A Deep Learning-Based Tool for Detection and Counting of Chloroplasts in Single-Cell Images

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Abstract

Chloroplasts are essential for plant photosynthesis, with densities varying across cell types and species. For single-cell spatiotemporal analysis, Deep-learning-based Detecting-and-Counting-chloroplasts (DeepD&Cchl) introduces an advanced AI tool for 3D chloroplast detection and counting. Built on the You-Only-Look-Once (YOLO) computer vision framework, it includes an Intersection Over Union (IOU) module to prevent double-counting across focal planes, ensuring accuracy in chloroplast quantification. DeepD&Cchl integrates seamlessly with Cellpose for single-cell segmentation, allowing for robust chloroplast detection across various imaging methods—light, electron, and fluorescence microscopy—without specific preparation or pre-training. Additionally, by plotting chloroplast counts relative to cell size, it supports cell type-specific clustering, providing critical morphological insights. DeepD&Cchl enhances plant science research by offering a versatile, accurate, and efficient solution for chloroplast identification, counting, and cell-type analysis, making it an invaluable tool for scientists and engineers studying plant development and adaptation.

Introduction

Chloroplasts are essential for photosynthesis and plant development, with their density varying across cell types and species. Accurate chloroplast counting is critical for understanding plant adaptability. At the stomatal level, chloroplast numbers serve as indicators for hybrid species identification and ploidy estimation (Fujiwara et al., 2019; Watts et al., 2023). Variations among genetic backgrounds further suggest their potential as classification markers (Pyke and Leech, 1994). However, traditional methods like manual counting, molecular staining, and 2D imaging are time-consuming, error-prone, or limited in accuracy, particularly for 3D analysis of living cells (Cole, 2016; Kubínová et al., 2014).

Recent advances in artificial intelligence, particularly deep learning, offer new possibilities for addressing these challenges. Tools like Cellpose enable rapid single-cell segmentation (Stringer et al., 2021), and the YOLO framework excels in real-time object detection (Redmon and Farhadi, 2017). However, their application to 3D subcellular structures, such as chloroplasts, in living cells remains limited, with challenges like double-counting errors yet to be resolved.

To achieve high-efficiency and high-accuracy chloroplast quantification, we developed DeepD&Cchl (Deep-learning-based Detecting-and-Counting-chloroplasts). Key innovations include:

- integration of the YOLO object detection algorithm with an Intersection Over Union (IOU) module to eliminate double-counting errors.
- seamless combination with Cellpose for precise 3D chloroplast detection in living and fixed cells.
- support for cell-type clustering by correlating chloroplast counts with cell size.

These features make DeepD&Cchl a powerful tool for plant research and agricultural innovation.

Related Work

Traditional Counting Methods

Chloroplast counting methods have evolved over time, yet traditional approaches still face significant limitations in efficiency, accuracy, and scalability. Manual counting, one of

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the earliest techniques, involves direct observation and annotation of chloroplasts in microscopic images. While simple and widely used, it is highly time-consuming, labor-intensive, and prone to human errors, particularly when analyzing large datasets or complex cell structures (Arena et al., 2017).

Semi-automated methods, such as those enabled by ImageJ or Fiji software, provide some improvements. These methods often rely on threshold-based segmentation and automated particle counting tools, such as the Analyze Particles feature in ImageJ. While they reduce manual effort, they require careful parameter tuning for each dataset, making them susceptible to user variability. Moreover, these methods struggle with overlapping structures and lack robust batch processing capabilities, limiting their efficiency for large-scale studies (Schneider et al., 2012; Rueden et al., 2017). Molecular staining coupled with flow cytometry represents another approach for chloroplast quantification. While this method offers high-throughput analysis, it is hindered by the isolation process, which can disrupt cellular integrity. Additionally, it cannot reliably determine the number of chloroplasts per cell, as the process lacks spatial context (Cole, 2016; Mattiasson, 2004).

3D imaging methods, such as confocal laser-scanning microscopy, provide more detailed insights into chloroplast distribution within thick cells. By examining chemically fixed 3D volumes, researchers have shown that nearly 90% of chloroplasts are missed when using 2D images alone (Kubínová et al., 2014). However, this approach requires extensive data collection and is subject to technical constraints and subjective interpretation. Its dependency on complex and expensive instrumentation also limits accessibility. Collectively, these traditional methods are limited in their ability to handle the complexities of chloroplast distribution in living cells, particularly for 3D datasets.

Deep Learning-based Counting Methods

Deep learning has emerged as a transformative approach in life sciences, offering automated and scalable solutions for cellular analysis. These methods have been successfully applied to segmentation, detection, and counting tasks, addressing many limitations of traditional approaches.

Cell segmentation tools, such as Cellpose, have demonstrated remarkable versatility and efficiency. This deep-learning-based framework enables rapid segmentation of individual cells across diverse imaging modalities, including brightfield and fluorescence microscopy, with minimal user input. Its adaptability to various cell types and imaging conditions has made it a popular tool for single-cell analysis (Stringer et al., 2021; Pachitariu et al., 2022).

Object detection frameworks, like YOLO (You Only Look Once), have shown exceptional performance in real-time detection and counting tasks. YOLO's speed and accuracy make it particularly well-suited for cell detection in dense or noisy microscopy images, enabling high-throughput analysis with minimal computational overhead (Redmon and Farhadi, 2017; Alam and Islam, 2019). However, YOLO's application in subcellular structure detection is still in its early stages.

Specialized deep-learning tools for biological research have further advanced organelle-level analysis. For example, DeepBind, a tool leveraging deep learning, predicts DNA and RNA binding specificities with high accuracy, demonstrating the adaptability of these techniques to molecular-level tasks (Alipanahi et al., 2015). For chloroplast quantification, DeepLearnMOR, a YOLOv5-based framework, offers fully automated counting of fluorescently labeled chloroplasts. While achieving high precision and speed, it is limited by its reliance on fluorescence imaging, reducing its versatility across different imaging modalities (Li et al., 2021).

Despite these advancements, existing deep-learning methods face challenges in 3D subcellular structure analysis. Current tools are primarily designed for 2D images, which often suffer from defocusing and out-of-focus errors in thick cells, leading to inaccuracies (Kubínová et al., 2014). Moreover, the lack of integration with 3D imaging workflows and the inability to address double-counting errors in multi-focal plane datasets limit their effectiveness for 3D chloroplast detection in living cells.

Methods

Workflow of the Method

DeepD&Cchl is a deep-learning tool built on the YOLO, designed to identify and count chloroplasts. The workflow can be referred to Figure 1. This innovative tool leverages various types of microscopy images to accurately detect chloroplasts under diverse conditions, ensuring high precision through meticulous manual labeling and robust training protocols. The integration of the Cellpose tool for single-cell segmentation further enhances its accuracy in 3D detection and counting.

Training Process

Firstly, the training process was executed in the YOLOv7 framework (Wang et al., 2023) using various types of chloroplast microscopy images to recognize chloroplasts under different conditions, as shown in Figure 1a. The YOLOv7 model was cloned from its GitHub repository (https://github.com/WongKinYiu/yolov7). The YOLOv7 model was cloned from its GitHub repository (https://github.com/WongKinYiu/yolov7). It consists of four main parts: the input section, which handles image preprocessing tasks such as data augmentation and resizing; the backbone feature extraction network, which extracts deep features from the image using Convolutional Block Attention (CBS) convolution, Max Pooling (MP) convolution, and Enhanced Leaky Aggregation Network (ELAN) layers; the neck feature fusion network, which integrates multi-scale features through CBS, Spatial Pyramid Pooling (SPP)

with Cross-Stage Partial Connections (CSP), MP, and ELAN structures; and the detection head, which employs an anchor mechanism for object detection, utilizing Complete Intersection over Union (CIOU) and Non-Maximum Suppression (NMS) processing to achieve precise prediction results.

To establish a robust foundation for DeepD&Cchl, we created a high-quality, manually labeled chloroplast dataset using LabelImg software (https://github.com/tzutalin/labelImg; Tzutalin, 2015). This dataset serves as the training data, providing real-world examples of chloroplasts under various conditions, enabling accurate identification in new images. To maintain clarity, we established a systematic naming protocol:

***DeepD&Cchl_L**:* Light microscope images
***DeepD&Cchl_E**:* Electron microscope images
***DeepD&Cchl_F**:* Fluorescent images

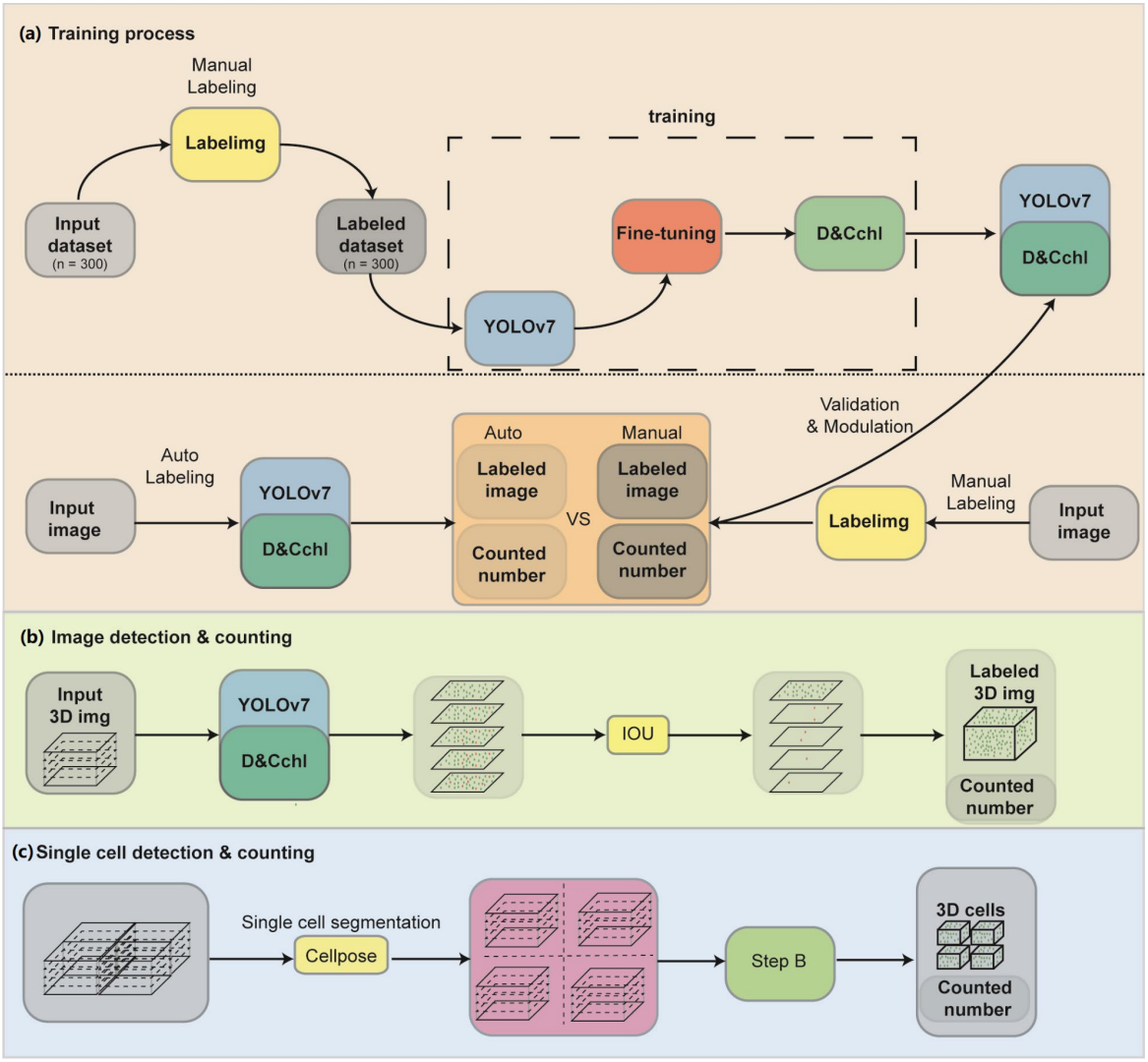

Figure 1: Workflow of the DeepD&Cchl for Chloroplast Detection and Counting. (a) Chloroplast detection with YOLOv7 using labelImg for image annotation, with data split into training and validation sets for DeepD&Cchl model training and manual annotations for comparison. (b) Chloroplast detection and counting in a 3D model from multi-layer stacks, using five focal plane images and IoU strategy to prevent repeated detections. (c) Chloroplast counting in individual cells, using Cellpose for cell segmentation and the trained DeepD&Cchl model for detection. Gray represents the dataset, yellow represents software, blue represents the neural network framework, and dark green represents models generated during training.

To train the DeepD&Cchl models, which learn to recognize chloroplasts from various types of chloroplast images. For instance, light microscope chloroplast images of three bryophyte (*Sphagnum squarrosum* [*S. squ*], *Physcomitrium patens* [*P. pat*], and *Ricciocarpos natans* [*R. nat*]) were obtained for training of DeepD&Cchl-L model, which specifically detect light microscope chloroplasts.

The manually labeled data were utilized as the ground truth reference. Any mislabeled or omitted annotations were meticulously counted by human inspection. As mentioned in Figure 1a, the accuracy mentioned refers to the proportion of correct annotations made by the artificial intelligence system relative to the manual annotations.

Counting in 3D cell and IOU

To construct a 3D cell model, sequential 2D images were captured at varying focal depths and stacked to form a comprehensive representation, with each layer contributing unique depth information. This approach allows for a detailed analysis of cellular structures in three dimensions. To achieve 3D accurate chloroplast detection, an IOU module for DeepD&Cchl was developed. It addresses the issue of repeated counting across consecutive 2D focal planes by identifying and merging overlapping detections (Figure 1b). The IOU value, ranging from 0 to 1, indicates the degree of overlap between the two detected boxes, and is calculated by the following formula:

$$\text{IOU}=\frac{\text{Area of overlap}}{\text{Area of union}} \quad (1)$$

Here:

- Area of Overlap refers to the shared region between the two bounding boxes.
- Area of Union is the total area covered by the two bounding boxes combined.

An IOU threshold was set to exclude potential lateral displacement effects during data acquisition at different focal depths. The threshold was chosen to be 0.5, which worked effectively throughout the process. The procedure began with an empty benchmark, and targets from the first image in the sequence were added to this benchmark. For each subsequent image, IoU calculations were performed between each newly detected target and the targets already present in the benchmark. If the IoU exceeded the preset threshold, the detected target was considered to be an existing chloroplast and was thus classified as such, preventing duplicate counting. If the IoU was below the threshold, the target was classified as a new chloroplast and added to the benchmark. This iterative process continued for each image, ensuring accurate tracking of chloroplasts while minimizing errors due to lateral displacement.

For precise 3D detection and counting of chloroplasts within each cell, the Cellpose tool was employed for single-cell segmentation (Fig. 1c). The pre-trained Cellpose model (https://github.com/MouseLand/cellpose; Pachitariu et al., 2022) was further trained with plant cell images to create the cyto2pro model, which enables efficient segmentation of single plant cells.

Data Preparation and Model Training

Approximately 300 bright-field light microscopy images, 119 fluorescence microscopy images, and 512 electron microscopy images were manually labeled using LabelImg. In total, over 20,000 chloroplasts were annotated for light microscopy, 3,600 for fluorescence microscopy, and 2,500 for electron microscopy. The dataset was divided into training and validation sets, with 90% allocated for training and 10% for validation.

For training the YOLOv7 model, a Python environment was configured with the required libraries. The Adam optimizer was employed with a learning rate of 0.001 and a batch size of 16. Training durations were 2.662 hours for light microscopy, 2.123 hours for fluorescence microscopy, and 3.216 hours for electron microscopy images. All experiments were conducted on a desktop computer equipped with an Intel Core i7-10700 CPU running at 3.80 GHz and an NVIDIA GeForce RTX 3060 GPU with 12GB of VRAM. The code was implemented using the PyTorch 2.0.0 framework with CUDA version 12.2 support.

Evaluation Metrics

The trained model was evaluated using standard object detection metrics, including Precision, Recall, Average Precision (AP), and Mean Average Precision (mAP). These metrics measure the model's accuracy, detection capability, and overall performance in detecting chloroplasts across different categories (Raza et al., 2023). Additionally, the F1 score, the harmonic mean of precision and recall, was used to assess the balance between these two aspects. These metrics provide a comprehensive evaluation of the model's effectiveness in chloroplast detection.

Plant Materials and Culture Conditions

The bryophyte plant materials (*S. squ, P. pat, and R. nat*) were provided by Prof. Ruiliang Zhu and Prof. Yue Sun (East China Normal University), while *Wolffia australiana* (*W. aus*) was gifted by Dr. Li Feng (High School Affiliated to Renmin University of China).

Bryophyte Culture: Surface soil was rinsed, followed by treatments with 0.05% Triton buffer and 5% NaClO solution. After sterilization, the thalli were placed on ½ GB5 medium (pH 5.7–5.8) with 1% sucrose and incubated at 22°C with a 16-h-light/8-h-dark cycle. Vibrant green thallus sections were propagated on the same medium, and growth was documented.

Arabidopsis thaliana (Col-0, WT): Seedlings were transferred to soil 14 days after cultivation on ½ MS agar medium. Plants were grown under a 16-h-light/8-h-dark photoperiod at 22°C and 70% relative humidity.

Wolffia australiana: Cultured in liquid ½ MS medium in a controlled growth chamber under similar conditions.

Imaging Techniques

Light Microscopy: Images were captured using an Olympus-BX43 microscope. Liverwort scales were dissected and

laid flat in water droplets on a clean slide, covered with a coverslip, and examined under low magnification. High-quality images of chloroplasts were obtained at 10×60 magnification from regions with dispersed chloroplasts. A series of images at different focal depths were collected by adjusting the sample stage to ensure clear visualization and accurate counting.

Fluorescence Microscopy: Images were acquired using a LEICA TCS SP8 confocal microscope with a 40× or 63× oil immersion objective at an excitation wavelength of 632 nm.

Serial Block Face Scanning Electron Microscopy (SBF-SEM): *S. squ* capitulum samples were fixed in 4% paraformaldehyde at 4℃ overnight, stained with osmium tetroxide, thiocarbohydrazide, uranyl acetate, and Walton's lead aspartate, then dehydrated in an acetone series. Samples were embedded in EPIN812 resin, sectioned into thin strips, and imaged using a 3VIEW-SEM (Zeiss) at 40 nm per slice to generate 3D reconstructions.

Results and Discussions

Chloroplast detection and counting with DeepD&Cchl Using light microscope images

To thoroughly assess the performance of DeepD&Cchl-L in chloroplast detection, we employed the comprehensive evaluation metric, mAP (Figure 2a). In the validation dataset, when the confidence level was set to 0.5, the model achieved an average precision of 0.877. Furthermore, the F1 curve peaked at 0.84, further demonstrating the model's excellent balance between precision and recall.

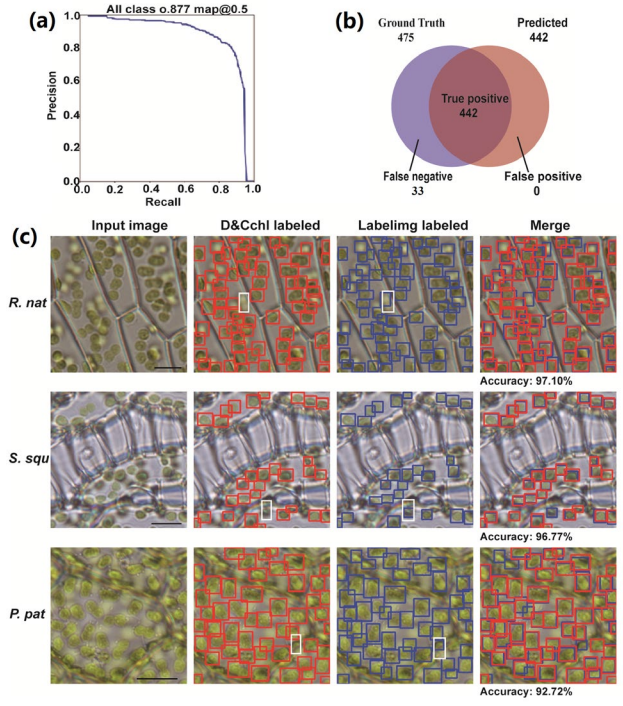

Figure 2: Assessment of the DeepD&Cchl for chloroplast detection in *S. squ, P. pat*, and *R. nat* images. (a) Precision-recall curve. (b) Venn diagram of true positives and predicted positives. (c) Comparison of detected chloroplasts (red boxes), manual labels (blue boxes) and missed detections (white boxes).

In combination with the IOU module, the DeepD&Cchl tool automatically detects and counts chloroplast in 3D volumes. To accurately calculate the chloroplast in 3D, we used DeepD&Cch on a multilayer of light-microscope images covering the entire cell (Figure 1b and Figure 3). Sequenced images of *S. squ*, *P. pat*, and *R.nat* leaf cells were obtained at various focal depths. The DeepD&Cchl was applied for each individual layer, and IOU was used for monitor the overlaps of every target between layers. The accurate counts of chloroplasts in different plants were obtained (Figure 3). The precision was significantly improved in multilayer statistics than that in the single-layer image. In single-layer images, only about 90% of the total chloroplasts could be detected, and in multilayer images, almost all of them could be detected.

To evaluate the efficiency of DeepD&Cchl in detecting chloroplast (Figure 1b), we manually labeled 9 unseen images (3 images from each different plant) as a test dataset. The DeepD&Cchl tool successfully detected 88 chloroplasts in *S. squ* (missing 3), 160 chloroplasts in *P. pat* (missing 10), 194 chloroplasts in *R.nat* (missing 20), and 475 chloroplasts in total images (missing 33), while falsely detected 0 chloroplasts (Figure 2b). The final counting results were calculated and the precision rates are 97.10%, 96.77%, and 92.72% respectively (Figure 2c). In all, the DeepD&Cchl-L tool has showed an expert performance on automatic chloroplast detection and counting with light microscopy images.

Detecting chloroplasts in various types of microscope images using DeepD&Cchl

To expand the application of the DeepD&Cchl tool, we have incorporated various types of chloroplast microscope images, including electron microscopy and fluorescent microscopy images (Figure 4). The same training strategies were used on various types of images, like those used for light microscope images. To assess the performance of the three DeepD&Cchl models, we conducted tests on three distinct sets of untreated images (Figure 4). The results revealed that 66, 5, and 73 chloroplasts were individually identified from fluorescent, electron microscopy, and light microscope images, respectively. The accuracies are approximately 93.75%, 100%, and 97.43%, respectively.

To better illustrate the performance of the DeepD&C tool across different microscopy modalities, Table 1 summarizes the training time and average test accuracy. It is evident that the method typically achieves a counting accuracy greater

than 90%. The tool may be particularly well-suited for SEM images, as the chloroplasts exhibit distinct features in this type of imagery.

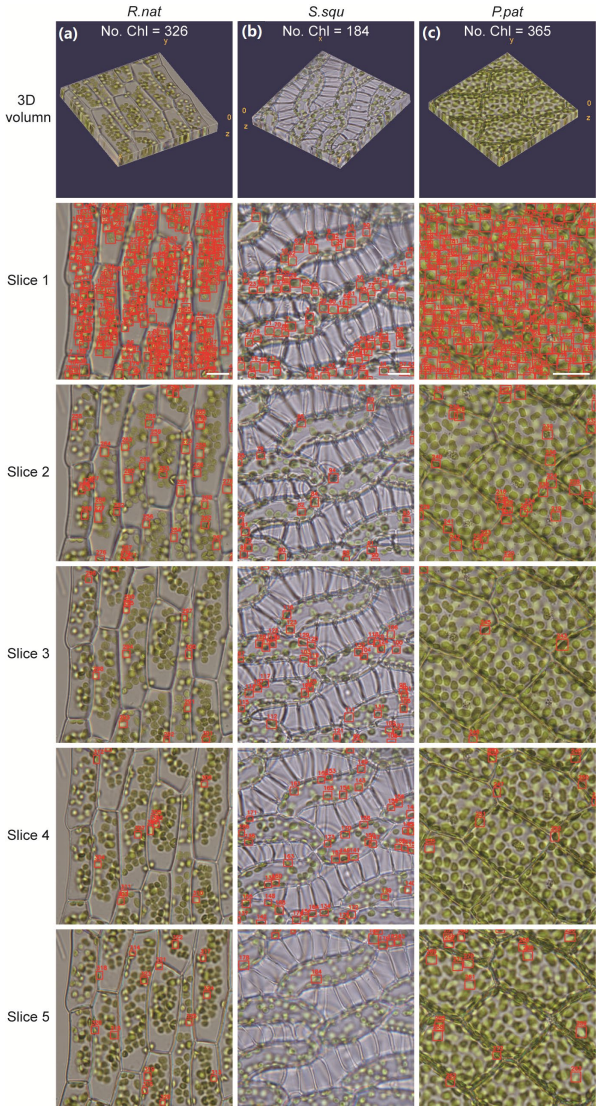

Figure 3: Chloroplast detection and counting using DeepD&Cchl in 3D volume (a)-(c) The microscopic image series and chloroplast detection results (signed by red squares) for S. squ, P. pat, and R. nat. The labels Slice1 to 5 indicate the first to fifth layer of chosen images from each sample's microscopic image series. Scale bars, 10 μm.

Notably, for the light microscope images, we also purposely utilized a set from multilayer cell leaves of *W. arr*, which exhibited inferior clarity compared to single-layered cell images (Figure 4). These results not only affirm the high adaptability and precision of the DeepD&Cchl tool in chloroplast detection and counting across various types and complexities of images, but also underscore the capability of deep learning methods in precise organelle quantification within diverse biological samples, highlighting their potential and universality in biological research.

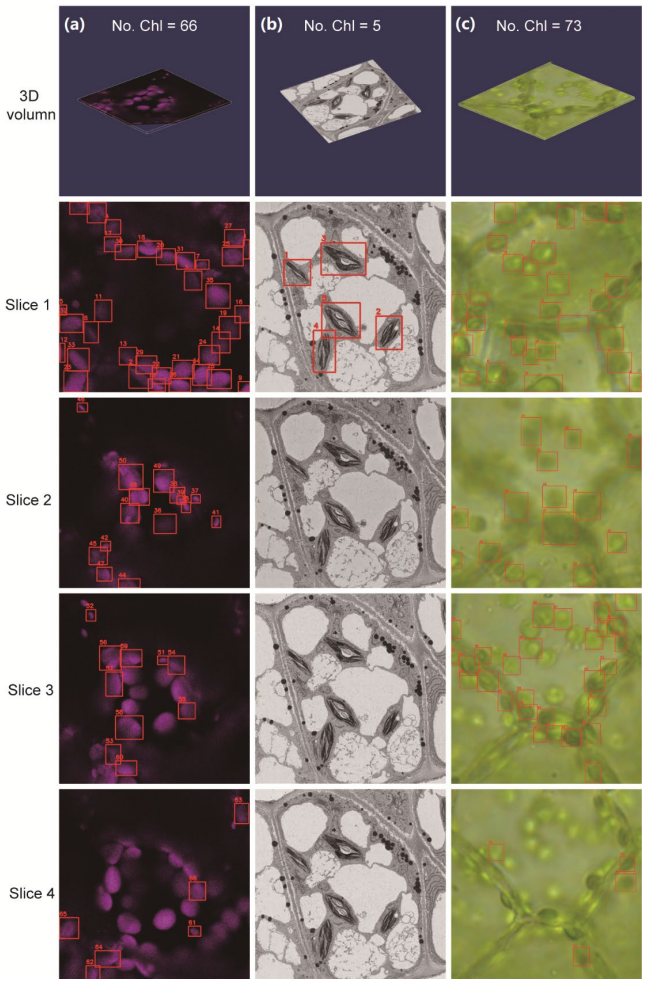

Figure 4: 3D chloroplast detection using DeepD&Cchl models across different image types: (a) fluorescent images of *Arabidopsis thaliana* [*A.tha*] (DeepD&Cchl_F), (b) electron microscopy images of *S. squ* (DeepD&Cchl_E), and (c) images of *W. arr* (DeepD&Cchl_L)

Table 1 comparison of the process time and performance for different Microscopy Modalities

	Light	Fluorescent	SEM
Number of Training/Validation Images	300	119	512
Training Time (h)	2.662	2.123	3.216
Accuracy (%)	96.01 ±1.91	93.75	100

DeepD&Cchl single-cell chloroplast detection function enables cell type clustering

To achieve single-cell counting of chloroplasts, we employed a Cellpose segmentation tool. We re-trained the original model cyto2 with our dataset and obtained a new model named 'cyto2pro', and then utilized it to segment optical microscope images. We specifically chose intact individual cells as inputs for object detection, subsequently conducting chloroplast detection and counting within those individual cells using DeepD&Cchl at both 2D and 3D levels (Figure 5). Plotting cell area against chloroplast count in single cells of *R. nat* (Type A-B) and *A. tha* (Type C-D) reveals cell type-specific clustering (Figure 5). This highlights the relationship between chloroplast count and cell size in determining cell type, providing valuable morphological information for single-cell studies. These results have significant engineering applications. The ability to accurately count chloroplasts and analyze their distribution relative to cell size can be used in synthetic biology and agricultural engineering. This method facilitates the development of genetically modified plants with optimized chloroplast distribution for enhanced photosynthetic efficiency and improved crop resilience.

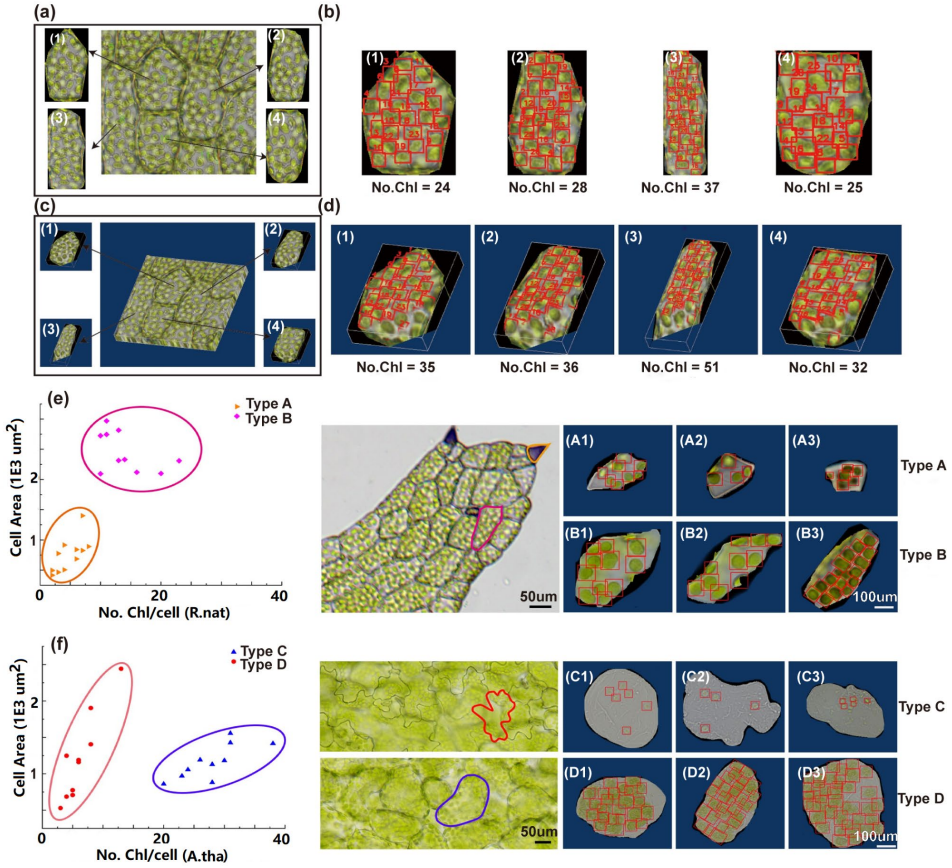

Figure 5: Cell type-specific clustering promoted by single-cell chloroplast detection using DeepD&Cchl. (a-b) Single-layer images segmented via Cellpose, labeling cells 1-4 and counting chloroplasts. (c-d) Multi-layer images forming 3D volumes with chloroplasts highlighted in red boxes. (e-f) Scatter plots show chloroplast count vs. cell area in *R. nat* (Type A-B) and *A. tha* (Type C-D), respectively. No.Chl refers to the number of detected chloroplasts, highlighted by red boxes.

Limitations and potential improvements for the DeepD&Cchl tool

Currently, within the DeepD&Cchl tool, we've trained three unique chloroplast detection models for images from light microscopy, fluorescence microscopy, and electron microscopy. While effective within their specific scopes, these models require specialized knowledge for selection and operation, which can hinder widespread adoption. To address this, further development of a multimodal model that can process diverse microscope imagery. One proposed solution is integrating the classification and detection capabilities of YOLO to create a model that can automatically recognize and process different microscope images. Future research

should focus on integrating various detection and classification techniques to create an efficient, accurate, and user-friendly chloroplast image analysis system. This will boost the model's adaptability and flexibility, ensuring high-quality results across various observation scenarios.

Another limitation of the DeepD&Cchl method is its effectiveness in handling highly dense or clustered chloroplasts, where overlap between chloroplasts can pose significant challenges. To address this issue, one potential solution is to employ digital refocusing techniques to differentiate chloroplasts at different depths. This approach could help to resolve the overlap problem by enhancing the clarity of chloroplasts located at various focal planes. Additionally, multi-scale analysis may be beneficial in improving the detection and separation of closely clustered chloroplasts. By analyzing the images at different scales, the method can more effectively distinguish individual chloroplasts, even in dense regions, thereby improving accuracy in these complex scenarios.

The DeepD&Cchl method, utilizing deep learning, provides an innovative solution for accurately counting chloroplasts in plant cell. Even in the presence of noise or poor image quality, the method effectively distinguishes chloroplasts from background noise by recognizing their unique features. Its effectiveness would be amplified when integrated with object detection frameworks like Faster R-CNN (Faster Region-CNN) or SSD (Single Shot MultiBox Detector), improving precision and segmentation accuracy (Liu et al.,2016; Girshick R et al.,2015). When combined with time series analysis tools like LSTM (Long Short-Term Memory), it allows real-time monitoring of chloroplast dynamics under various environmental conditions (Hochreiter and Schmidhuber,1997). The addition of multimodal data and technologies like autoencoders and VAEs (Variational Autoencoders) enables deep extraction of cellular features, offering a more efficient and comprehensive approach to plant cell research.

The DeepD&Cchl method enhances plant cell type classification accuracy, aiding in understanding plant growth characteristics and adaptability to environmental changes. While it is primarily designed for chloroplast detection, the method has the potential for broader applications within plant cells. Moreover, it can be easily extended to the recognition, classification, and counting of other organelles. Its robust and accurate image analysis makes it suitable for detecting and quantifying other cellular structures and organelles with further training. This could enrich our understanding of cellular mechanisms and processes, advancing plant cell biology as well as agriculture engineering.

Conclusion

In conclusion, DeepD&Cchl offers a precise and efficient method for detecting and counting chloroplasts, contributing to our understanding of their function and behavior in plant cells. It can also elucidate how chloroplast density affects plant processes like photosynthesis and energy production. The tool's ability to analyze cells from various species may lead to discoveries about species-specific adaptations and evolutionary developments. Additionally, its applications in synthetic biology and agricultural engineering could enhance crop yields and resilience. Future research should explore integrating various detection and classification techniques with spatial omics methods to create an efficient, accurate, and user-friendly chloroplast image analysis system.

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