Automated image analysis in large-scale cellular electron microscopy: A literature survey

Anusha Aswath\textsuperscript{a,b,*}, Ahmad Alsahaf\textsuperscript{a}, Ben N. G. Giepmans\textsuperscript{b}, George Azzopardi\textsuperscript{a}

\textsuperscript{a}Bernoulli Institute of Mathematics, Computer Science and Artificial Intelligence, University Groningen, Groningen, The Netherlands
\textsuperscript{b}Dept. Biomedical Sciences of Cells and Systems, University Groningen, University Medical Center Groningen, Groningen, The Netherlands

Abstract

Large-scale electron microscopy (EM) datasets generated using (semi-) automated microscopes are becoming the standard in EM. Given the vast amounts of data, manual analysis of all data is not feasible, thus automated analysis is crucial. The main challenges in automated analysis include the annotation that is needed to analyse and interpret biomedical images, coupled with achieving high-throughput. Here, we review the current state-of-the-art of automated computer techniques and major challenges for the analysis of structures in cellular EM. The advanced computer vision, deep learning and software tools that have been developed in the last five years for automatic biomedical image analysis are discussed with respect to annotation, segmentation and scalability for EM data. Integration of automatic image acquisition and analysis will allow for high-throughput analysis of millimeter-range datasets with nanometer resolution.

Keywords: Electron microscopy, segmentation, supervised, unsupervised, machine learning, deep learning, AI

1. Large-scale cellular EM

Electron microscopy (EM) is widely used in life sciences to study tissues, cells, subcellular components and (macro) molecular complexes at nanometer scale. Two-dimensional (2D) EM aids in diagnosis of diseases, but routinely it still depends upon biased snapshots of areas of interest. Automated pipelines for collection, stitching and open access publishing of 2D EM have been pioneered for both transmission EM (TEM) images (Faas et al., 2012) as well as scanning TEM (STEM) (Sokol et al., 2015) for acquisition of areas up to $1\text{mm}^2$ at nanometer-range resolution, Table 1. Nowadays, imaging of large areas at...
Figure 1: Typical large-scale EM allows to analyze tissue at a high resolution. Overview and snapshots of several cellular, subcellular and macromolecular structures of which up to a million can be present per dataset (de Boer et al., 2020). Bars: red 10 µm; green 1 µm; white 0.5 µm. Full access to digital zoomable data at full resolution is via http://www.nanotomy.org.

High resolution is entering the field as a routine method and is provided by most EM manufacturers. This we term nanotomy, for nano-anatomy (Ravelli et al., 2013; de Boer et al., 2020; Dittmayer et al., 2021). The large-scale images allow for open access world-wide data sharing; see nanotomy.org for more than 40 published studies and the accessible nanotomy data.

A typical nanotomy dataset has a size of 5-25GB at 2.5nm pixel size. Nanotomy allows scientists to pan and zoom through different tissues or cellular structures in a Google Earth-like manner (Fig. 1). Large-scale 2D EM provides unbiased data recording to discover events such as pathogenesis of diseases at the supra-cellular level for morphological changes. Moreover, nanotomy allows for the quantification of subcellular hallmarks. With state-of-the-art 2D EM tech-

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1www.nanotomy.org
2https://myscope.training/
Table 1: Major large-scale EM techniques. Note that the biomaterial for the techniques below is stained with heavy metals to generate contrast. For further information see the MyScope website\(^2\) and the papers reviewed by Peddie and Collinson\(^{2014}\) and Titze and Genoud\(^{2016}\).

| 2D EM                              | Methodology                                                                 |
|------------------------------------|----------------------------------------------------------------------------|
| Transmission Electron Microscopy (TEM) | A widefield electron beam illuminates an ultra-thin specimen and transmitted electrons are detected at the other side of the sample. The structure that is electron dense appears dark and others appear lighter depending on their (lack of) scattering. |
| Scanning Electron Microscopy (SEM) | The raster scanning beam interacts with the material and can result in back scattering or formation of secondary electrons. Their intensity reveals sample information. |
| Scanning Transmission Electron Microscopy (STEM) | SEM on ultrathin sections and using a detector for the transmitted electrons. |

| 3D EM                              |                                                                                           |
|------------------------------------|--------------------------------------------------------------------------------------------|
| Serial section TEM (ssTEM) or SEM (ssSEM) | Volume EM technique for examining 3D ultrastructure by scanning adjacent ultrathin (typical 60-80 nm) sections using TEM or SEM, respectively. |
| Serial Block-face scanning EM (SB SEM) | The block face is scanned followed by removal of the top layer by a diamond knife (typical 20-60 nm) and the newly exposed block face is scanned. This can be repeated thousands of times. |
| Focused Ion Beam SEM (FIB-SEM)     | Block face imaging as above, but sections are repeatedly removed by a focused ion beam that has higher precision than a knife (typically down to 4 nm) suitable for smaller volumes. |
and heterogeneous appearances, further surrounded by complicated structures. 2D EM datasets only have neighbouring (X/Y axes) context as reference for structural analysis. The vEM datasets leverage knowledge from adjacent sections (Z-axis) that share high resemblance, and therefore aids in better reconstruction of the volume. Additionally, large-scale 2D EM images are more spatially distributed across cells than traditional EM micrographs of a selected region or vEM that occupy a smaller spatial area, despite both large-scale 2D EM and vEM containing gigabytes of information. It requires global image processing without losing coherent information.

Here, we review automated methods for large scale EM image segmentation and analysis.

2. Deep learning and segmentation

Deep learning has become the state-of-the-art methodology for many computer vision tasks, including segmentation. This has been a paradigm shift when compared to traditional segmentation methods, which were generally performed using machine learning classifiers such as Adaboost, Random forests, and Support Vector Machines (SVMs), which required domain-oriented, hand-crafted features as inputs (Table 2). The introduction of Deep Neural Networks (DNNs) has enabled automatic extraction of hierarchical representations from raw image data [LeCun et al. 2015]. Convolutional Neural Networks (CNN) have significantly advanced image-related tasks such as classification, detection, and segmentation with end-to-end learning from feature extraction to prediction.

A typical CNN consists of convolutional filters or weight sharing filter banks in each layer of the neural network. Convolutional filters are linear functions that are used for low-level basic operations, such as blurring and edge detection. A 2D convolutional filter uses a 2D matrix, referred to as a kernel, centered on a local region in a given image and applies a linear operation between the kernel coefficients and the respective pixel values in the local region concerned. The resulting scalar value is the response of the filter to the considered region. The filter is then slid across the whole image to compute the responses at every location. All responses form what is known as a response or a feature map which has the same size as the input image if the sliding is done one pixel at a time. 3D filters operate in a similar way but use 3D kernels and are applied to 3D volumes of vEM images. In a CNN, the filters allow for weight sharing throughout the image capturing structured data using translational and rotational invariance [Krizhevsky et al. 2012] [Yamashita et al. 2018]. The spatial extent of the connectivity of a filter with local pixels is called the receptive field or the kernel size. The response maps obtained by such filters are then processed by a non-linear activation function, before being downsampled by a pooling unit in order to learn abstract representations [LeCun et al. 2015]. Finally, the response maps are fed to a fully connected layer which determines a label for the given image.
Challenges with DNNs

Deep Learning

Vanishing gradient

Convolutedal DNNs with convolutional layers. Convolutional layers consist of nodes that apply linear filters by scanning a given image in overlapping blocks of pixels to extract features. Besides convolutions each layer includes a nonlinear activation function followed by down sampling. Examples include AlexNet [Krizhevsky et al., 2012], an early deep CNN designed to classify a thousand categories of images (ImageNet). VGG [Simonyan and Zisserman, 2014] reduced the number of parameters by using smaller filters multiple times between layers. Residual Neural network (ResNet) [He et al., 2016] trains deeper networks efficiently using residual blocks that connect outputs of stacked layers to the block’s input layer with identity skip connections.

Degradation in DNNs

Deeper additional layers that provide the network power to calculate a more complex function without increasing the training set may lead to a performance saturation and eventually degradation. ResNets allow for uninterrupted flow of information from previous layers to subsequent ones, realising a complex function with less parameters and hence less risk of both degradation and vanishing gradients [He et al., 2016].
Progress in the development of CNNs has led to a plethora of applications including the automatic analysis of medical images. The CNN designed by Ciresan et al. (2012), for instance, was used for the segmentation of neuronal membranes in stacks of EM images. The images were segmented by predicting the label of each local region or patch covered by a convolutional filter in a sliding window approach. Despite the method’s success - winning the 2012 ISBI EM segmentation challenge - the method has two major limitations. First, the sliding window approach is slow as it suffers from redundancy due to the processing of large overlaps between adjacent patches. Second, there is always a trade-off between the size of the patches (context) and full-resolution prediction. It turns out that localisation ability decreases with an increasing context due to downsampling by the many max pooling layers. Improvements in the semantic segmentation of EM images continued with the development of the Fully Convolutional Network (FCN) (Long et al., 2015). FCN allows for variable sized images as input by replacing the fully connected layers of a standard CNN with fully convolutional maps (Fig. 2). Now, the spatial maps in the last layer of an FCN correspond to certain local patches or pixels of an input image depending on the network depth.

Encoder-decoder architectures of the FCN type have been catalysts in providing better localization and use of larger context. The decoder also captures multi-scale features using skip connections to fuse feature maps from shallow layers, providing larger context. Skip connections bypass some of the neural network layers and take the output of one layer as the input to the subsequent ones. As a result, an alternative and shorter path is provided for backpropagating the error of the loss function, which also contributes in avoiding the vanishing gradient and the degradation problems in deep networks (Table 2). In principle, skip connections allow for a better upsampling in higher layers. For instance, the symmetric U-Net architecture transfers full feature maps from the encoder to the decoder paths, achieving the best segmentation results of neuronal membranes in EM images in the ISBI 2015 challenge (Ronneberger et al., 2015). SegNet was proposed to transfer only the pooling indices between the encoder and decoder paths to reduce the load on memory (Badrinarayanan et al., 2017). Nevertheless, U-Net captures more complex information from the stored encoder layer outputs concatenated using skip connections for upsampling and thus outperforms SegNet in terms of accuracy.

The concept of convolutional output layers in FCNs enables the conversion of popular DNNs, such as AlexNet, VGGNet, ResNet and GoogleNet (Inception-v1) (Krizhevsky et al., 2012; Simonyan and Zisserman, 2014; He et al., 2016; Szegedy et al., 2015), into encoder-decoder architectures for semantic segmentation. DNNs are universal approximators that can realise a complex function with even two layers of the network. Shallow neural networks with a few layers are not adequate to learn robust models due to overfitting. Deeper networks with small receptive fields, such as VGG-16 and AlexNet, became popular for
Figure 2: Encoder-decoder networks for FCN and U-Net. Each of the first four convolutional layers in the encoder are followed by the nonlinear activation function ReLU and max pooling. The last layer uses a softmax function to assign a probability class score to each pixel. The FCN decoder includes an upsampling component that is linearly combined with the low-level feature maps in the third convolutional layer of the encoder. The sizes of these feature maps are 4 times less than the size of the input image $I$ (denoted by $I/4$). Finally, there is a direct upsampling from $I/4$ to the original size of $I$ followed by softmax for classification. The symmetrical U-Net architecture shares the features maps in the encoder with the decoder path together with skip connections.

Image classification due to their generalization ability. DNNs have a large number of parameters to learn using the loss function (or error) that quantifies the penalty of the predicted values with respect to the desired ones. The addition of layers to make an architecture deeper brings other scientific challenges, such as the vanishing gradient and network degradation (Table 2). Methods to address these challenges with training deeper networks include different strategies in initializing network parameters (Glorot and Bengio, 2010), training networks in multiple stages (Simonyan and Zisserman, 2014), and using companion loss functions or auxiliary supervision in the middle layers (Szegedy et al., 2015). The most recent and important networks in computer vision are called the Residual Neural Networks or ResNet that overcame the vanishing gradient and the degradation issues simultaneously (Table 2).

Spatial pyramid pooling and dilated (or atrous) convolutions were introduced in the DeepLab family of segmentation architectures for the purpose of larger context capture. DeepLab models addressed the challenges of achieving robustness for different scales of classes and considering larger context without increasing computational complexity (Chen et al., 2014, 2017a,b, 2018). Moreover, multi-scale context aggregation using the Pyramid Scene Parsing Network.
(PSP-Net) or spatial attention using the attention U-Net have also gained popularity for their ability to capture larger context (Zhao et al., 2017; Oktay et al., 2018). The ability to deal with varying scales while capturing more context is particularly important in EM analysis where the neighbourhood of a structure may have an impact in determining a precise delineation.

3. Literature search

The following search query was used in both Pubmed and Web of Science on words in titles (TI) only, restricted to 2017-2021: TI=((electron microscopy OR EM) AND (segmentation OR supervised OR unsupervised OR self-supervised)) NOT cryo. Cryo-EM (Kucukelbir et al., 2014) was excluded because it involves molecule datasets as opposed to cellular EM. Results from the query that are beyond our scope were excluded. A detailed review of the resulting 28 papers (Table 3) is given in terms of the data annotation and studied structures, the segmentation approaches, and computational scalability.

4. Data, anatomical structures and annotation

Early examples of automated segmentation include the reconstruction of brain tissues for connectomics, which is the map of neuronal cell bodies and their connections, the synapses. The pioneer work on automated neuronal membrane segmentation by Ciresan et al. (2012) showed the success of neural networks on EM images in the ISBI 2012 challenge. This has motivated more research activity in this direction, resulting in key segmentation architectures such as U-Net (Ronneberger et al., 2015). Properties of the most commonly used datasets are shown in Table 4.

Large-scale connectome datasets mostly focused on sub-cellular components related to brain cells such as synapses, pre- and post-synaptic sites, axons and mitochondria (Takemura et al., 2015; Kasthuri et al., 2015). The open organelle project provides free access to high resolution 3D EM datasets and their segmentations for analysing intracellular sub-structures and organelles. With the challenge to automatically reconstruct tissues at cellular level, several organelles besides mitochondria, namely nucleus, plasma membrane, endoplasmic reticulum, nuclear envelope, vesicles, lysosomes, endosomes and microtubules have now become the focus in open-source datasets (Heinrich et al., 2021).

Detailed annotation of large-scale EM images is required for automatic image segmentation and analysis of structures. Three approaches of annotations are considered: 1) human annotators; 2) software tools for biologists; and 3) specialized microscopy imaging modalities.

https://www.openorganelle.org/
| Method                                      | Type  | Dataset | 2D | 3D | F | SS | SB | TEM | Structures                 | Study                          |
|--------------------------------------------|-------|---------|----|----|---|----|----|----|-----------------------------|--------------------------------|
| Residual Deconvolutional Network (RDN)     | S     | -       | ✓  | ✓  | ✓ | -  |    |    | Membranes                   | Fakhry et al. (2016)           |
| DeepEM3D                                   | S     | -       | ✓  | ✓  | ✓ | -  |    |    | Membranes                   | Zeng et al. (2017)             |
| Feature selection and boosting             | S     | -       | ✓  | ✓  | ✓ | -  |    |    | Mitochondria, synapses, membranes | Cetinaa et al. (2018)          |
| Pre-trained networks                        | S     | -       | ✓  | ✓  | ✓ | -  | ✓  |    | Neurophil, axons             | Drawitsch et al. (2018)        |
| 2D Convolutional Neural Network (CNN)       | S     | ✓       | -  | ✓  | ✓ | ✓  | -  | -  | Mitochondria                 | Oztel et al. (2018)            |
| 3D Residual FCN                             | S     | -       | ✓  | ✓  | ✓ | ✓  | -  | -  | Mitochondria                 | Xiao et al. (2018)             |
| Two-stream U-Net                            | UN    | -       | ✓  | ✓  | ✓ | ✓  | -  | -  | Mitochondria, synapses       | Bermúdez-Chacón et al. (2019)  |
| Random forest                              | S     | ✓       | -  | -  | - | ✓  | -  | -  | Glomular basement membrane   | Cao et al. (2019)              |
| Fully Convolutional Network (FCN)          | S     | ✓       | -  | ✓  | ✓ | -  |    |    | Mitochondria                 | Dietmeier et al. (2019)        |
| Fully Residual U-Net (FRU-Net)             | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Membranes                    | Gómez-de Mariscal et al. (2019) |
| 3D Convolutional Neural Network (CNN)       | S     | ✓       | ✓  | ✓  | ✓ | ✓  | -  | -  | Cells, mitochondria, membranes | Guay et al. (2019)             |
| Residual Neural Network (ResNet)           | S     | ✓       | -  | -  | ✓ | ✓  | -  | -  | Neural cell body, cell nucleus | Jiang et al. (2019)            |
| Morphological operators/superpixels        | UN    | -       | ✓  | ✓  | ✓ | -  |    |    | Mitochondria, membranes      | Karabag et al. (2019)          |
| Random forest                              | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Mitochondria                 | Peng and Yuan (2019)           |
| U-Net, ResNet, HighwayNet, DenseNet        | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Membranes                    | Urakubo et al. (2019)          |
| DenseUNet                                  | S     | ✓       | -  | ✓  | ✓ | -  | -  | -  | Mitochondria                 | Cao et al. (2020)              |
| Fully residual CNN (FR-CNN)                | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Membranes                    | He et al. (2020)               |
| HighRes3DZMNet                              | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Mitochondria, endolysosomes  | Mekuc et al. (2020)            |
| U-Net, autoencoder                         | UN    | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Mitochondria                 | Peng et al. (2020)             |
| DeepACSON                                  | S     | -       | ✓  | ✓  | ✓ | -  | -  | -  | Axons                        | Abdollahzadeh et al. (2021)    |
| 2D-3D hybrid network                       | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Cells, granules, mitochondria | Guay et al. (2021a)            |
| 3D U-Net                                   | S     | -       | ✓  | ✓  | ✓ | -  | -  | -  | Up to 35 sub-cellular structures | Heinrich et al. (2021)         |
| CDeep3EM, EM-Net, PReLU-Net, ResNet         | S     | ✓       | -  | ✓  | ✓ | -  | -  | -  | Mitochondria                 | Khadangi et al. (2021b)        |
| Hierarchical encoder-decoder (HED-Net)     | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Mitochondria                 | Luo et al. (2021)              |
| Generative adversarial network (GAN)       | UN    | ✓       | -  | -  | - | ✓  | -  | -  | Cytoplasmic caspids          | Shaga Devan et al. (2021)      |
| Annotation-crowd-sourcing, ‘Etch a Cell’   | S     | ✓       | -  | -  | - | ✓  | -  | -  | ER, mitochondria, nucleus    | Spiers et al. (2021)           |
| U-Net                                      | SS    | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Membranes                    | Takaya et al. (2021)           |
| Hierarchical view ensemble (HIVE) Net       | S     | ✓       | ✓  | ✓  | ✓ | -  | -  | -  | Axons, nuclei, mitochondria  | Yuan et al. (2021)             |

Table 3: Survey papers. Abbreviations used - S (Supervised), UN (Unsupervised), SS (Semi-supervised), F (FIB-SEM), SS (Serial section), SB (SB-SEM).
### Table 4: Commonly re-used 3D EM datasets for image analysis improvement.

| Acquisition | Dataset | Region | Imaged tissue parameters (x,y,z) | Reference |
|-------------|---------|--------|----------------------------------|-----------|
| ssTEM       | ISBI 2012 / Drosophila | Nervous cord (Drosophila) | 2 × 2 × 1.5, 512 × 512 × 30, 4 × 4 × 50 | Ciresan et al. (2012) |
| ssSEM       | ISBI 2013 / SNEMI3D | Cerebral cortex (Mouse) | 6 × 6 × 6, 1024 × 1024 × 100, 6 × 6 × 30 | Arganda-Carreras et al. (2013) |
| ssSEM       | Kasthuri dataset | Neocortex (Mouse) | 10^3 × 10^3 × 130, 1024 × 1024 × 2000, 3 × 3 × 30 | Kasthuri et al. (2015) |
| SB SEM      | HeLa cells | Cultured cells (Human) | 1 × 1 × 0.2, 8192 × 8192 × 518, 10 × 10 × 50 | Iudin et al. (2016) |
| FIB-SEM     | EPFL Mouse Hippocampus | CA1 Hippocampus (Mouse) | 5 × 5 × 5, 2048 × 1536 × 1065, 5 × 5 × 5 | Lucchi et al. (2013) |
| FIB-SEM     | FIB-25 | Optic lobe (Drosophila) | 64 × 66 × 81, 6426 × 6623 × 8090, 10 × 10 × 10 | Takemura et al. (2015) |

#### 4.1. Human annotators

Human annotation is split into two categories, annotations by one or a few experts, and collaborative annotations by large groups of experts and non-experts, so-called crowd-sourced annotation. Projects such as Etch-a-Cell enable annotation through crowd-sourcing [Spiers et al., 2021] that allows volunteers to participate in large-scale annotation tasks digitally, with the aid of tutorials and other guided workflows. For example, volunteers are invited to annotate certain structures, such as endoplasmic reticulum (ER), mitochondria or nuclei, in order to collect ground truth segmentation labels for supervised machine learning algorithms. As human segmentations can be erroneous due to imprecise delineation of structures or bias, consensus between various volunteer segmentations is often used as the ground truth. Expert annotations are more accurate but require more time and resources. For example, labeling structures in 30 platelets from a small fraction of the imaged samples required 9 months from two human annotators [Guay et al., 2021a]. Also, the large scale connectomics project required extensive labelling of ground truth data [Plaza and Funke, 2018], and the proofreading of the ground truth dataset took around 5 years in the work of [Takemura et al., 2015].

#### 4.2. Biomedical image analysis software

Biomedical image analysis software applications are black box tools for many users to annotate or proofread EM datasets without having to know the under-

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Proofreading refers to the manual correction of segmented (manual or automatic) image data.
lying mechanisms. Large-scale connectome reconstruction prompted the development of various tools for proofreading and analysis of the reconstructed data. All the tools are mostly developed for connectome proofreading and analysis for serial sectioned image stacks and block-face images. Such tools mostly come with 2D viewers and 2D annotations using brush/flood-fill functions with 3D viewers for visualization (Table 5). Generally, the reconstructed or annotated maps are corrected by reading data from external servers. The data storage format in the server and the mechanism of data distribution to the client determine how well the tool can scale for annotation or proofreading of larger volumes.

No single software tool is typically enough for the entire automated image analysis pipeline. UNI-EM bundles several off-the-shelf tools for annotation, segmentation, proofreading or multi-view rendering into a single package (Urakubo et al., 2019). Entire segmentation workflow is included using DOJO, CNN models such as U-Net, ResNet, Highway Net, DenseNet and flood filling networks (Srivastava et al., 2015; Huang et al., 2017; Januszewski et al., 2018) and an additional 3D surface-mesh based renderer. DOJO is a web-based tool for large-scale annotation and proofreading (Haehn et al., 2014). The package also includes monitoring tools such as TensorBoard for performance monitoring (Mané et al., 2015). The user does not need programming skills to perform segmentation.

TrackEM2 is a software tool for optimized manual and semi-automatic reconstruction of neuronal circuits (Xiao et al., 2018; Jiang et al., 2019; Ciresan et al., 2012; Li et al., 2018). A companion tool for TrackEM2 is CATMAID, which is for data storage, management and collaborative annotation of large-scale 3D ssTEM datasets using a client-server model (Saalfeld et al., 2009). However, 3D neurite tracing to the next lateral 2D image plane obtained from a server can be less optimal due to low bandwidth and network latency issues. To overcome this challenge, webKnossos uses a 3D SB SEM dataset storage and transmission in the form of small 3D voxels, as used in Knossos (a standalone data annotation application for connectomics) (Helmstaedter et al., 2011). Web-Knossos is a cloud/browser-based 3D annotation tool for large-scale distributed data analysis (Boergens et al., 2017). An example of open-source demo for dense connectome reconstruction using webKnossos is given by Motta et al. (2019). 3D voxel based storage and access is also provided by VAST (Volume Annotation and Segmentation Tool) to handle petabyte range image data. Rich metadata based information can be generated for segmented objects and be grouped or organised in a tree structure for importing. Similar to CATMAID, VAST has a data management framework with specific file format conversion before analysis (Berger et al., 2018). A VAST format compatible dataset is publicly available to annotate the mitochondria dataset by Kasthuri et al. (2015).

The FlyEM project provides open-source tools which aim to fully reconstruct the neural connectivity of the Drosophila fly brain using EM imaging (Scheller et al., 2015).
Table 5: Reviewed biomedical image analysis software for manual or semi-automatic image segmentation, annotation and proofreading.

| Feature/Software | Visualization client | Annotation client | Collaborative annotation | Data conversion format | Scalability | Train/Test Inclusion of custom algorithm |
|------------------|-----------------------|-------------------|--------------------------|-----------------------|-------------|-----------------------------------------|
| UNI-EM           | 2D, 3D/DOJO, DOJO, 3D/DOJO | 3D Mesh, Web-based | PNG/TIFF High | DNN | Python script |
| webKnossos      | 2D, 3D/webKnossos      | Web-based, Knossos | High | - | Python script |
| FlyEM           | 2D, 3D/NeuTu, 2D/NeuTu | Web-based, DVID   | High | - | - |
| TrackEM2        | 2D, 3D/TrackEM2, 2D/TrackEM2 | Web-based, CATMAID | Moderate | - | - |
| VAST            | 2D, 3D/VAST, 2D/VAST | Central server, VAST | High | - | - |
| Ilastik         | 2D, 3D/ Ilastik       | Web-based, Ilastik | HDF5 | Low | Random forest |
| Weka            | 2D/ ImageJ, 2D/ ImageJ | - | PNG/TIFF Low | Random forest | Java script |
| IMOD            | 2D/ IMOD              | - | Camera specific | Undefined | - |

et al., 2020). One of the tools, NeuroProof (Plaza, 2014), introduces focused and faster proofreading using automated segmentation and prior information (synapse connectivity). Other tools, like Raveler and NeuTu, allow interactive proofreading in a distributed and scalable manner (Farm, 2014; Zhao et al., 2018). Distributed, Versioned, Image-oriented Dataservice (DVID) provides a web-based API for key-value based image labels for efficient storage and faster access. DVID facilitates the distribution and access of data through a common format to access all datasets using a higher-level API (Katz and Plaza, 2019).

Ilastik and Trainable Weka Segmentation (TWS) are plugins in Fiji to segment synaptic junctions and mitochondria (Sommer et al., 2011; Arganda-Carreras et al., 2017). Ilastik originally focused on the work of segmenting synaptic junctions and was further developed into a platform that includes fast interactive training through shallow classifiers (random forests) (Berg et al., 2019). Trainable Weka Segmentation (TWS) allows training of a random forest classifier for binary segmentation and provides options to select the features, load a stored classifier to visualize results on test images along with its performance metrics. Both are extensively used for labeling neuronal 3D images. IMOD can semi-automatically register 3D EM serial sections and was used to segment structures such as small extra-cellular vesicles (Gomez-de Mariscal et al., 2019).

The annotation tools show a trend towards semi-automated segmentation on web frameworks for scalable data access along with providing collaborative proofreading. For further information on accelerating computing and scalable data access for data analysis refer Section 6.
4.3. Correlative Light and Electron microscopy (CLEM)

Fluorescence microscopy can help to provide identifying information to EM images and can be used to improve automated analysis. CLEM is used to identify structures targeted with fluorescent probes in \( \mu m \) resolution images at (sub)cellular context from EM (de Boer et al., 2015). Drawitsch et al. (2018) performed CLEM for 40-50\( \mu m \) EM sections for a 3D connectome dataset. The workflow uses the full multi-color space of LM to identify the most-likely axon out of all reconstructed axons in large scale EM to best match with the LM-imaged axonal fluorescent signal. Besides webKnossos, which they used for manual reconstruction of axons that took 5300 work hours for a \( 1 \times 1 \times 0.1 \ mm^3 \) layer of the mouse neocortex, further speedup using partially automatic methods proposed in (Berning et al., 2015; Dorkenwald et al., 2017; Staffler et al., 2017; Januszewski et al., 2018) is conceivable. Other attempts for 2D CLEM are based on EM workflows or markers to speed up image registration or alignment.

5. Segmentation approaches

The taxonomy (Fig. 3) illustrates the main segmentation methodologies reviewed in this section.

5.1. Supervised learning

Supervised learning in segmentation refers to the family of machine learning algorithms that use a set of annotated images (training data) to create a computational model that can segment structures in unseen images (test data). The training set is used by the algorithm to determine the model’s parameters in such a way as to to maximize the model’s generalization ability.

5.1.1. End-to-end learning

End-to-end learning refers to the use of gradient-based methods to adjust the parameters of a complex deep neural network based on a loss function applied on the network’s prediction (Glasmachers, 2017). In principle, increasing network complexity - e.g. by increasing the number of layers - allows for more complex functions to be learned. In practice, however, there are technical challenges that limit a network’s learning capacity, like vanishing gradients and network degradation (Table 2). The ResNet architecture attempts to counter such limitations with residual blocks (He et al., 2016). Skip connections enable the design of very deep networks while propagating the gradient of the loss function through a lower number of layers. Encoder-decoder architectures, especially U-Nets, have enabled end-to-end learning for segmentation. ResNet blocks have become a standard in encoder and decoder networks for biomedical image segmentation.
Neuronal membranes and ResNets. Various variants of the U-Net architecture using residual blocks have been proposed for segmenting neuronal structures such as membranes, neural cell bodies and cell nucleus. FusionNet and Fully residual CNN (FR-CNN) use residual blocks at each level outperforming the standard U-Net architecture and FCN for membrane segmentation (Quan et al., 2016; He et al., 2020). Dense skip connections that connect each layer to every other layer in a feed-forward manner used in DenseUNets, a combination of U-Net and DenseNet (Cao et al., 2020), achieved competitive segmentation performance on the ISBI 2012 EM dataset. The deconvolutional network of Noh et al. (2015) introduced learnable unpooling layers, was used in combination with residual blocks in the in the Residual deconvolutional network (RDN) (Fakhry et al., 2016). RDN shows minimal inconsistencies in continuity of membrane detection across the 3D slices on the ISBI 2013 dataset. ResNet with atrous convolutions was used for the segmentation of neural cell bodies and nuclei. Additionally, the multi-scale contextual feature integration outperforms U-Net and Deeplab v3+ (Jiang et al., 2019).

Mitochondria and efficient 3D networks. Mitochondrial distribution inside a cell and alterations in its shape are related to degeneration or cellular death. High-resolution automatic analysis is required to study the physiological changes in mitochondria. Both 2D and direct 3D networks for reconstruction work well on isotropic FIB SEM images. A 2D image analysis pipeline for mitochondria segmentation from FIB-SEM dataset was performed by direct upsampling of the encoder features (Oztel et al., 2018). A hybrid 2D-3D network by Xiao et al. (2018) refers to the use of 3D convolutions at the start and end of an encoder-decoder network. Using 2D max-pooling instead of 3D in the same network...
helped in capturing anisotropy for SB SEM datasets. Auxiliary supervision in the mid-level features was key for better deep supervision to avoid the vanishing gradient problem. As compared to architectures like U-Net and 3D U-Net, lesser proofreading was needed due to better accuracy.

The 3D networks have millions of parameters to train and are computationally intensive. Multiple views of a 3D stack, three orthogonal views and a final branch to calculate context information from one of the views was used in HIVE-Net, a multi-task pseudo 3D residual network \cite{yuan2021hivenet}. Experiments show that the HIVE-Net with lesser number of parameters achieves state-of-the-art performance compared to deep learning models such as U-Net, 3D U-Net or the hybrid 2D-3D network \cite{xiao2018efficient}.

**Shape-based prior for segmentation.** Shape information is used for faster and regularized segmentation of axons, mitochondria and nuclei to take into account the heterogeneity in large volumes of 3D EM datasets. DeepACSON, a Deep learning-based Automatic Segmentation of axONs, achieves segmentation of lower resolution larger field of view images that miss distinctive image features using shape information \cite{abdollahzadeh2021deepacson}. Faster analysis of low resolution images on par with the high-resolution ones was made possible using shape information, such as ovality of mitochondria, tubularity of axons and circularity of nuclei.

Two-stage networks for shape-based discriminative feature training is performed by using the Hierarchical Encoder Decoder (HED) network \cite{luo2021hivo}. Based on the eccentricity (elliptic or circular shape) of mitochondria, ground-truth labels are sub-categorized into two sets for training: a first stage multi-task network and a second network for the full labels. Shape information priors used in such networks show less false positives and fewer missed detections in the segmentation of mitochondria when compared with U-Net, 3D U-Net, and HIVE-Net \cite{yuan2021hivenet}.

**Whole cell 3D reconstruction.** Robust and scalable automatic methods for whole-cell 3D reconstruction are required to study intricate organisation of thousands of structures inside a cell. FIB-SEM blocks from 5 different cell types and EM preparation methods were trained for around 35 different cellular organelles. The results show that a diverse set of all samples used for training improves generalisability more than training on only one specific target sample. Such comprehensive datasets and the trained models for 3D reconstruction of cells are made open-source \cite{heinrich20213dcell} to allow the exploration of local cellular interactions and their intricate arrangements.

### 5.1.2. Ensemble learning

Ensemble learning methods combine outputs of multiple predictions for better robustness. Pixel- or voxel-wise averaging and majority or median voting are amongst the main aggregation methods. DeepEM3D uses deep inception-residual modules in the encoding path and multi-scale contextual feature aggregation in the decoding path \cite{zeng2017deepem3d}. Variants of the DeepEM3D
architecture use ensemble learning to combine several models that are trained on neuronal boundaries with different thicknesses. Voxel-wise averaging of the predictions account for misalignment and anisotropy in the ssSEM image stacks.

Random forests (Table 2) use ensemble learning for improved generalization. A stack of Random forests was investigated for the segmentation of glomerular basement membrane from TEM images (Cao et al., 2019). Zoom-view random forests based on $N$ groups of membrane intensity images and one full-view random forest taking $M$ pixels sampled from all $N$ groups were trained to capture different imaging conditions. The method of using two-level integrated Random forests enhances generalization on different gray-scale intra-image variations and different morphologies of the membrane. Cetina et al. (2018) used the PIBoost algorithm (Fernandez-Baldera and Baumela, 2014), a multi-class generalization of AdaBoost with weak binary learners, for simultaneous segmentation of synapse with mitochondria and mitochondria with membranes. Better accuracy was obtained for both isotropic and anisotropic stacks (SB SEM) due to better representation ability and robustness to class imbalance.

Structured prediction are modelling techniques that forecast a set of values rather than a scalar value. In the segmentation context, structured prediction methods seek the joint prediction of the label of the pixel under consideration as well as the labels of the extended neighbourhood. The hierarchical approach by Peng and Yuan (2019) uses an iterative procedure to fine-tune the segmentation based on handcrafted features preserving neighborhood structure and class labels determined in previous iterations. Structured and cascaded approaches tend to improve the segmentation results by reducing the number of false positives and false negatives.

An ensemble of randomly initialized instances of the same network with each trained on less than 1% of the SB-SEM volume for seven classes in platelet cells yielded the best results to structural variations in large datasets, and outperformed individual 2D and 3D U-Net approaches (Guay et al., 2021a). Multiple network outputs were also combined using a workflow for binary EM segmentation provided by the EM-stellar platform (Khadangi et al., 2021b). The network architectures chosen for experimentation were CDeep3EM, EM-Net, PReLU-Net, ResNet, SegNet, U-Net and VGG-16 (Haberl et al., 2018; Khadangi et al., 2021a; He et al., 2015). A cross-evaluation using a heatmap of different evaluation metrics and networks shows that configuring an ensemble of various architectures is required to obtain the best results. Khadangi et al. (2021b) also demonstrated that no single deep architecture performs consistently well, and that is why ensemble approaches may have an edge over individual methods.

5.1.3. Transfer learning

Transfer learning adapts the knowledge acquired from one dataset to another, and is used when an application has insufficient amount of training samples. The pre-trained model is fine-tuned, usually in the final layers, with the training samples of the new dataset. Through transfer learning, the same neural network segmentation pathways have been used across biological systems in 3D (Guay et al., 2019). A three-class segmentation was proposed by Mekuc et al.
for the delineation of mitochondria, endolysosomes, and background. The domain information of the larger number of mitochondrial structures with texture similar to endolysosomes were used to learn a binary classifier against background. The weights were initialized for training a three-class model where only the last layer was fine-tuned to distinguish between the classes. Transfer learning thus enables the modeling of a dataset even when they are limited.

Fine-tuning a pre-trained network comes with the risk of over-fitting to the few labeled training examples of the new application. This challenge has opened up new research avenues, namely, few-shot learning and domain adaptation. Few-shot is a meta-learning approach that “learns to learn” from a given pre-trained model (Shaban et al., 2017). A pre-trained model is made to learn the similarity or difference between classes on the available few labeled samples, referred to as the support set (Dong and Xing, 2018). Complex non-linear mitochondrial morphology was captured using active feature selection and boosting (Dietlmeier et al., 2019). The VGG-16 model pretrained on the ImageNet dataset was used as a feature extractor for extracting hypercolumns that contain the activations of all CNN layers for each pixel. Hypercolumns were passed through a linear regressor for actively selecting features. Only 20 patches or blocks were used from a FIB-SEM stack for training a gradient based boosting classifier (XGBoost). By actively selecting features and learning using far less training data or even using a single training sample (single-shot) one can obtain competitive segmentation accuracy.

Domain adaptation is another form of transfer learning, where the source to target datasets share the same labels (classes) but have a different data distribution. Changes in data distribution can be due to slightly different experimental parameters during EM imaging or due to the imaging of different tissue types or body locations. Roels et al. (2019) aimed to learn a latent space with a shared encoder in such a way that the source (annotated samples) and target (few annotated samples) representations are aligned in that feature space. Transferring knowledge from isotropic to anisotropic SEM images is possible using learning in latent space for domain adaptation. In contrast, Bermúdez-Chacón et al. (2018) proposed a two networks, jointly trained using a differential loss function to regularize two U-Nets (one for each domain) to avoid domain shift. Only 10% of labeled target data was required for domain adaptation to achieve state-of-the-art performance when compared to a U-Net trained on fully annotated data.

5.2. Semi-supervised learning

Semi-supervised approaches use unlabeled data for training along with a small set of labeled data (Zhu and Goldberg, 2009). The distribution patterns from unlabeled data used in training models help to generalize more than supervised learning from the few labeled samples. Semi-supervised methods learn to improve the model performance in subsequent iterations using pseudo labels generated from the output of the pre-trained model. Incremental learning selects best features by iteratively adding decision trees to the classifier. (Utgoff
1989) demonstrated that adding only relevant decision trees can incrementally improve the prediction without needing to go back and retrain the model.

Label propagation in images of a 3D stack using pseudolabels from predictions of a trained network was performed in an incremental setting (Takaya et al., 2021). The experimental results conclude that the generalization performance using supervised learning on 3D EM does not perform well when compared with a sequential semi-supervised segmentation (4S) approach. The 4S approach improved the performance of the network by reducing false positives along with improving segmentation accuracy when compared with U-Net. Such a semi-supervised approach applied on sequential EM images (having strong correlation between images in stacks) reduced the annotation effort by experts.

5.3. Unsupervised approaches

Unsupervised approaches are categorized into two groups: the more traditional relies on image processing and thresholding without involving learning algorithms, i.e., by Karabağ et al. (2019) who used traditional image processing algorithms to detect nuclear envelopes. Low-pass filtering, edge detection, dilation to connect disjoint edges, super-pixel analysis followed by smoothing and filling of holes form an unsupervised pipeline for nuclear envelope detection. The pipeline performed better than the four deep learning models VGG16, ResNet18, U-Net and Inception-ResNetv2 investigated by Szegedy et al. (2017). The cell nucleus that becomes smaller at the edges than the middle 2D slices lead to a highly imbalanced images for training the deep architectures. However, the learning-free unsupervised approach assumes that the cell nucleus is always located at the center of the three dimensional stack and that nuclear envelope is darker than nucleus and surrounding background, and therefore such learning-free approaches may not be sufficiently robust in generalization.

The more advanced group of unsupervised approaches configure models from unlabeled data. Unsupervised domain adaptation with self-supervision (self-generated labels) was used to determine pivot locations in the target dataset with no labels that characterise regions of mitochondria or synapse (Bermúdez-Chacón et al., 2019). The target domain locations from the correspondences of similar structures were converted to heatmaps, for adapting the model based on a two-stream U-Net (Bermúdez-Chacón et al., 2018). The results were consistent with those obtained under fully annotated samples trained on U-Net or semi-supervision (use of transfer learning). No new annotation effort in case of domain shifts was required for volumes of FIB-SEM from different mouse specimens.

Adversarial learning trains networks to predict the same output for two datasets, one source and the other being the adversarial perturbed data, for which the latter gives a different output in spite of belonging to the same class. Each algorithm can use a different approach, such as sharing weights across domains (Peng et al., 2020) or use of generative samples from a generator to confuse the discriminator as is done by Generative Adversarial Networks, or GANs for short (Goodfellow et al., 2014). Adversarial learning is also used for domain adaptation to learn non-discriminating features for robustness to shift in
data distributions (Peng et al., 2020). Domain-invariant features in the encoder are learnt through a reconstruction auto-encoder in an unsupervised manner. As the target has no labels, the shared decoder features are still not discriminative to the target domain for which the proposed method uses adversarial learning in the decoder stage. GANs use adversarial learning to generate synthetic training samples with the same statistics as the source training data. Image synthesis using a GAN generates images with similar distribution but varied object configuration for a three-class detection from a TEM dataset (Shaga Devan et al., 2021). Synthetic images speed up automatic image analysis even when large training datasets are not available, thus improving the performance significantly.

5.4. Performance evaluation metrics

Segmentation is evaluated using pixel-based matching or segment-based matching for binary segmentation. When the segmentation gives a unique index to each object it is called instance segmentation, evaluated by penalising overlaps with other individual segments.

Common pixel-based matching measures are accuracy, true positive rates and false positive rates (Jiang et al., 2019). Precision, recall (sensitivity), specificity and F1-score (harmonic mean of precision and recall) are the most basic performance measures used in various studies to quantify the effectiveness of 2D and 3D segmentation methods (Xiao et al., 2018; Dietlmeier et al., 2019; Khadangi et al., 2021; Takaya et al., 2021). Most binary image segmentation tasks suffer from class imbalance as the background class is much larger than the objects of interest. To address class imbalance, methods such as Intersection over Union (IoU) or Jaccard index, which determine the similarity between the ground-truth and predicted sets, are more appropriate. The Dice similarity coefficient (DSC) addresses class imbalance by only considering the segmentation class for evaluation. The DSC and JAC are the most commonly used measures for performance evaluation in EM (Mekuč et al., 2020; Xiao et al., 2018; Yuan et al., 2021; Luo et al., 2021; Cao et al., 2019; Peng and Yuan, 2019; Bermúdez-Chacón et al., 2018; Peng et al., 2020). The conformity coefficient used by Xiao et al. (2018) is a global similarity score with more discrimination capabilities than Jaccard or DSC (Chang et al., 2009). To calculate how far the segmented structure is from the ground truth, besides the Jaccard index, the Hausdorff distance is another sensible measure (Karabağ et al., 2019). The latter is the spatial distance between two sets, and apart from matching segments, it also takes into account the pixel/voxel localisation. Common metrics used for instance segmentation in (Yuan et al., 2020; Luo et al., 2021) are the Aggregated Jaccard Index (AJI) and the Panoptic Quality (PQ) (Kumar et al., 2017; Kirillov et al., 2019), which account for under- and over-segmentation more accurately than the Jaccard index and DSC. The Jaccard curve, which was inspired by the precision/recall and receiver operating characteristic (ROC) curves, is a measure that quantifies the quality of a segmentation result without involving any thresholds (Cetina et al., 2018). Fig. 4 illustrates how such measures are computed.
Figure 4: Common performance metrics for binary segmentation. For semantic segmentation, the overall overlap of the ground truth (GT) mask with prediction (PR) is compared without differentiating between objects of the foreground class. For instance segmentation, each GT component is matched with only one PR component, the one with which it has the largest intersection. The PR component \( A \) overlaps with two GT components, \( a \) and \( b \), but is matched only with \( a \) due to a larger overlap. The Aggregated Jaccard Index (AJI) takes the sum of the intersections of all matched GT and PR components divided by the sum of their unions plus the unmatched PR components. The Panoptic Quality (PQ) is the sum of the IoU ratios of all associated GT-PR pairs (i.e. TPs) divided by the sum of all TPs and half of the unmatched GT and PR components. The symbol \(|.|\) indicates the area of the component concerned.

Boundary matching and information theoretic scores have emerged as two important metrics to evaluate neuronal boundary maps (Unnikrishnan et al., 2007; Arbelaez et al., 2010; Arganda-Carreras et al., 2015). The most popular ones are similarity-oriented measures between two clusters for paired labels instead of pixel-wise errors. Boundary maps are transformed to segmentations by finding connected components. The rand index quantifies the similarity between the results of two clusters by taking the ratio of the sum of the total number of pairs of points in agreement and pairs of points in disagreements with respect to the total number of pairs between the two clusters. Another measure is based on what are known as the split and merge errors. The split error is computed by taking two randomly selected voxels belonging to the same segment in the ground truth and assessing them based on the joint probability of belonging to the same region in the segmentation result. The merge error is based on whether two voxels predicted as belonging to the same segment do actually belong together. The Rand F-score is then the weighted harmonic mean of the merge and split errors. Metrics like foreground-restricted rand scoring and foreground-restricted information theoretic scoring after border thinning are the state-of-the-art metrics for neuronal boundary segmentation (Cao et al., 2020; Zeng et al., 2017; He et al., 2020; Cetina et al., 2018; Khadangi et al.)
6. **Scalability and performance matters**

Bioimage analysis software tools use high-performance computing for scalable processing. Scalability refers to the data processing frameworks and computational resources that can handle big data. Scalable machine or deep learning is not limited by the algorithms involved, but the supporting infrastructure which is vast and complex (Sculley et al., 2015).

6.1. **Distributed computing**

Distributed computing divides a single problem into many parts, controlled by a master node but processed in different computing units (worker nodes). More databases or processing nodes can be added to the system, rather than using a single server with many nodes that is not used at all times. Fault tolerance nodes address hardware failures known as worker nodes or master replica (Fig. 5). Users or clients access and process data remotely in different systems of the network. Biomedical image analysis tools for neuronal reconstruction such as VAST, NeuTu, webKnossos, TrackEM2 (CATMAID) use distributed storage and processing.

Detailed metrics to evaluate for synapse connectivity were introduced in VAST for evaluating petabyte range datasets for 3D EM. A software ecosystem in VAST is used to evaluate two large datasets (Takemura et al., 2015, 2017) deployed in a scalable cluster-based solution using Apache-Spark (Zaharia et al., 2010). The latter is an open source data processing framework to store and process data in batch or real-time across clusters of computers. Distributed Versioned Image-oriented data service (DVID) provides branched or distributed versioning in connectomics reconstruction workflow for collaborating proofreaders from any part of the world (Katz and Plaza, 2019). NeuTu is a client program that uses DVID as its scalable image database for large-scale, collaborative 3D neuronal reconstruction proofreading. A backend Hadoop framework used by Yuan et al. (2020) allows for distributed data storage of large amounts of data. The scaling of clusters with more generated EM data and redundancy of data in various clusters provides reliable data management and integrity.

Decentralized computing, modeled after Google Maps (Rasmussen, 2005), was introduced in the Collaborative Annotation Toolkit for Massive Amounts of Image Data (CATMAID) (Saalfeld et al., 2009). It uses in-browser decentralized annotation of large biological stacks. Immediate or message passing from farther nodes in the network make images accessible to the user (Fig. 5). Projects, image stack information, and annotations (metadata) are stored in a centralized server for cross-referencing and collaboration. WebKnossos uses decentralized systems for image storage of 3D cubes and is implemented for an efficient in-browser access and reconstruction (Boergens et al., 2017).

http://www.biii.eu/
6.2. Cloud computing

Cloud computing is the on-demand provision of servers, applications, networking capabilities, and hardware resources on the internet (Kagadis et al., 2013). There are three models of cloud service. The infrastructure as a service (IaaS) model only includes computing resources, networking, and storage. The platform as a service (PaaS) model includes the application design, testing and development tools, middleware, operating systems, and databases. Finally, the software as a service (SaaS) model facilitates the availability of all application services to users from any device with an internet connection.

CDeep3EM (Haberl et al., 2018), a pre-configured cloud-based implementation of the DeepEM3D CNN for image segmentation, is publicly available on Amazon Web Services (AWS). EM-stellar (Khadangi et al. 2021b) is a Jupyter notebook platform hosted on Google Colab with ready access to cloud computing resources. Colab is a framework for developers to interface with the existing
notebooks and cloud infrastructure to evolve networks for specific microscopy image processing. The aim of the ZeroCostDL4Mic project by von Chamier et al. (2021) is to make deep learning for microscopy analysis accessible to users with no coding experience by leveraging the open, cloud-based Google Colab resources. State-of-the-art networks are provided as notebooks for segmentation, object detection, denoising, and super-resolution microscopy, along with quantitative tools to analyse model performance and optimize it along with data augmentation and transfer learning options.

7. Challenges and future trends

CNNs for segmentation are the most popular methods for automatic feature extraction and pixel-wise reconstruction in EM images. A notable example is the U-Net architecture. Techniques for structural segmentation improve robustness through additional supervision or ensemble techniques for detecting less false positives and false negatives. Whole cell 3D reconstruction for segmenting many organelles in volume EM datasets provide public datasets for reuse (Heinrich et al., 2021; Guay et al., 2021a). Large public datasets are not available for training and new datasets generated from EM techniques lack labels for supervised learning. Few-shot learning for segmentation has shown promising results in context of noisy labels and for incremental learning (Dong and Xing, 2018; Liang et al., 2022; Tao et al., 2020). Newer methodologies of semi-supervised and unsupervised learning techniques are becoming more appealing. Semi-supervised and unsupervised methods that can segment new datasets with minimal annotations allow these methods to scale to larger EM datasets. Larger datasets scale well on deeper networks such as transformers that use built-in self-attention between patches which can prove useful for large-scale 2D EM segmentation (Dosovitskiy et al., 2020; Zheng et al., 2021).

Self-supervised methods with the contrastive learning framework have been used to learn similar or dissimilar pairs from data, namely SimCLR (Simple Framework for Contrastive Learning of Visual Representations) and MoCo (Momentum Contrastive Learning) (Chen et al., 2020; Ciga et al., 2020). Networks that are initialized either randomly or by being pretrained on large datasets, such as ImageNet, do not perform better as compared to pre-training using MoCo (Guay et al., 2021b; Casser et al., 2018; Perez et al., 2014; Mekuč et al., 2020). Self-supervised methods can also benefit from multi-modal EM data, such as CLEM. In fact, Seifert et al. (2020) give further insight on the potential of deep learning for automatic image registration for CLEM. Other multi-modal training data that can be used for EM segmentation in the future is ColorEM or EDX information (Pirozzi et al., 2018).

The ability to segment new datasets with minimal annotations allows these methods to scale to larger EM datasets generated from state-of-the-art technologies, such as multi-beam scanning EM. Other aspects of scalability are improving due to the widespread use of distributed or cloud computing. Accessibility by users to such computing resources has also improved through the continued development of bioimage analysis software. Pathologists, for instance, can now
use off-the-shelf cloud applications for data access and analysis. Distributed computing can also be performed on the cloud which makes it even more appealing as all required resources can be remotely scaled with ever increasing EM data. Large-scale EM can also benefit more from the Google Maps based decentralized processing approach in a similar manner to improved data access for vEM.

Indexing of segmented structures is important for fast retrieval purposes in practical applications, but it is hardly addressed in this literature. The idea is to enable a domain expert to query a database in three different ways, namely label-based, image-based, and proximity-based. A label-based query would require the user to specify the label of a structure of interest, image-based would require the submission of an image example of a structure of interest and, proximity-based would require the specification of the distance and direction between a set of structures of interest. Such functionality would enable domain experts to enhance their interaction with a database with large volumes of large-scale EM images.

All research data should be Findable, Accessible, Interoperable and reusable (FAIR) for both machine and people (Wilkinson et al., 2016). Findability requires globally identifiable data resource associated with rich metadata, whereas accessibility provides an open, free and universally implementable protocol along with authorization mechanisms for accessing protected data. Interoperability is the data format or knowledge representation language that helps machines understand or be compatible with a service operating on the digital resource. Reusability requires that an individual or even machine can decide if the resources are useful for any task based on its license terms.

8. Conclusion

Automated image analysis techniques for EM are evolving in accordance with the recent advancements in imaging technologies. For instance, automated large-scale 2D EM makes greater demands on the capture of global context by segmentation algorithms, without the aid of 3D information available in volume EM. Given that the lack of fully annotated data in medical imaging will persist and is likely to be compounded by the exponential generation of image data, we suspect that semi-supervised and self-supervised approaches will play a bigger role in the segmentation of EM data in the future.

9. Acknowledgement

This project has received funding from the Centre for Data Science and Systems Complexity at the University of Groningen[1]. Part of the work has been sponsored by ZonMW grant 91111.006; the Netherlands Electron Microscopy

[1]www.rug.nl/research/fse/themes/dssc/
Infrastructure (NEMI), NWO National Roadmap for Large-Scale Research Infrastructure of the Dutch Research Council (NWO 184.034.014); the Network for Pancreatic Organ donors with Diabetes (nPOD; RRID:SCR_014641), a collaborative T1D research project sponsored by JDRF (nPOD: 5 – SRA – 2018 – 557 – Q – R) and The Leona M. & Harry B. Helmsley Charitable Trust (Grant 2018PG – T1D053). The content and views expressed are the responsibility of the authors and do not necessarily reflect the official view of nPOD. Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed in http://www.jdrfnpod.org/for-partners/npod-partners/.

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