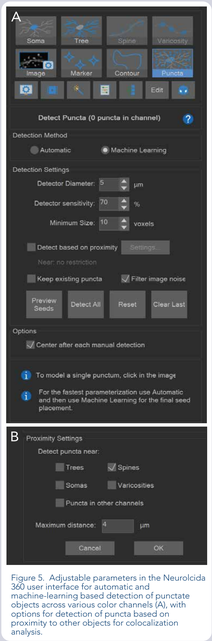

Figure 5. Adjustable parameters in the Neurolucida 360 user interface for automatic and machine-learning based detection of punctate objects across various color channels (A), with options for detection of puncta based on proximity to other objects for colocalization analysis.

Automatic Puncta Detection

Neurolucida 360's advanced puncta detection tools automatically model and quantify the morphology of a wide variety of biological punctate objects – such as mRNAs, synapses, boutons, proteins, cell nuclei or nucleoli, and whole cells. The detector utilizes three user-defined criteria: detector size, detector sensitivity, and minimum size. The detector size parameter indicates the diameter of the detector "seeds". Seeds of the designated diameter are automatically placed throughout the 3D image on local regions of high intensity across a given color channel. Of these identified potential punctum, only the objects that fit within the detector sensitivity and detector size criteria are detected. The 3-dimensional boundaries of each individual puncta are calculated and modeled as a unique 3D surface.

Machine-Learning Precision

By employing the machine-learning method during detection, Neurolucida 360 applies a trained classifier to refine the result. The machine learning method combines the user-defined size and sensitivity criteria with a specialized appearance classifier, leading to a more accurate selection of puncta of interest.

Object-Based Colocalization

As an alternative to global puncta detection, we implemented a method for object-based colocalization. This option enables the user to detect puncta based on their proximity to biological objects – somas, trees, spines, varicosities, or puncta in other color channels. This option has significant relevance to analyzing ExM imaging as it can produce high-order channel counts with specific and non-specific puncta alongside highly-specific morphological labels, especially with multiple L/I/D rounds. The Neurolucida 360 image handling system is compatible with hundreds of image channels, each of which can be isolated to run channel-specific object segmentation, reconstruction, and analysis.

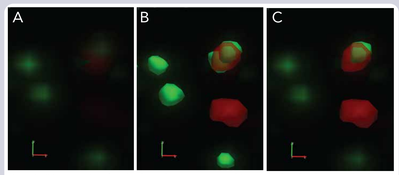

Figure 6. XY perspective of a subvolume showing punctate objects in both red and green color channels before detection (A), after machine-learning based detection of both channels (B), and after machine-learning based and object-based proximity detection of puncta from other channel (C).

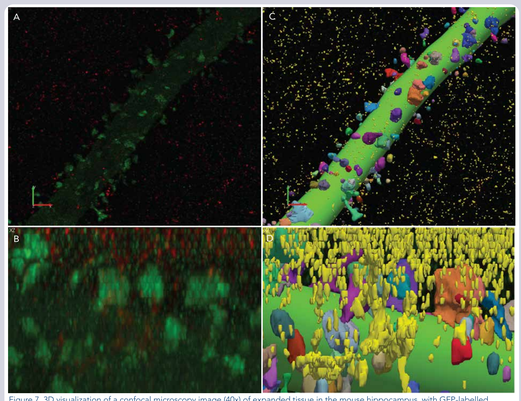

Figure 7. 3D visualization of a confocal microscopy image (40x) of expanded tissue in the mouse hippocampus, with GFP-labelled dendritic segments and spines (green) and Arc 28S RNAs (red) from XY and XZ perspectives (A,B). Automatically-derived model of the GFP dendrite segment (green) and dendritic spines (many colors) using U-net segmentation and reconstruction algorithms. The Arc 28S RNAs have been detected and modelled (yellow) automatically using Neurolucida 360's puncta detection tool (C, D).

Discussion

Structural and Spatial Morphological Analysis

A large and varietal portion of neuronal mRNAs are found in the axonal and dendritic processes of the neurons. Most excitatory synapses in the CNS are localized to the dendritic spines. Within a single presynaptic or postsynaptic site in the central nervous system, more than 1,000 different proteins can be found. Not only are they present in such magnitude, but there is considerable variation between types of synaptic proteins. A healthy nervous system relies on functional synaptic transmission. When transmission goes awry, synaptic dysfunction is a significant contributing factor to a plethora of neurological afflictions.

With innovative ExM and Neurolucida 360, researchers can fully explore the spatial transcriptome and proteome of the central nervous system on the micro and nanoscale. Together, these technologies will be an invaluable system for developing and evaluating novel treatments against neurodevelopmental, neuropsychiatric, neurodegenerative, neuroimmune, and neurological diseases.

The advanced image registration, segmentation, and reconstruction tools in Neurolucida 360 produce data models of expanded biologies that can be exported to Neurolucida Explorer for robust morphological analysis of the structure and orientation of neurons - but also the local distribution and colocalization of surrounding mRNA and protein populations.

Analysis of Expanded Dendritic Spines

- Automatic classification of filopodia, mushroom, stubby, and thin spines
- Volume, surface area, density
- Individual spine analysis:
  - Plane angle
  - Backbone length
  - Head to neck length
  - Head position and diameter
  - (ExM) neck diameter

Puncta Morphology and Distribution

- Number, volume, surface area
- Distance to additional structures
- Post-detection object-based colocalization (% overlap) of puncta to trees, spines, varicosities, and other puncta
- Nearest neighbor
- Puncta within regions of interest, across layers

Future Directions

- Develop novel morphometric analyses for further interrogation of ExM and ExR data
- Implement advanced model data storing mechanisms to support fast spatial queries
- Implement analysis of mRNA multiplexed signals
- Implement interactive 3D visualizations of spatial distributions across large domains
- Develop registration of multiple imaging rounds with complex spatial distortions
- Implement non-circular cross-section modeling of dendritic processes

References & Acknowledgements

[1] [Add ExM protocol reference]

[2] [Add ExR protocol reference]

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Conflict of interest: MBF Bioscience is a commercial entity, and the authors affiliated with it are company employees.

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