

CryoVesNet: A Dedicated Framework for Synaptic Vesicle Analysis in Cryo-Tomograms

This manuscript ([permalink](#)) was automatically generated from aseedb/deep-prepyto-paper@edd83ad on August 15, 2023.

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Abstract

Cryo-electron Tomography (Cryo-ET) has the potential to reveal cell structure down to atomic resolution. Nevertheless, cellular cryo-ET data is often highly complex, and visualization, as well as quantification, of subcellular structures require image segmentation. Due to a relatively high level of noise and anisotropic resolution in cryo-ET data, automatic segmentation based on classical computer vision approaches usually does not perform satisfactorily. For this reason, cryo-ET researchers have mostly performed manual segmentation.

Communication between neurons relies on neurotransmitter-filled synaptic vesicle (SV) exocytosis. Recruitment of SVs to the plasma membrane is an important means of regulating exocytosis and is influenced by interactions between SVs. Cryo-ET study of the spatial organization of SVs and of their interconnections allows a better understanding of the mechanisms of exocytosis regulation. Extremely accurate SV segmentation is a prerequisite to obtaining a faithful representation of SVs state of connectivity. Hundreds to thousands of SVs are present in a typical synapse, and their manual segmentation is a time-consuming exercise, which has been recognized as a bottleneck by the community.

Several attempts to automate vesicle segmentation by classical computer vision or machine learning algorithms have not yielded very robust results. We addressed this problem by designing a workflow consisting of a U-Net convolutional network followed by post-processing steps. This combination yields highly accurate results. Furthermore, we provide an interactive tool for accurately segmenting spherical vesicles in a fraction of the time required by available manual segmentation methods. This tool can be used to segment vesicles that were missed by the fully automatic procedure or to quickly segment a handful of vesicles while bypassing the fully automatic procedure. Our pipeline can in principle be used to segment any spherical vesicle in any cell type as well as extracellular vesicles.

Introduction

The fine architecture of cells can be investigated by cryo-electron tomography (cryo-ET) [1]. Cellular structures are preserved down to the atomic scale through vitrification and observation of the samples in a fully hydrated state. When a macromolecule is present in a sufficient number of copies in the cells imaged by cryo-ET, it is possible to obtain its atomic structure *in situ* using subtomogram averaging [2,3]. Cellular cryo-ET datasets are usually extremely complex, making them difficult to analyze. This is aggravated by the sensitivity of biological samples to electron radiation, which limits the signal-to-noise ratio in cryo-ET datasets [4]. Tomographic reconstructions are generated from a series of images of the sample acquired at different viewing angles. The geometry of the samples prevents acquisition at certain angles, resulting in anisotropic spatial coverage. The resolution in the directions close to the axis of the electron beam incident on the untilted sample is strongly reduced. This effect, commonly referred to as the missing-wedge artifact, further complicates data analysis. In particular, organelles fully bounded by a membrane appear to have holes at their top and bottom (relative to the electron beam axis) [4].

The synapse is a specialized cellular contact at which information is transmitted from a neuron to another. The former neuron is called presynaptic and the latter is postsynaptic. In most cases, the signal is transmitted by the release of neurotransmitters into the intercellular space.

Neurotransmitters are stored in SVs and are released following the fusion of an SV with the presynaptic plasma membrane. A synapse contains hundreds of SVs and their mobility and recruitability for neurotransmitter release depends on inter-vesicle interactions through so-called connector structures [5]. The characterization of these interactions can be performed automatically with the Pyto package, which implements a hierarchical connectivity approach to segment connectors

[6]. For accurate connector segmentation, accurate segmentation of SVs is a prerequisite. To date, SV segmentation has been performed manually, but given the large number of SVs per dataset, it is an extremely time-consuming process. Typically, one person spends 3 to 8 working days segmenting a single dataset. Attempts to perform this task automatically based on classical computer vision algorithms have not yielded sufficiently accurate performance [7].

To alleviate this situation, we decided to develop an approach based on deep learning.

Convolutional neural networks (CNN) have been successfully employed to segment cryo-ET data [8]. Although entirely satisfying for visualization purposes, this approach has not met the requirements of Pyto. A recent publication described accurate SV segmentation of transmission electron microscopy images using CNN, but it is limited to 2-dimensional (2D) images of resin-embedded synapses [9]. In the first study, cryo-ET data are decomposed in individual 2D slices, which are handed as separate inputs to the CNN. The independent output 2D prediction images are reassembled in a 3-dimensional (3D) stack [8]. As discussed above, membranes oriented approximately parallel to the plane of the 2D tomographic images are not resolved. In the absence of contextual knowledge of the other 2D images, the CNN fails to segment these regions of the vesicles. Hence, spherical vesicles appear open, whereas we expect closed spherical objects. A recent publication addressed this issue by implementing a downstream fitting step based on the Gaussian process, allowing for the smooth closure of the membranes [10]. We followed a different approach and used a 3D U-Net CNN that takes 3D images as input [11]. Weigert et al. [12] implemented a U-Net for content-aware restoration (CARE) of 3D fluorescence microscopy datasets. They showed that it can restore information from anisotropic and very noisy datasets. Such networks have been used in the last couple of years in cryo-ET analysis, mainly to perform denoising and object detection [13,14,15]. We implemented a 3D U-Net based on CARE building blocks and trained it with manually segmented datasets. This method provided good accuracy and was only slightly affected by the missing wedge artifact. Nevertheless, it was not sufficient for our downstream Pyto analysis. Hence, we developed a post-processing method, which transforms the segmented objects into spheres and refines their radius and center location. The procedure includes an outlier detection procedure. This leads to a substantial improvement in accuracy, which is reflected in Pyto performances comparable to those obtained after manual SV segmentation. We also introduce a semi-automatic method to quickly fix wrongly segmented or missed SVs.

Although our set of procedures was developed with the use case of SV segmentation in mind, it can be used to segment any other types of biological spherical vesicles, such as transport vesicles, secretory vesicles, endocytic vesicles, and extracellular vesicles.

Results

In view of the effort required for the manual segmentation of SVs, we decided to develop an automatic segmentation procedure. Since we had previously manually segmented a number of tomograms with the program IMOD, we could use these segmentations as the ground truth [16]. We trained a U-Net with a set of 9 segmented tomograms of rat synaptosomes (see Materials and Methods).

We sought to further improve segmentation accuracy by feeding the probability mask output by the U-Net to a series of postprocess steps (Figure 1). Three sets of tomograms were used to assess the performances of the pipeline:

1. Training dataset : The 9 rat synaptosome tomograms that had been used for U-Net training
2. Testing dataset: Another 9 rat synaptosome tomograms

3. Testing dataset for generizability: 12 mouse astrocytic and primary neuronal culture tomograms).

Each tomogram was split into patches of 32^3 voxels. These patches were fed in the U-Net, which output a probability mask for those patches. To obtain a complete probability mask, the patches are stitched back together. The probability mask was binarized first with a global threshold value and then with an adaptative localized thresholding steps (Figure 1, Figure 2).

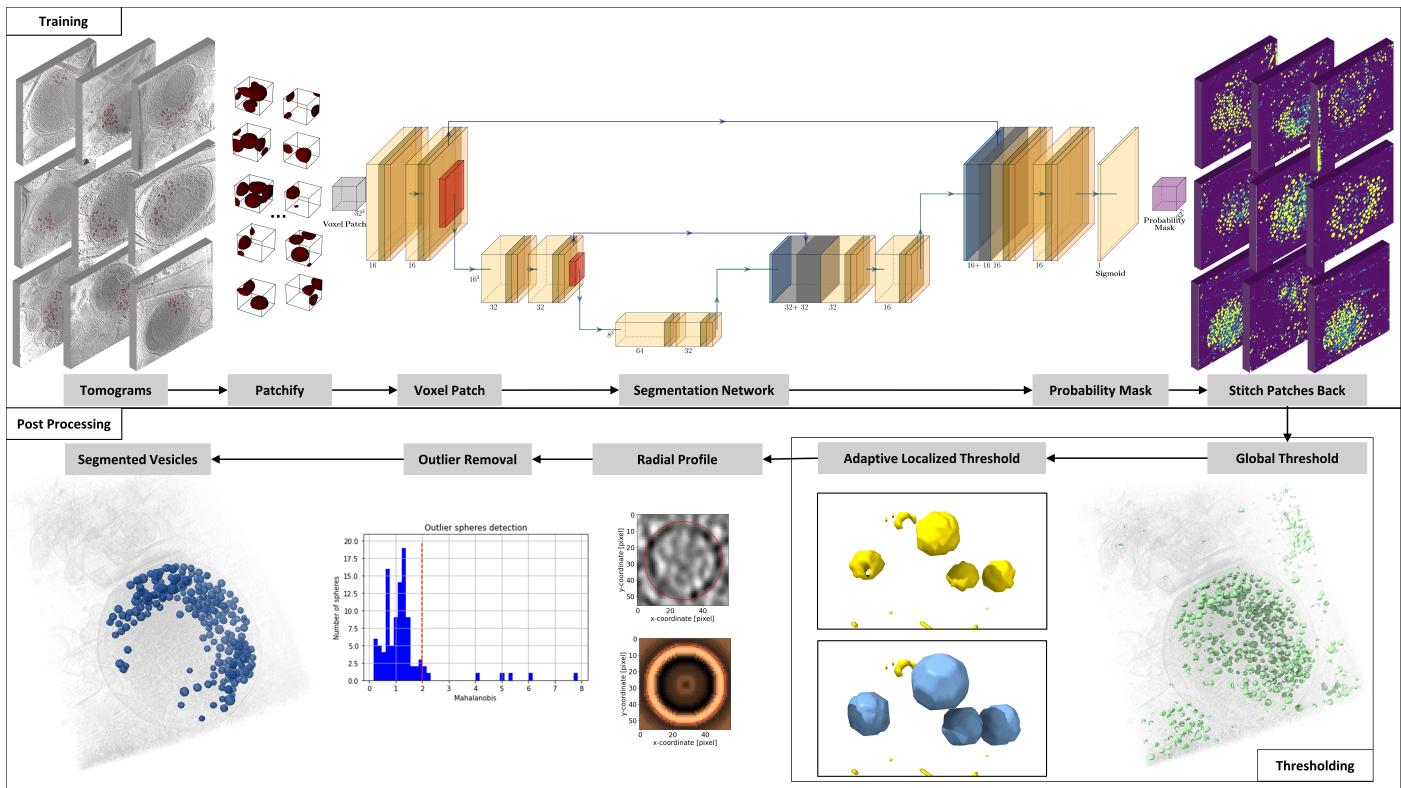


Figure 1: Pipeline of automatic segmentation. a) Tomograms b) Splitting in 3D patches c) Segmentation Network/ trained U-Net d) probability masks e) stitching patches back together f) thresholding g) radial profile h) outlier removal i) Segmented Vesicles

While a global threshold serves as an initial step in vesicle segmentation, it often falls short in complex scenarios, especially when vesicles are in close proximity or exhibit overlap. To enhance the segmentation accuracy in such cases, we employ an adaptive thresholding methodology that capitalizes on the probability mask generated by our deep learning architecture.

Initial Segmentation and Thresholding:

The segmentation journey commences with the alignment of the image with its associated deep labels and the probability mask. To ensure a comprehensive segmentation, the probability mask's edges are meticulously cropped, safeguarding against inadvertent truncation of boundary-residing vesicles. An optimal threshold, derived from the image and the probability mask, undergoes fine-tuning through a coefficient adjustment. This refined threshold then serves to binarize the mask, with connected components subsequently labeled, delineating individual vesicles.

Collision Resolution through Adaptive Thresholding:

In scenarios where vesicles are densely packed, collisions become a challenge, often leading to the amalgamation of multiple vesicles into a singular segmented entity. Addressing this, potential collision vesicles are pinpointed based on specific geometric attributes, such as their extent and area z-score. For each vesicle identified under this criterion, a localized region surrounding the vesicle is extracted.

This region is then subjected to a masking process, ensuring focus remains solely on the vesicle in question.

The essence of collision resolution revolves around the iterative modulation of the threshold to attain a more granular segmentation. Commencing from the established threshold, it's progressively augmented until the connected components within the localized region amplify, signaling a potential vesicle separation. This adaptive modulation persists until a threshold is identified that adeptly segregates the vesicles or until a set precision threshold is attained.

Expansion and Removal of Minuscule Vesicles:

Post-segmentation, certain vesicles might be represented as notably smaller than anticipated. To address this, an expansion strategy is employed, iteratively lowering the threshold to encapsulate more of the vesicle's structure. If expansion fails to achieve the desired size, these diminutive vesicles are removed, ensuring the final segmentation remains representative of genuine vesicle structures. In conclusion, our adaptive thresholding technique, combined with collision resolution, ensures a more accurate and refined segmentation of vesicles in cryo-ET data. This approach capitalizes on the rich information present in the probability mask, providing a robust solution to the challenges posed by closely packed vesicles.

_more detail about global and adaptive localized threshold* For further optimization of the mask, outliers were removed. Removed outliers mostly consisted of vesicles which were only partially segmented, and vesicles which masks were adjacent due to proximity. The removed masks, which only partially traced the vesicles, were reevaluated by reducing or expanding their radius (Figure 3). (_was the center of the vesicles also reevaluated?* _Its not clear or might be false sentence we didnt remove or re evaluate the mask?*

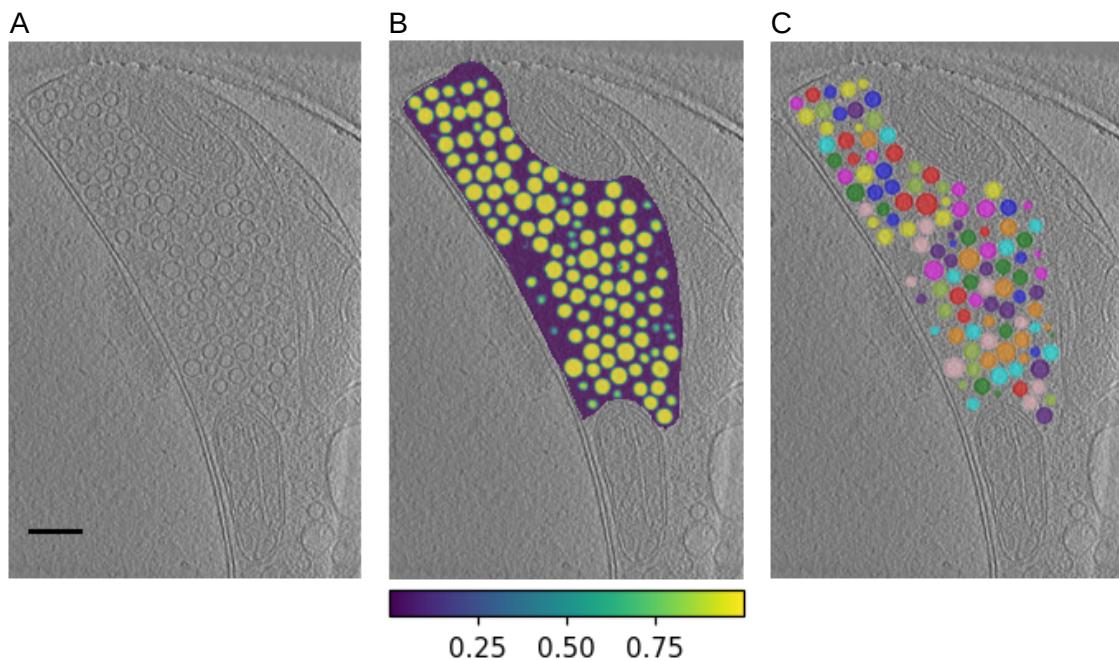


Figure 2: A 2D slice of an automatically segmented dataset. A) A section through a presynaptic terminal in a neuron tomogram. B) Predicted probability mask restricted to the segmentation region. Purple corresponds to a low SV probability and yellow to a high SV probability. C) Instance mask of the vesicles after post processing.

Each segmented vesicle undergoes a radial profile refinement. Representing vesicles as spheres, their position and radius are iteratively adjusted to mirror their actual structure in the tomogram. By computing the radial average and updating the vesicle's radius, this method ensures the segmented vesicles are a true representation of their form in the tomogram.

The radial profile serves as a pivotal tool in refining the segmentation of vesicles. By analyzing the radial distribution of intensity around the vesicle's centroid, we can discern the vesicle's true boundary. This method ensures that the segmented vesicles closely match their actual structure in the tomogram by iteratively adjusting their position and radius. The radial average is computed, and the vesicle's radius is updated based on the radial distance of the vesicle membrane's center and its thickness. This iterative refinement continues until the vesicle's representation aligns with its true structure.

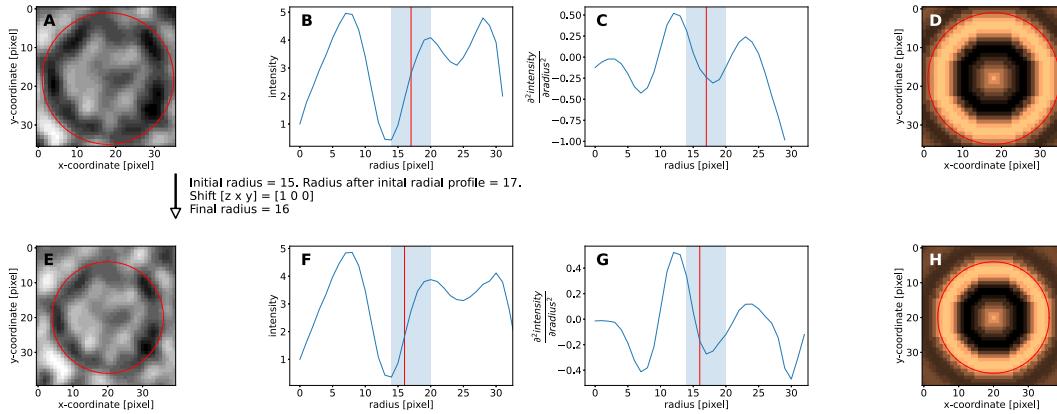


Figure 3: Vesicle radius and position through radial profile and cross-correlation Radial Profile Refinement A) couple of vesicles are not centered B) Radial Profile. Blue range is from membrane center to outer white halo center, this is the search range for the optimal radius. (smoothed by gaussian filtering) C) second derivative of radial profile E, F, G) Same as above columns after refinement

The adjacent vesicle masks were separated (Figure _missing*). _how?*

_**missing Figure- Splitting adjacent vesicles. A) Examples of tomogram, no labels; B) raw label with connected vesicle-labels; C) modified label with seperated vesicles ---> for software: IMOD**

The Dice coefficient was used to track the global congruence between the manually segmented mask and the predicted mask within the different tomograms (Figure 4). To quantitatively assess the accuracy of our segmentation approach, we employed the Dice coefficient. This metric gauges the similarity between the manually annotated vesicle masks and the masks predicted by our model. A higher Dice coefficient indicates a closer match between the manual and predicted segmentations, providing a measure of the model's performance. The results, visualized in Figure 4, showcase the global congruence across different tomograms, highlighting the efficacy of our segmentation pipeline.

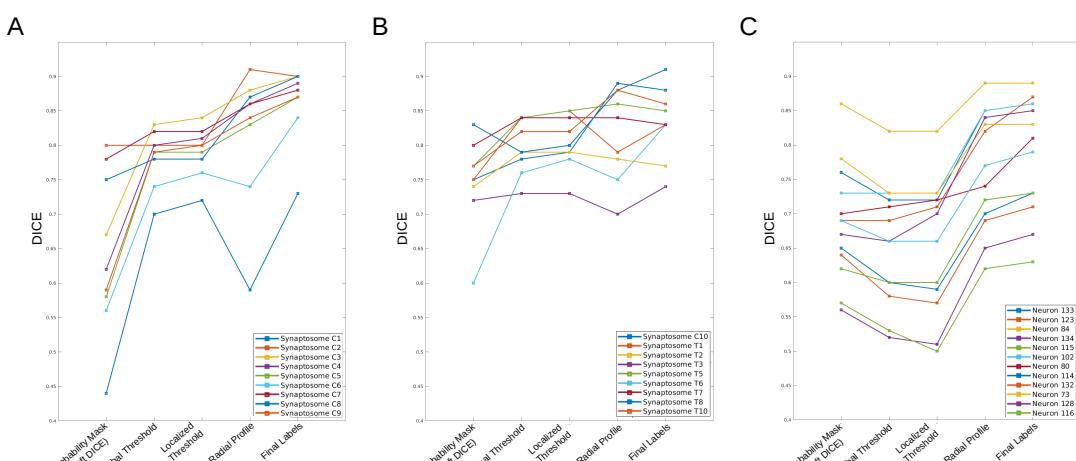


Figure 4: Dice developement during post-processing Dice developement at different post processing steps of initial predicted mask (different colors correspond to different tomograms): A) synaptosomal training datasets B) synaptosomal test datasets c) neuron test datasets

Table 1: Evaluation of the segmentation on the synaptosome train set. Mask Dice: Mask Dice coefficient for the predicted mask; Final Label Dice: Dice coefficient after post-processing; δd : diameter error on correctly detected vesicle; Δc : average error center (nm); Number of Vesicles: number of expected vesicles; TP: True Positive; FN: False Negative; FP: False Positive

Dataset	Mask Dice	Final Label Dice	δd	Δc (nm)	Number of Vesicles	TP	FN	FP
SC 1	0.44	0.73	0.07	$2.55 \pm 1.5_6$	223	198	26	49
SC 2	0.8	0.9	0.05	$2.12 \pm 1.0_6$	105	103	2	1
SC 3	0.67	0.9	0.05	$1.86 \pm 1.2_4$	128	127	1	6
SC 4	0.62	0.89	0.03	$1.78 \pm 0.9_2$	144	141	3	4
SC 5	0.58	0.87	0.04	$1.86 \pm 1.0_0$	214	209	5	13
SC 6	0.56	0.84	0.04	$1.92 \pm 1.0_5$	104	102	2	16
SC 7	0.78	0.88	0.06	$1.86 \pm 0.9_0$	184	184	0	16
SC 8	0.75	0.9	0.05	$1.70 \pm 0.9_3$	132	126	6	1
SC 9	0.59	0.87	0.05	$1.87 \pm 0.9_1$	135	132	3	14
Average	0.64 ± 0.11	0.86 ± 0.05	0.05	$1.95 \pm 1.0_8$	152.22	97.00%	3.00%	7.30%

Table 2: Evaluation of the segmentation on the synaptosome test set (same sample type as the train set). For the meaning of the columns, see Table 1.

Dataset	Mask Dice	Final Label Dice	δd	Δc (nm)	Number of Vesicles	TP	FN	FP
SC 10	0.75	0.88	0.07	1.86 ± 1.18	129	123	6	5
ST 1	0.75	0.83	0.11	2.66 ± 1.52	699	687	12	33
ST 2	0.74	0.77	0.11	2.27 ± 1.84	122	117	5	2
ST 3	0.72	0.74	0.11	3.64 ± 2.22	434	397	37	57
ST 5	0.77	0.85	0.08	2.20 ± 1.26	535	526	9	25
ST 6	0.6	0.83	0.07	2.02 ± 1.12	373	353	20	42
ST 7	0.8	0.83	0.06	2.22 ± 1.14	110	107	3	9
ST 8	0.83	0.91	0.04	2.09 ± 1.04	100	99	1	2
ST 10	0.77	0.86	0.05	1.96 ± 1.04	77	74	3	6
Average	0.75 ± 0.06	0.83 ± 0.05	0.08	2.32 ± 1.43	286.56	96.30%	3.70%	6.10%

Table 3: Evaluation of the segmentation on the neuron test set (different sample type as the train set). For the meaning of the columns, see Table 1.

Dataset	Mask Dice	Final Label Dice	δd	$\Delta c (nm)$	Number of Vesicles	TP	FN	FP
N 133	0.76	0.86	0.05	2.16 ± 1.3 2	523	467	56	8
N 123	0.64	0.71	0.05	2.05 ± 1.1 8	66	58	8	2
N 84	0.86	0.89	0.06	1.44 ± 0.7 5	498	484	14	1
N 134	0.56	0.67	0.09	2.87 ± 2.5 0	638	384	254	63
N 115	0.57	0.63	0.08	3.56 ± 3.2 3	170	123	47	32
N 102	0.73	0.86	0.05	1.47 ± 0.7 9	103	86	17	1
N 80	0.7	0.81	0.07	2.67 ± 2.0 0	111	102	9	3
N 114	0.65	0.73	0.07	2.68 ± 1.7 9	131	93	38	9
N 132	0.69	0.87	0.03	1.65 ± 1.2 6	135	129	6	32
N 73	0.78	0.83	0.06	2.93 ± 2.0 0	526	483	43	2
N 128	0.67	0.85	0.04	2.33 ± 1.7 0	252	232	20	19
N 116	0.62	0.73	0.07	2.38 ± 1.8 2	296	207	89	35
Average	0.69 ± 0.09	0.79 ± 0.09	0.06	2.35 ± 1.8 3	287.42	83.60%	16.40%	7.90%

Our method transfers well across datasets even without fine-tuning which shows robustness and generalization.

Comparison of manual segmentation with automatic deep-learning based segmentation

In the realm of cryo-electron tomography (cryoET), accurate segmentation of synaptic structures is paramount for understanding their spatial organization and function. Traditional manual segmentation, while precise, is time-consuming and often limited in scope. The advent of deep learning-based segmentation offers a promising alternative, providing both speed and scalability.

The traditional approach to manual segmentation in cryoET studies often restricted analyses to specific distances from the active zone, typically up to 250 nm. This limitation inherently narrows the scope of synaptic or neuronal analyses. However, with the integration of deep learning-based segmentation, it's now feasible to analyze entire synaptosomes or neurons comprehensively. Not only does this method expedite the segmentation process, but it also enhances the accuracy and breadth of the analysis. Furthermore, interactive tools like Napari significantly augment this process, allowing researchers to swiftly rectify false positives and negatives by adding or removing vesicles as needed, ensuring the highest level of accuracy in the final segmented output.

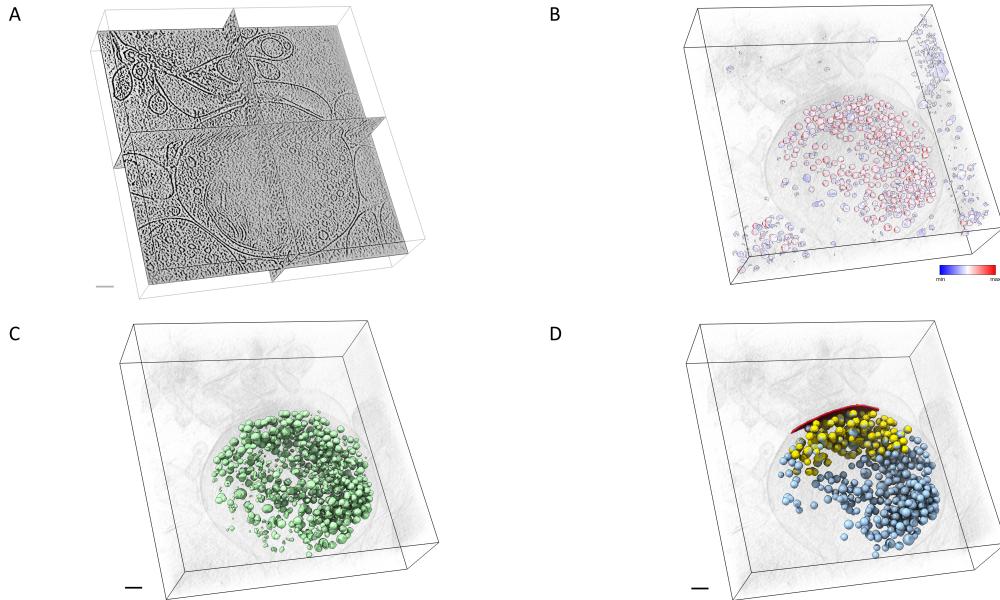


Figure 5: A: A 3D box representation of a synaptosome, visualized in an orthogonal view. The image showcases the intricate details of the structure with a scale bar indicating 100 nm.B: A probability map derived from the 3D U-Net segmentation. The map is presented schematically, with a color gradient ranging from blue (0.9968) to red (1), indicating the likelihood of synaptic vesicle presence.C: A tomogram post optimal global thresholding. This image provides a detailed view of the segmented structures, highlighting the precision of the automated segmentation process.D: The final representation of synaptic vesicle segmentation post-processing. Synaptic vesicles are depicted in light blue, overlaid on the ground truth obtained from expert annotations. The active zone is distinctly marked in red. The scale bar for this image is also set at 100 nm.

#!#[3D model of manual segmented and automatically segmented synaptosome.](#)

Hierarchical connectivity segmentation of presynaptic terminals

Pyto is a software package designed for the analysis of pleomorphic membrane-bound molecular complexes in 3D images, particularly in the context of cryo-electron tomograms of neuronal synapses. A key feature of Pyto is its ability to accurately segment connectors and tethers within the pre-synaptic terminal, a task that requires a high level of precision. This segmentation process is hierarchical and connectivity-based, detecting densities interconnecting vesicles (connectors) and densities connecting vesicles to the active-zone plasma membrane (tethers).

In the study of synaptic vesicles regulation, the precision of segmentation is crucial. Accurate segmentation of connectors and tethers is essential as these structures play a significant role in the regulation of synaptic vesicles. Software that segments subcellular compartments, such as Pyto, provides a valuable tool in this process.

We have developed a software that is designed to be compatible with programs like Pyto. This compatibility allows us to extract more detailed information from Cryo-Electron Tomography (Cryo-ET), even in densely populated *in situ* conditions. By using our software in conjunction with programs like Pyto, we can gain a deeper understanding of synaptic vesicles regulation. Importantly, our software aims to eliminate the need for manual segmentation, enhancing the efficiency and accuracy of the process.



Figure 6: 3D Model of Cultured Mouse Neuron: Left - Tomogram; Left Center - Schematic segmentation with cell outline (light blue), mitochondria (dark blue), lysosomes (green), microtubules (dark magenta); Right Center - Vesicles segmented using VesPy and connectors via Pyto; Right - 3D representation of vesicles in the presynaptic terminal..

Discussion

While the Dice coefficient is a good global measure to assess the predictions in comparison to the ground truth, it is difficult to assess local segmentation accuracy. For example, a single generated vesicle label containing several close connected vesicles would not be practical for further analysis for the researcher although it could have almost the same dice value. What is important for actual usage of the software would be the number and percentage of true-detected vesicles, false-positive and false-negative rates.

Center error: If we measure each axis error it will reveal that human bias in segmentation is more affected on the Z-axis. [we didn't show it in number but its checked the hypothesis] _we cannot claim something without showing it. ---> this would belong into results*

3d unet good for 3D processing recent Nature methods paper by Ben Engel, DeepFinder -> Relion for STA creates mask to find more using dl -what are they doing, maybe compare that in the text, different aims; we might compare results we achieve (keep as bonus, revision)

Outlook

implement automatic cell-outline and active zone segmentation as deep learning workflow using UNet
implement automatic connector and tether segmentation as a deep leaning workflow using UNet

Materials and methods

Cryo-electron Tomography Datasets

Two datasets of different origin were used as input and test subjects for the automatic segmentation pipeline, respectively. They consisted in rat synaptosomes primary neuron cultures derived from mice. The preparation procedure of the samples from which the datasets were obtained as well as the biological analysis of these datasets was previously reported. [17].

Synaptosome preparation

Preparation of astrocytic and neuronal culture

Plunge freezing and spray mixing (Vitrification)

Cryo-electron microscopy (Cryo-ET)

Tomogram reconstruction

Ground truth annotation for organelles, cytoplasm, and membranes

Ground truth vesicles annotation

CNN Preprocessing

Binarization of probability masks

Post Processing on Binarized map (adaptive threshold, radial profile, outlier removal)

Evaluation metrics

Computational setup

Manual segmentation and automatic interboundary segment detection

Manual segmentation of SVs, mitochondria, the active zone PM, and of the segmentation region was done in IMOD (??Figure S4A&B??) [16]. SVs were segmented as spheres. The segmentation region marked the region to be analyzed by Pyto [6]. The analysis by Pyto was essentially the same as described previously [5,6]. In short, the segmented region is divided in 1 voxel thick layers parallel to the active zone for distance calculations. A hierarchical connectivity segmentation detects densities interconnecting boundaries. The boundaries were synaptic vesicles and the active zone PM. Detected intervesicular segments are termed connectors and segments connecting vesicles to the active zone PM are called tethers (Figure _add figure number*). Distance calculations respective to SVs were done from SV center. The segmentation procedure is conservative and tends to miss some tethers and connectors because of noise. Consequently, the numbers of tethers and connectors should not be considered as absolute values, but rather to compare experimental groups. As it was done before, an upper limit was set between 2100 and 3200 nm³ on segment volume. The tomograms that were used for this analysis were binned by a factor of 2 to 3, resulting in voxel sizes between 2.1 and 2.4 nm.

Pre-processing of manual segmentation outputs from IMOD for further use (jupyter notebook pre-pyto)

probably not necessary to mention output from IMOD to prepyto input label file procedure

put this somewhere else

Dataset description

The used datasets included a total of 30 tomograms with heterogeneous pixel sizes, defocus and resolution.

1. 9 synaptosome datasets were used for training and validation.
2. 9 synaptosome datasets was used for test.
3. 12 Neuron dataset were used for assessing transfer learning potential.

Training Dataset Construction

In the preparation of our training dataset, we utilized segmented 3D image volumes. The primary volume was systematically divided into 32x32x32 sub-volumes. To ensure the relevance and richness of the data, only those sub-volumes that were sufficiently occupied by vesicles, specifically containing more than 1000 voxels, were retained. This meticulous approach was adopted to ensure that the training set effectively captured the intricate cellular structures, optimizing it for subsequent deep learning analyses.

Stiching Pathces Back Together (building probability map?)

In deep learning segmentation for cryoET tomograms, smaller patches are segmented for computational efficiency. Our U-Net model, trained on 32-voxel patches, utilizes a 24-voxel region of interest (ROI). To mitigate tiling effects during testing, the network input can be expanded to larger volume like 64 voxels. The tomogram undergoes padding to align with the ROI, ensuring reduced edge artifacts. Segmentation is executed in tiles, where the U-Net predicts the synaptic vesicle probability for each tile. Only the central part of the segmented patch, corresponding to the ROI, is retained. This approach guarantees consistency in overlapping regions between adjacent tiles. Finally, the segmented tiles are reassembled, yielding a continuous synaptic vesicle probability map of the entire volume.

Network architecture and training procedure

We used a U-Net with two downsampling stages and two convolutional layers per stages, with a kernel size of 3, and ReLU activation function based on the open-source CARE framework (Figure 8 [12]). Datasets were prepared by splitting the 3D tomographic volume of synaptosomes into 32^3 -voxel subvolumes and keeping only subvolumes occupied by a sufficient amount (> 1000 voxels) of binarized vesicle label. 860 subvolumes were used for training and 100 subvolumes were used for validation. We used the Adam optimizer on a binary cross-entropy loss function.

The learning progress was tracked by calculating the Dice coefficient and the loss value after each training epoch (Figure 2). The Dice coefficient for the training set was initially ~0.25 and rose to over 0.9 after approximately 50 epochs, while for the validation set, it rose to 0.8. The loss for the training set went from 0.55 and to values below 0.05 after 50 epochs while for the validation set it went from 1 and to slightly below 0.3 after the initial 50 epochs and then rose slightly.

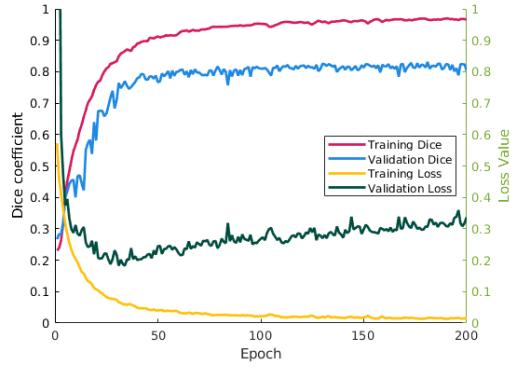


Figure 7: Dice coefficient and loss value for training and validation set.

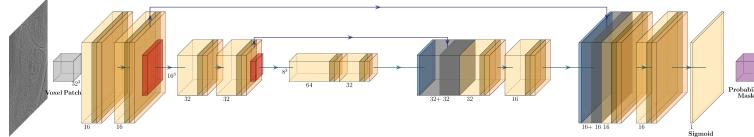


Figure 8: Network architecture used for vesicle segmentation. We used a U-Net based on the CARE framework [12]. The input is a cubic volume of 32^3 voxels. The output is a per-pixel probability cube of the same size as the input.

Global Threshold

In the pursuit of refining the output of our deep neural network, a systematic approach was employed to determine the optimal threshold for the probability mask. The objective was to ascertain a threshold that minimizes the values at the vesicle borders, ensuring precise delineation. By iterating through potential threshold values ranging from 0.8 to 1 in increments of 0.01, a binary mask was generated for each threshold. Subsequently, an erosion operation was applied to the binary mask, and the difference between the original and eroded masks produced the vesicle shell. The pixel values of the original image corresponding to this shell were recorded for each threshold. This iterative process aids in identifying the threshold that offers the sharpest and most accurate representation of vesicle borders.

U-Net output threshold refinement

The probability mask output by the U-Net was first made binary by determining a global threshold value. To this end, the mask was binarized for a range of threshold values comprised between 0.8 and 1. For each probability value a 1-voxel thick label shell was computed. The shell mask was then applied on the input data and the average masked voxel intensity was computed. Since the shell of correctly segmented vesicles corresponds to the vesicle membrane, we expect low intensity pixels. The threshold value resulting in the minimal average intensity of the shell masked voxels was used as the global threshold.

Amin please write the equations for these steps. The probability mask was binarized using the global threshold and was each separate segment was assigned an individual label with `scikit-image label` method.

A majority of vesicles were correctly segmented but we noticed some segments included two vesicles. We therefore evaluated each segment with two criteria based on the fact that synaptic vesicles have a homogenous size and are spherical. Firstly we calculated the volume z-score z for each segment:

$$z(S_i) = \frac{V(S_i) - \mu}{\sigma} \quad (1)$$

where S_i is the segment i , $V(S_i)$ is the volume of S_i , μ the average volume of all segments, and σ the standard deviation of the segment volumes. Secondly, we computed the segment extent e :

$$e(S_i) = \frac{V(S_i)}{B(S_i)} \quad (2)$$

where $B(S_i)$ is the volume of segment i bounding box. The extent of a sphere equals $\frac{\pi}{6}$. Segments with both a z-score $z > 1$ and an extent $e < 0.25$ were considered as potentially comprising two vesicles. For each of these segments, the probability mask threshold was increased until two distinct segments were generated. Subsequently, the extent and volume of all segments was evaluated again. Any segment with $e < 0.25$, or $e > 0.75$, or $V < k$ was discarded. This ensured that segments deviating highly from spherical shape and segments with a volume smaller than an acceptable volume k were removed.

Even if most synaptic vesicles were detected and well segmented, segmentation accuracy was not sufficient for our downstream application. To improve accuracy, each segment was converted to a spherical segment and its radius and position was refined. Initial spherical conversion was done by setting the center of the sphere C at the position of the centroid of the segment, while the radius r was defined as half the length of the bounding box longest edge. The segment position and radius was iteratively refined as follows. 1. The radial average $\langle I(d) \rangle$ was computed:

$$\langle I(d) \rangle = \frac{1}{4\pi^2 r^2} \int_0^{2\pi} \int_0^{2\pi} I(d, \theta, \phi) d\phi d\theta \quad (3)$$

where d is the radial distance from the segment center, θ the polar angle, and ϕ the azimuthal angle. 2. The radius of the vesicle r was updated as:

$$r = d_m + \frac{t_m}{2} \quad (4)$$

where d_m is the radial distance of center of the vesicle membrane, and t_m the thickness of the vesicle membrane. d_m was defined as the radial distance for which the radial average was minimal. $\frac{t_m}{2}$ was calculated as the distance between the center of the vesicle membrane and the minimum of the second derivative of the radial profile in the interval between the center of the vesicle membrane and the maximum of the Fresnel fringe outside the membrane. 3. The radial average was back projected in 3-dimension:

$$I(x, y, z) = \langle I(\sqrt{x^2 + y^2 + z^2}) \rangle \quad (5)$$

where $(x, y, z) = (0, 0, 0)$ is the coordinate of the segment center. 4. We computed by cross-correlation the shift between the obtained 3-D average and the 3-D image in the cubic box with central coordinates C and edge length $l = 2r + c$, where c is a constant. C was updated by subtraction of the shift. 5. Steps 1 to 4 were repeated for a maximum of 10 iterations until convergence or until a total shift of $\frac{1}{2}\sqrt{3l_o^2}$, where l_o is the edge length of the initial box. The feature space of predicted vesicle labels was computed, containing membrane thickness t_m , membrane intensity ρ , and vesicle radius r . ρ was defined as the mean intensity of the radial average within the radial distance interval $[d_m - \frac{t_m}{2}, d_m + \frac{t_m}{2}]$.

Outlier Detection and refinement

Based on the radial profile of the data, key features, namely thickness, radius, and membrane density, are extracted. Using these criteria, the Mahalanobis Distance is calculated for each data point to quantify its distance from the distribution of these features. The significance of the Mahalanobis Distance is interpreted using the chi-squared distribution. The p-value is derived by employing the cumulative distribution function (CDF) of the chi-squared distribution, with the Mahalanobis Distance

as the input and the length of the criteria as the degrees of freedom. The resulting p-value provides a measure of the statistical significance of each data point's distance.

With the computed p-values, outlier detection and refinement are conducted. If the p-value of a specific vesicle is not within an acceptable range (0.3), indicating it might be an outlier, its radial profile is recalculated using a broader margin to find the optimal profile. We perform this recalculation for up to 10 iterations. Typically, vesicles are either adjusted within the initial iterations or remain unchanged throughout. Vesicles that, even after recalibration, do not meet the p-value criteria are entirely removed from the dataset. This iterative process, involving recalculating the radial profile and adjusting parameters, continues until all vesicles either conform to the acceptable p-value range or are excluded. The refined dataset is then ready for subsequent analyses. This iterative approach ensures that all vesicles in the dataset either meet the p-value criteria or are excluded. Outlier detection and refinement are initiated based on the computed p-values.

Using this multivariate feature space, we detect outliers by computing Mahalanobis Distances (MD) on normalized variables using the covariance matrix of observation and obtaining the p-value on MD.

The sphere segments with a p-value higher than a defined threshold were discarded and the process was repeated iteratively. If the MD p-value of a specific vesicle was not in a specific margin range (0-10), their radial profile was recalculated, and the label entirely removed if they again failed to pass the margin of the p-value. Amin, please check what exactly was done with the outliers. And p-value cannot be higher than 1, while you wrote (0-10)

Evaluation Metrics (Analysis of Results)

The evaluation framework was designed to assess the capabilities of the proposed toolbox for automatic synaptic vesicle segmentation. The framework was not only designed to evaluate quantitatively performance of the neural network, but rather assay the segmentation of vesicles in practice (_unsure what the last part means*. _I tried to say we develop the software rather than an algorithm paper with ablation study kinda more trasnfer learning but however for tranfer learning we might need add finetunning the network or say this sentence in other way*). The pipeline also generates a specific output format, which is necessary to further analyze the presynaptic tomograms via another pre-developed toolbox (Pyto), which segments small molecular filaments associated to the synaptic vesicles, titled tethers and connectors.

DICE

The _general form??* DICE coefficient for probabilistic subvolume maps was calculated after each epoch as a performance quantification while _during?* training. The probabilistic mask subvolumes were stitched back together, creating a probabilistic map of the whole tomogram. The Soft-DICE for the whole tomogram was calculated to evaluate the similarity of the predicted probability mask with ground truth. Note that soft-dice is equivalent to dice, when the input is binarized (which we will do at the end of the post-processing).

$$1 - \frac{2 \sum_{voxels} y_{true} y_{pred}}{\sum_{voxels} y_{true}^2 + \sum_{voxels} y_{pred}^2}$$

_shouln't it be voxels instead of pixels??* _yes voxel is right* _fixed*

THE DICE was also employed to monitor all stages of post-processing on the eventual label file, to observe the effect of each post-processing step.

Vesicle Diameter Deviation Analysis

Vesicle Diameter Deviation Analysis

A critical characteristic of a vesicle is its diameter, predetermined as per specific criteria (refer to the Outlier Exclusion section). To assess the precision of our diameter estimation for accurately identified vesicles, a deviation metric is introduced.

Denote the diameter of a manually segmented vesicle by d_{GTi} and the estimated diameter of the corresponding vesicle as d_{Si} . The deviation in diameter estimation for each vesicle can be expressed as:

$$\delta d = 1 - \frac{\min(d_{Si}, d_{GTi})}{\max(d_{Si}, d_{GTi})} \quad (6)$$

This formula offers an insight into the congruence between our estimated diameter and the manual segmentation, with a diminished value of δd signifying a closer approximation.

The diameter of a vesicle is one of its relevant characterizations, and it is predefined (see Outlier Removal). _which diameters are we using in this evaluation as input, pre- or post-outlier removal?* _after! I meant from that parenthesis that radius or diameter we assume as one characteristic of vesicle we might can write it better
* The error of diameter estimation of true-detected vesicle is defined as 1 minus the proportion of diameters

$$\delta d = 1 - \frac{\min(d_{Si}, d_{GTi})}{\max(d_{Si}, d_{GTi})} \quad (7)$$

where d_{GTi} is the diameter of each true manual segmented vesicle, and d_{Si} is the diameter of its estimation.

Center error

The center error is an euclidean distance of ground truth and corresponds to true predicted vesicles _true pos or true neg? True Predicted means true positive*. A vesicle was defined as a true-detected vesicle if the predicted center was located inside the hand-segmented vesicle or the other way around the center of prediction was located inside the predicted vesicle. _isn't this a bit too general, shouldn't this be a tighter evaluation?* _we assume this as hard condition to be true positive we could define like some % like 50% intersection but this condition is generally harder This means the volume of intersection of the estimated vesicle with the distance of d to a ground truth vesicle with radius R is:

$$V = \frac{1}{12}\pi(4R + d)(2R - d)$$

Computational Setup

All experiments were conducted using 4 x NVIDIA 2080 Ti GPUs with CUDA 10.1. [_Ioan's Grant Acknowledgement*](#) The software environment was set up with Python 3. Key libraries and packages utilized include TensorFlow 2.4.1 with GPU support and Keras 2.4.3. For visualization neurons with tethers and connectors and regarding video, Amira 2022.2 was employed. Image visualizations were achieved using both UCSF Chimera and ChimeraX. Surface rendering was performed by the volume tracer and color zone in UCSF ChimeraX. For detailed configurations and parameters, users are referred to the accompanying source code.

Manuscript preparation

The manuscript was written with the open and collaborative scientific writing package Manubot [18]. The source code and data for this manuscript are available at https://github.com/aseedb/synaptic_tomo_ms.

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